Induction of ErbB2 by ultraviolet A irradiation: Potential role in malignant transformation of keratinocytes

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Ultraviolet (UV) A (320–400 nm), which constitutes more than 90% of UV radiation in the sunlight that reaches the earth's surface, is considered a major cause of human skin photo-aging and skin cancer. Exposure of keratinocytes to UVA has previously been reported to lead to the activation of a variety of epidermal growth factor receptors (EGFR), including ErbB2, and ErbB2 activation is involved in skin tumor development. Here, we demonstrate that ErbB2 expression is enhanced by low-energy UVA (300–3000 mJ/cm^2) irradiation in the skin tissues of both hairless mice and HaCaT keratinocytes. Luciferase reporter-gene activity using the 756-bp flanking region of the human *erbB2* gene was increased by UVA irradiation. UVA irradiation also selectively increased the levels of activator protein (AP)-2α, but not AP-2β and AP-2γ. The increase in the reporter gene activity of HaCaT cells exposed to UVA was abolished by mutation of the two AP-2 binding sites in the promoter region of the *erbB2* gene. Inhibition of cAMP-dependent protein kinase caused complete blockage of ErbB2 induction and AP-2α activation by UVA irradiation. Finally, we reveal that pre-exposure of HaCaT cells to UVA potentiates EGF-inducible anchorage-independent growth of the keratinocytes, which is significantly suppressed by ErbB2 inhibition. These results support the hypothesis that UVA enhances the expression of ErbB2 via cAMP- and protein kinase-dependent AP-2α activation in keratinocytes, which may serve as a key mechanistic basis for the malignant transformation of keratinocytes exposed to UVA irradiation. (*Cancer Sci* 2008; 99: 502–509)

Radiation from the sun can be divided into infrared, visible, and UV light. The UV spectrum can be further divided into three classifications: UVC (200–280 nm), UVB (280–320 nm), and UVA (320–400 nm). Because a large portion of UVB and all UVC wavelengths are absorbed by the ozone layer,[1] UVA constitutes more than 90% of the UV radiation to which human skin is exposed.[2] UV radiation leads to the development of skin cancers and pathogenesis of other skin damage, and the cell proliferation signaling pathways initiated by UVA radiation are believed to contribute to skin tumor promotion and progression.[3] In general, epidermal cells exposed to UVA radiation undergo apoptosis, but cell survival can be promoted by the activation of proliferation signals in conjunction with the suppression of apoptosis, allowing the potential for malignant transformation. However, the molecular mechanisms of cell survival or malignant transformation after UVA exposure are not fully understood.[4]

Activating mutations of various oncogenes play an important role in tumorigenesis. One of the most important activating mutations involves the *erbB2*/*HER-2*/*neu* gene, which is a member of the EGFR family of tyrosine kinase receptors. ErbB2 has been shown to be important for skin tumor formation as well as normal skin development. It has been reported that constitutive expression of ErbB2 in the epidermis of transgenic mice results in epidermal hyperproliferation and spontaneous skin tumor development,[5] and higher expression of ErbB2 was found in most human squamous cell carcinomas.[6] ErbB2 activation is also involved in the tumor promotion process of phorbol ester-treated mouse epidermis.[7]

Increasing evidence suggests that growth factor receptor tyrosine kinases, including EGFR, are activated in response to UV irradiation.[8] In murine skin, UV-induced proliferation of keratinocytes, epidermal hyperplasia, and tumorigenesis were blocked by EGFR tyrosine kinase inhibitors.[9,10] Although it is clear that UV radiation is capable of activating EGFR signaling, the contributions of UVA to EGFR activation are still unclear.[11] Moreover, whether UVA irradiation changes the expression of ErbB2 in keratinocytes has not been studied. Therefore, we investigated whether UVA affects ErbB2 expression in both hairless mice and the HaCaT human keratinocyte cell line, and examined the mechanistic basis for its effect on ErbB2 expression. In order to define the pathological implications of UVA-stimulated ErbB2 overexpression in human skin carcinogenesis, we also studied the transforming potential of EGF-treated or untreated HaCaT cells after pre-exposure to UVA irradiation and further assessed the possible role of ErbB2 using a chemical inhibitor.

Materials and Methods

Materials. Antiphospho-ERK, anti-ERK, antiphospho-p38 kinase, and anti-p38 kinase antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-ErbB2 and anti-AP-2α antibodies were purchased from DakoCytomation (Glostrup, Denmark) and Upstate (Charlottesville, VA, USA), respectively. The antibodies against AP-2β and AP-2γ were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alkaline phosphatase or HRP-conjugated donkey antimouse, antirabbit, and antigoat IgG were acquired from Jackson ImmunoResearch (West Grove, PA, USA). 5-Bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium and the phRL-SV40 plasmid were obtained from Promega (Madison, WI, USA). The antiactin antibody, as well as other reagents for the molecular studies, was purchased from Sigma Chemical (St Louis, MO, USA). The p756-Luc construct, which contained 756 bp of the human ErbB2-promoter region, and the p756m-Luc construct, in which the AP-2 binding site was mutated, were kindly provided by Dr R. Winkler (University of Liege, Belgium).[12] Hairless mice (HR-1, 20–25 g) were provided by Dr H. K. Choi (Chosun University, Gwangju, South Korea). Throughout the experiments, mice were housed at

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Abbreviations: AP, activator protein; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ErbB2-HaCaT, ErbB2-overexpressing HaCaT; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; GFP-HaCaT, GFP-overexpressing HaCaT; HRP, horseradish peroxidase; MAP, mitogen-activated protein; MSCV, murine stem cell viral; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PKA, cAMP-dependent protein kinase; UV, ultraviolet.

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five per laminar flow cage maintained at 22 ± 2°C, 50–60% relative humidity, under a 12:12 h L : D cycle. The animals were kept in these facilities for at least 1 week before the experiment. The animal care committee at Chosun University approved the present study.

Cell culture and UVA irradiation. The HaCaT human keratinocyte cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For UVA irradiation to HaCaT cells, the culture medium was removed, and cells were washed with sterile PBS. UVA exposure was carried out using fluorescent lamps (UVP, Upland, CA, USA) with the dish lid off. In selected experiments, cells were preincubated with inhibitors for 10 min prior to irradiation.

Preparation of nuclear fraction. The cells in dishes were washed with ice-cold PBS, scraped, and transferred to microtubes. The cells were allowed to swell after addition of 100 μL lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The cell membranes were disrupted by vortexing, and the lysates were incubated on ice for 10 min and centrifuged at 7200*g* for 5 min. Pellets containing the crude nuclei were resuspended in 60 μL extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. The samples were centrifuged at 15 800*g* for 10 min to obtain the supernatants containing the nuclear extracts. The nuclear extracts were stored at –80°C until needed.

Immunoblot analysis. Sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblot analysis were carried out according to previously published procedures.[13] The cells were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/mL leupeptin. The skin tissues obtained from the back of hairless mice were removed and homogenized using EBC lysis buffer. The cell lysates or the skin tissue homogenates were centrifuged at 10 000*g* for 10 min to remove the cell debris. The proteins were fractionated using 10% separating gels. The fractionated proteins were then transferred electrophoretically to nitrocellulose papers, and the proteins were immunoblotted with each specific antibody. The secondary antibodies were either HRP- or alkaline phosphatase-conjugated anti-IgG antibodies. The nitrocellulose papers were developed using either 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium chloride or an ECL chemiluminescence system. For chemiluminescence detection, the LAS3000-mini (Fujifilm, Japan) was used.

Immunohistochemistry. A universal immunoenzyme polymer method was used for immunostaining. Sections 4-μm thick were cut from formalin-fixed, paraffin-embedded tissue blocks, mounted on polylysine-coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol. After deparaffinization, antigen retrieval treatment was carried out at 121°C for 15 min in 10 mM sodium citrate buffer (pH 6.0), and the sections were then treated with 3% hydrogen peroxide in methanol solution for 20 min in order to quench endogenous peroxidase activity. To block intrinsic avidin–biotin capabilities, the tissue slides were treated with avidin–biotin blocking kit reagents (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 15 min. The anti-ErbB2 and anti-AP-2α antibodies were used as the primary antibodies. The final products were visualized using the 3-amino-9-ethylcarbazole substrate system (DakoCytomation). All experiments were carried out in duplicate.

Reporter gene assay. Promoter activity was determined using a dual-luciferase reporter assay system (Promega). Briefly, cells were transfected transiently with 1 μg of either p756-Luc or pm756-Luc plasmid and 10 ng of pRL-SV plasmid (*Renilla* luciferase expression was used for normalization) (Promega) using the Genejuice (Novagen, Madison, WI, USA) or Hilymax reagent (Dojindo Molecular Technologies, MD). The cells were then incubated in serum-free culture medium for 18 h. The firefly and *Renilla* luciferase activities in the cell lysates were then measured using a luminometer (TD-20, CA, or LB941; Turner Designs, Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activity was calculated by normalizing the promoter-driven firefly luciferase activity to the *Renilla* luciferase activity.

cAMP determination. A commercial chemiluminescent enzyme-linked immunosorbent assay kit (Applied Biosystems, Bedford, MA) was used to determine 3′,5′-cAMP levels in cell lysates, according to the manufacturer's protocol.

Colony-formation assay (soft agar assay). HaCaT cells were exposed to UVA in the presence or absence of the ErbB2 inhibitor AG825 and incubated for 20 h in a 5% CO_2 incubator. Then, the cells (8×10^3/mL) were maintained in 1 mL of 0.3% basal medium Eagle's agar containing 10% fetal calf serum with or without EGF (10 ng/mL) at 37°C for 14 days. After the incubation, cell colonies were scored using a microscope.

Construction of the ErbB2 retroviral plasmid and infection of HaCaT cells. ErbB2 was overexpressed stably in HaCaT cells using an MSCV-GFP retrovirus system. Human ErbB2 cDNA was subcloned into the MSCV-GFP retroviral vector and phoenix cells were transfected with MSCV-GFP (control) or MSCV-hErbB2-GFP plasmid. Supernatants containing amphotrophic replication-incompetent retroviruses were collected and then stored at –80°C until required. HaCaT cells at 30% confluence were infected eight times with retrovirus particles. The intensities of infection were monitored by western blot analysis using a specific antibody.

Data analysis. Scanning densitometry was carried out using an Image Scan and Analysis System (FLA-7000, Fujifilm). One-way analysis of variance (ANOVA) was used to assess significant differences between the treatment groups. The Newman–Keuls test was used to compare multiple group means for each significant treatment effect. The criterion for statistical significance was set at either $P < 0.05$ or $P < 0.01$.

Results

Induction of ErbB2 expression by UVA in both HaCaT keratinocytes and hairless mice. To investigate whether UVA irradiation affects the expression of ErbB2 in HaCaT human keratinocytes, we measured the ErbB2 levels after exposure of the cells to UVA irradiation (30–3000 mJ/cm^2). The ErbB2 protein levels were increased by UVA exposure in an energy-dependent manner (Fig. 1a). Furthermore, we examined whether ErbB2 induction by UVA also occurs *in vivo* in hairless mice. After hairless mice were exposed to UVA irradiation (300–3000 mJ/cm^2), we extracted total cell lysates from mice skin, and then detected the ErbB2 expression levels by western blot analysis. As expected, ErbB2 expression was also increased by UVA exposure. In contrast, only a slight amount of ErbB2 protein was detected in the control (Fig. 1b). Immunohistochemical analyses were then carried out to confirm this result. ErbB2 antibody-positive staining was found mainly in the plasma membrane of the epidermis (Fig. 1c). Compared to the control sample, the intensity of plasma membrane ErbB2 staining was higher in the skin tissues from UVA (3000 mJ/cm^2)-exposed hairless mice (Fig. 1c). These results show that UVA exposure induces ErbB2 in both the HaCaT cells and the skin of hairless mice.

Essential role of AP-2 in transactivation of the *erbB2* gene by UVA. The AP-2 family has been shown to play a key role in the transcriptional regulation of *erbB2*.[14,15] Our previous studies

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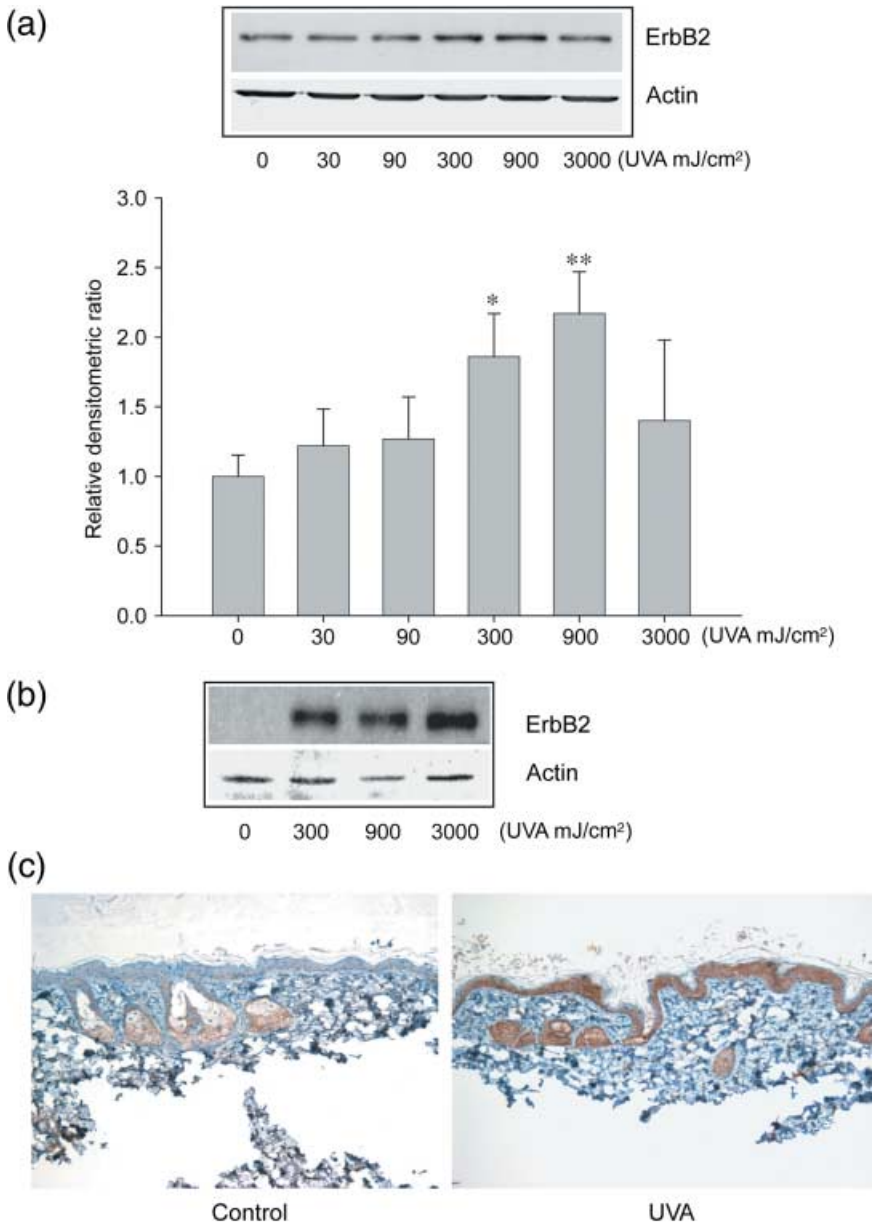

Fig. 1. Effect of ultraviolet (UV) A irradiation on the expression of ErbB2 in both HaCaT keratinocytes and the skin tissue of hairless mice. (a) Immunoblot analysis of ErbB2 in HaCaT keratinocytes. HaCaT cells were exposed to UVA (30–3000 mJ/cm^2) and the total cell lysates were obtained 24 h after UVA irradiation. Each lane was loaded with 20 μg of the cell lysates. Equal loading of the proteins was verified by actin immunoblot. Scanning densitometry was used to assess the relative change in ErbB2. The data represent the mean ± SD of three separate experiments (significant compared with the control, $*P < 0.05$, $**P < 0.01$; control level = 1). (b) Immunoblot analysis of ErbB2 in the skin tissue lysates of hairless mice. A representative immunoblot shows the ErbB2 protein in the tissue lysates of hairless mice exposed to UVA (300–3000 mJ/cm^2) (24 h). Each lane was loaded with 20 μg of the tissue lysates. The results were confirmed by repeated experiments. (c) Immunohistochemical analysis of ErbB2 in the skin tissues (×200). Hairless mice skin tissues were removed 24 h after exposure to UVA (3000 mJ/cm^2). The brown-colored staining represents plasma membrane ErbB2 expression.

showed the relationship of AP-2 transcription factors with the stimulation of *erbB2* gene transactivation in both primary cultured mammary epithelial cells and human breast cancer cells.[13,16] An Ets binding site has also been identified in the proximal promoter region of the *erbB2* gene,[17] and PEA3, a member of the Ets family of transcription factors, downregulates ErbB2 expression, allowing preferential inhibition of tumorigenesis.[18] Thus, we measured the nuclear levels of AP-2 family members and PEA3 in HaCaT keratinocytes after UVA irradiation in a time-dependent manner (1–24 h). UVA irradiation (300 mJ/cm^2) increased the nuclear level of AP-2α at 3 and 6 h, but not of AP-2β, AP-2γ, or PEA3 (Fig. 2a). These results indicate that UVA irradiation selectively causes accumulation of AP-2α in the nucleus, and suggest that AP-2α activated by UVA exposure mediates transcription of the *erbB2* gene by binding to the AP-2 binding site of the target gene.

Given the role of AP-2 in ErbB2 expression and the activation of AP-2α by UVA irradiation, we further assessed whether the *erbB2* gene was activated transcriptionally via AP-2 binding elements in HaCaT cells exposed to UVA. Reporter gene assays were carried out using HaCaT cells transfected with p756-Luc, which contains luciferase cDNA and the 756-bp *erbB2* promoter. Reporter activity was increased significantly by 90 and 300 mJ/cm^2 UVA irradiation (Fig. 2b). In the ErbB2 immunoblot experiments, 90 mJ/cm^2 UVA did not cause a significant increase in ErbB2 protein (Fig. 1a). The discrepancy of the required UVA energy for ErbB2 induction may result from the difference of experimental conditions (e.g. cell confluence or use of transfection reagents). To confirm the role of AP-2 activation in UVA-mediated transcriptional activation of the *erbB2* gene, pm756-Luc, which contains two mutated AP-2 binding sites in the p756-Luc wild-type plasmid, was used.[12,13] The enhanced reporter activity caused by UVA irradiation, as well as the basal level, was significantly decreased in pm756-Luc (Fig. 2c). These results indicate that transactivation of the *erbB2* gene induced by UVA irradiation is mediated by activation of AP-2.

Requirement for cAMP/PKA signaling in AP-2-mediated ErbB2 induction. Various extracellular stimuli can affect the activity and

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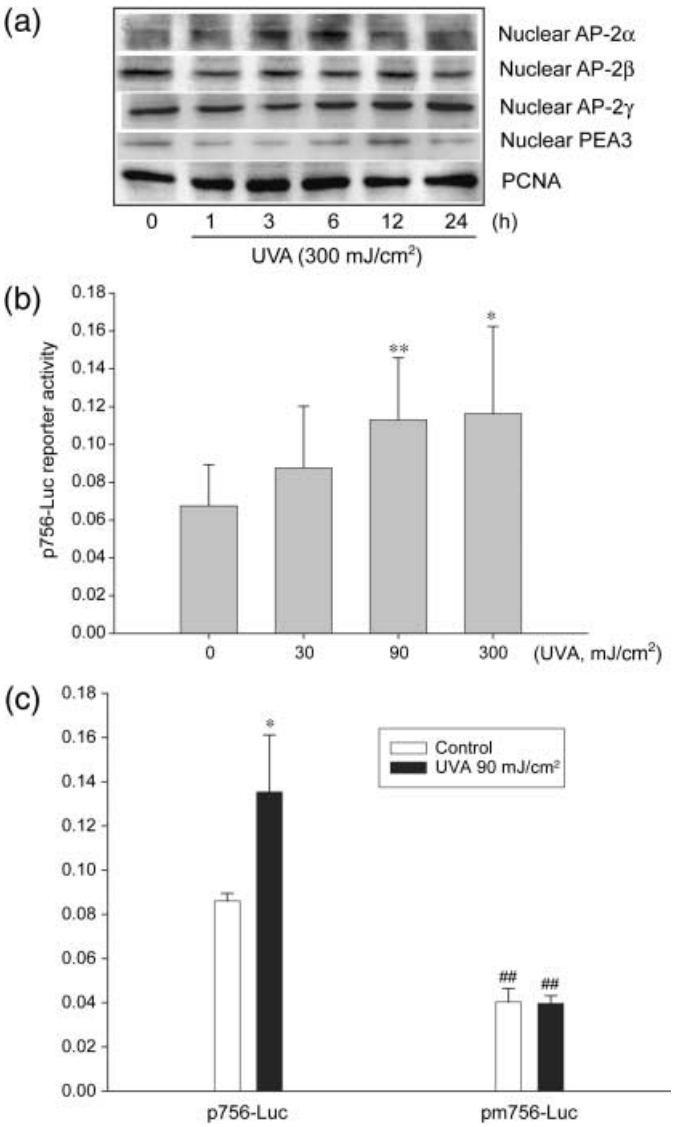

Fig. 2. Role of activator protein (AP)-2α activation in the induction of ErbB2 by ultraviolet (UV) A. (a) Western blot analyses of AP-2α, AP-2β, AP-2γ, and PEA3 in the nuclear fractions. The nuclear fractions were obtained from the HaCaT cells exposed to UVA (300 mJ/cm²) (1–24 h), and the AP-2 forms and PEA3 in each fraction were immunoblotted with the appropriate antibodies. The nuclear levels of proliferating cell nuclear antigen (PCNA) were determined as relative loading controls. The results were confirmed by repeated experiments. (b) Induction of luciferase activity by UVA in HaCaT cells transfected transiently with the human erbB2 chimeric gene construct, p756-Luc, which contained the 756-bp promoter region of the human *erbB2* gene and luciferase cDNA. A dual luciferase reporter assay was carried out on the lysed HaCaT cells cotransfected with the erbB2-luciferase gene construct, p756-Luc (firefly luciferase) and pRL-SV (*Renilla* luciferase) (ratio of 100:1) 18 h after exposure to UVA (30–300 mJ/cm²). Activation of the reporter gene was calculated as a relative change in the *Renilla* luciferase activity. The data represents the mean ± SD of five or six separate samples (significant compared with the control, $*P < 0.05$, $**P < 0.01$). (c) UVA (90 mJ/cm²)-inducible luciferase activity in the HaCaT cells transfected with pm756-Luc containing two mutated AP-2 sites in the p756-Luc reporter vector. The data represent the mean ± SD of five separate samples (significant compared with the control, $*P < 0.05$; significant compared with p756-Luc-transfected groups, $**P < 0.01$).

expression of transcription factors by regulating distinct members of the kinase family.[19] ERK, p38 kinase, and cAMP-dependent PKA are associated with activation of the AP-2 transcription factor.[20–23] Our previous study also revealed that PKA has a key role in AP-2 activation by p53 inactivation.[13] We first measured the levels of cAMP, phosphorylated ERK, and phosphorylated p38 kinase after UVA irradiation. The levels of cAMP necessary for PKA activity were increased in response to UVA (300 mJ/cm²) at early time points (30 min or 1 h) (Fig. 3a). However, UVA marginally affected the levels of phosphorylated p38 kinase and ERK (Fig. 3b). In order to investigate whether these signal transduction pathways are involved in UVA-mediated

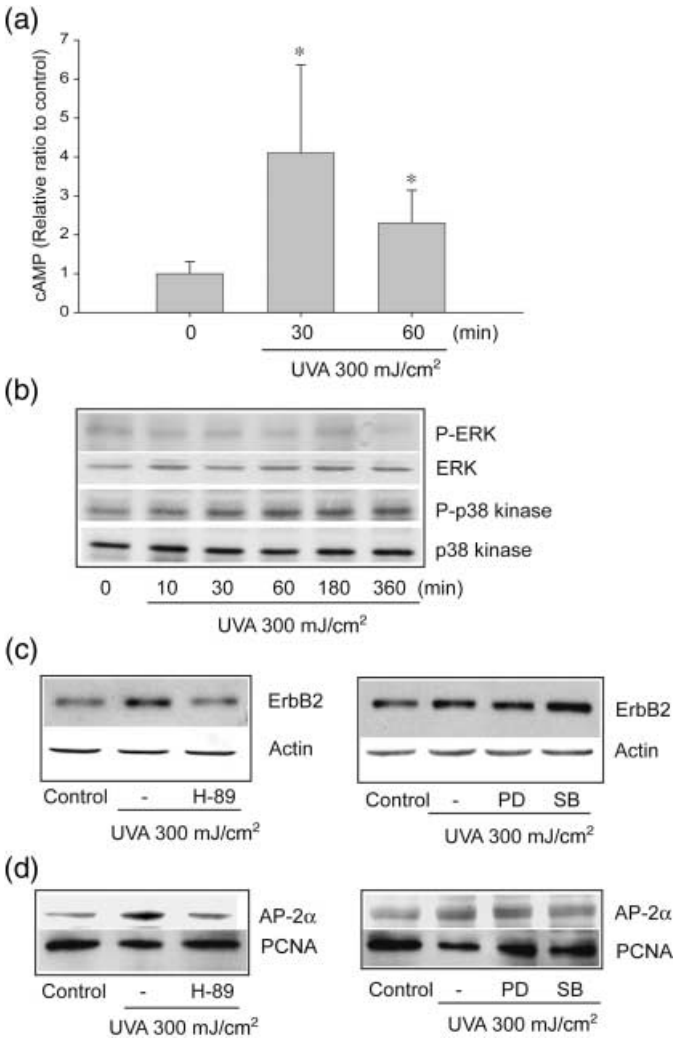

Fig. 3. Role of the cAMP–cAMP-dependent protein kinase (PKA) pathway in ultraviolet (UV) A-inducible ErbB2 expression. (a) The cellular levels of cAMP in HaCaT cells exposed to UVA (300 mJ/cm²). The cell lysates were obtained at 30 and 60 min after UVA exposure. cAMP levels were determined using an enzyme-linked immunosorbent assay kit (significant compared with control group, $*P < 0.05$). (b) The activities of extracellular signal-regulated kinase (ERK) and p38 kinase after exposing the HaCaT cells to UVA (300 mJ/cm²). The activity of ERK or p38 kinase was assessed by immunoblotting the respective phosphorylated kinase. The effects of PKA, ERK, and p38 kinase inhibitors on the UVA-mediated increase in the levels of (c) ErbB2 and (d) nuclear activator protein (AP)-2α. The HaCaT cells were preincubated with H-89 (PKA inhibitor, 10 μM), PD98059 (PD, ERK inhibitor, 30 μM) or SB203580 (SB, p38 kinase inhibitor, 20 μM) for 10 min and exposed to UVA (300 mJ/cm²). Nuclear extracts (AP-2α) or total cell lysates (ErbB2) were obtained 6 or 24 h after UVA exposure, respectively. The levels of actin and PCNA were determined as relative loading controls.

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ErbB2 induction, we carried out additional experiments using specific kinase inhibitors. Pretreatment of HaCaT cells with H-89, a specific PKA inhibitor, diminished the ErbB2 protein levels induced by UVA irradiation (Fig. 3c, left panel). In contrast, the increased ErbB2 protein levels in response to UVA were not affected by inhibition of ERK (PD98059) or p38 kinase (SB203580) (Fig. 3c, right panel). The data show that UVA-induced ErbB2 expression is dependent on the PKA signaling pathway.

We further determined the nuclear levels of AP-2α in cells pretreated with each kinase inhibitor to confirm whether cAMP–PKA is related specifically to UVA-mediated AP-2α activation. In comparison, PD98059 or SB203580 did not affect the level of AP-2α (Fig. 3d, right panel) whereas preincubation of HaCaT cells with H-89 abrogated UVA-mediated AP-2α activation (Fig. 3d, left panel). These data indicate that PKA signaling is required for AP-2α activation. Hence, UVA-induced transient activation of the cAMP–PKA signaling pathway seems to be responsible for AP-2α activation and subsequent ErbB2 induction.

Pathological role of UVA-inducible ErbB2 expression: Increase in the transforming potential. ErbB2 activation is considered an important factor contributing to malignant transformation. Soft agar colony-formation assays were carried out to examine the potential pathological implications of UVA-inducible ErbB2 overexpression during skin tumorigenesis. A typical characteristic of malignant transformed cells is anchorage-independent growth, that is, the ability to survive and form a colony in soft agar. After HaCaT cells were exposed to UVA irradiation with or without AG825, a selective inhibitor of ErbB2, the cells were incubated in soft agar plates for 14 days. We could not find significant colony sizes in control HaCaT cells, but single exposure of UVA (900 mJ/cm^2) caused clonal growth of HaCaT cells in soft agar plates (Fig. 4a,b). Moreover, UVA enhanced the

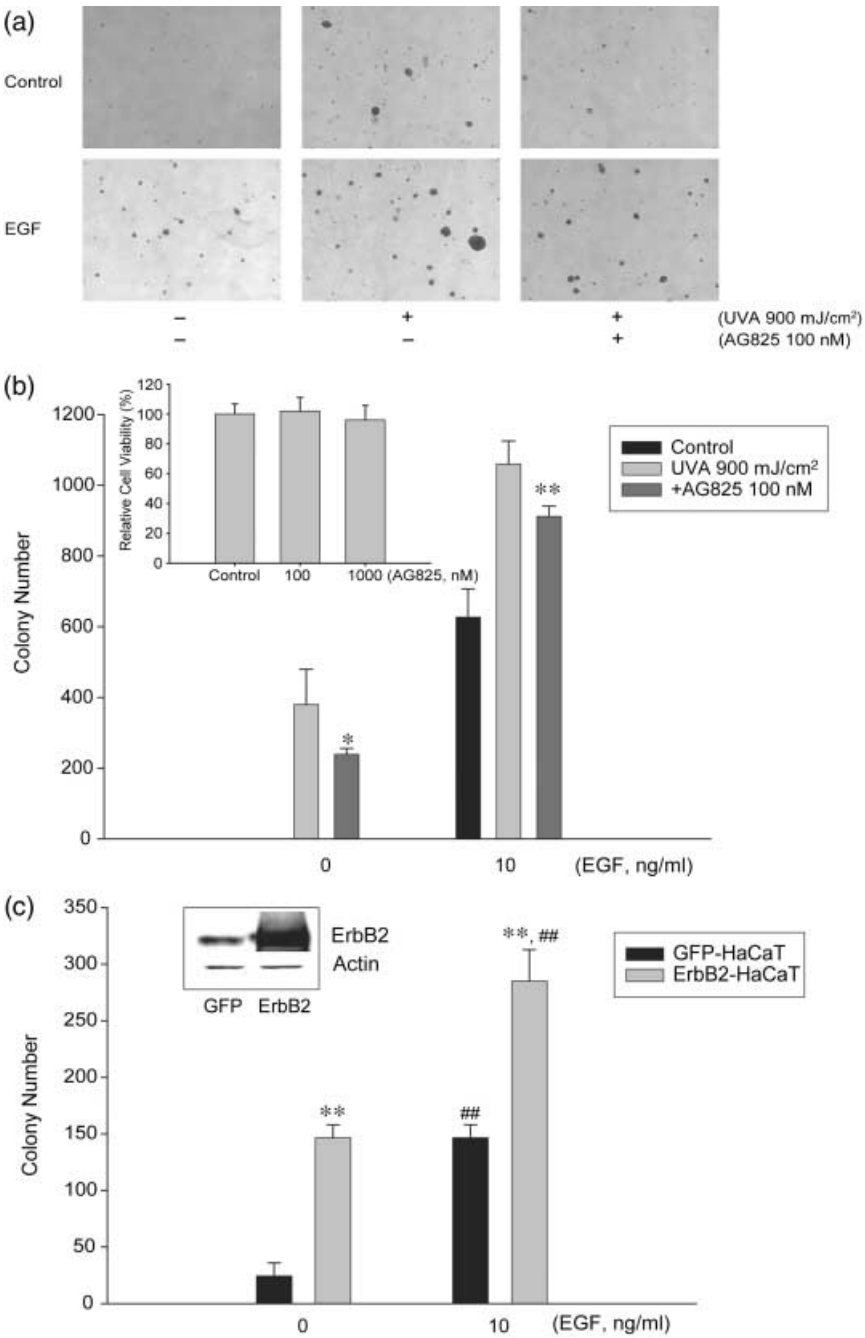

Fig. 4. Role of ErbB2 overexpression in ultraviolet (UV) A-stimulated transformation of HaCaT cells. (a) Representative figures of the colony-formation assay. HaCaT cells exposed to UVA (900 mJ/cm^2) were incubated for 14 days with or without AG825 (100 nM) and 10 ng/mL epidermal growth factor (EGF) in soft agar plates. (b) The quantitative colony numbers in each group (significant compared with UVA-exposed group, $*P < 0.05$; $**P < 0.01$). The upper insert in the figure indicates MTT assay results showing AG825 cytotoxicity. HaCaT cells were incubated with 100 nM or 1000 nM AG825 for 24 h. (c) The quantitative colony numbers in both green fluorescence protein (GFP)-overexpressing HaCaT (GFP-HaCaT) (GFP) and ErbB2-overexpressing HaCaT (ErbB2-HaCaT) (ErbB2) cells (significant compared with GFP-HaCaT cells, $**P < 0.01$; significant compared with EGF-untreated cells, $\#\# < 0.01$). The upper insert in the figure indicates the western blot result of ErbB2 protein expression in both of the cell types.

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frequency and size of the colonies promoted by EGF (10 ng/mL) stimulation (Fig. 4a,b), whereas pretreatment with AG825 (100 nM) significantly abrogated the stimulatory effect of UVA on colony formation (Fig. 4a,b). In particular, the size of colonies was decreased by AG825 treatment (Fig. 4a). To exclude the possibility that AG825 had non-specific cytotoxicity, we determined cell viability after exposure of HaCaT cells to AG825 for 24 h. AG825 in our experimental conditions (100 nM) and even at 10-fold-higher concentration (1000 nM) did not cause any cytotoxic effects in HaCaT cells (Fig. 4b, insert). Hence, the decrease in colony formation caused by AG825 seems to result from its ErbB2 inhibitory effect, but not from its non-specific cytotoxic effect. Moreover, we established HaCaT cells stably overexpressing ErbB2 using retroviral infections in order to test whether ErbB2 potentiates EGF-dependent colony formation in HaCaT cells. In comparison to GFP-HaCaT cells, the basal ErbB2 level was obviously increased in ErbB2-HaCaT cells (Fig. 4c, insert). As shown in Figure 4c, the anchorage-independent growth was enhanced in the ErbB2-HaCaT cells, compared to the GFP-HaCaT cells. These results suggest that UVA stimulates anchorage-independent growth, providing for the potential of malignant transformation in keratinocytes, and that ErbB2 overexpression by UVA exposure has a role in skin carcinogenesis.

UVA-mediated cAMP increase and AP-2α activation in skin tissues of hairless mice. To confirm that cAMP–PKA pathway-dependent AP-2α activation is upregulated in UVA-exposed hairless mice, we determined the cAMP levels in the lysates of skin tissues and compared AP-2α staining intensities using immunohistochemical analysis. The cAMP levels were 19-fold enhanced 30 min after UVA (3000 mJ/cm^2) irradiation (Fig. 5a). Immunohistochemistry showed that AP-2α antibody-positive staining was found only in the nuclei of epidermal cells (Fig. 5b). Compared to the control, the AP-2α staining intensity was higher in the skin tissues from UVA (3000 mJ/cm^2)-exposed hairless mice (Fig. 5b).

Discussion

Skin exposure to UVA or UVB is believed to be an important pathological challenge in the development of human skin cancers.[3] Although both UVA and UVB radiation can penetrate the epidermal layer of skin, UVB mainly causes direct DNA damage and subsequent mutations. Compared to UVB, the harmful effects of UVA have been regarded to be associated with either indirect DNA damage via the generation of reactive oxygen species or impairment in membrane signaling.[3] UVA irradiation persistently activates growth factor receptor tyrosine kinases, such as EGFR and its downstream kinase ERK,[4,8] which might positively correlate with skin tumor development. In contrast, pre-exposure of cells to higher-energy UVA (>1.5 J/cm^2) has been shown to ameliorate EGF-inducible ERK activation.[24] Here, we demonstrate that low-energy UVA (300–900 mJ/cm^2) irradiation causes a potent induction of ErbB2 and this activation of ErbB2 is associated with an increase in the transformation potential of HaCaT keratinocytes. Moreover, UVA-stimulated ErbB2 induction was more prominent in the skin of hairless mice; the basal ErbB2 levels in the mice skin samples were minimal, but the protein levels were enhanced drastically by UVA exposure. Compared with previous studies, we used relatively low-energy UVA (30–3000 mJ/cm^2). Thus, the data shown in the present study might more closely reflect environmental conditions.

The transcription factor AP-2 plays a key role in regulating *erbB2* gene transcription.[25,26] In the human *erbB2* gene, two putative AP-2 binding sites are involved in expression of the *erbB2* gene as potent enhancer elements.[12] Hence, the role of AP-2 in UVA-inducible ErbB2 expression was examined in both HaCaT cells and hairless mice. The present results show that the nuclear levels of AP-2α, but not of AP-2β and AP-2γ, were enhanced by UVA irradiation. It has been shown that AP-2α and

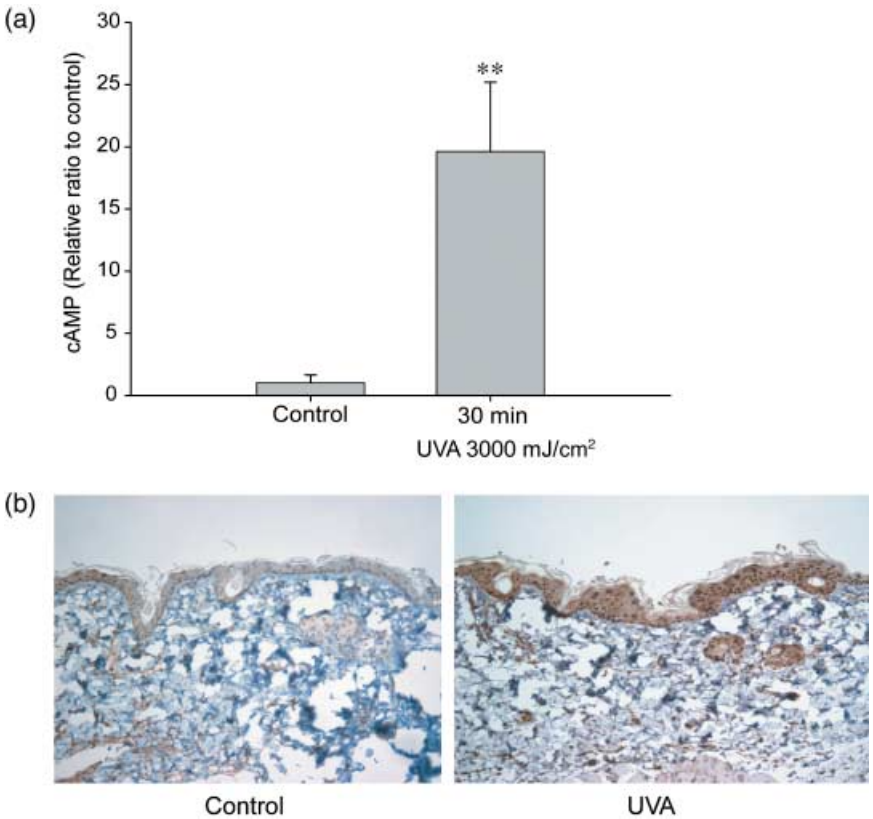

Fig. 5. Ultraviolet (UV) A-mediated cAMP increase and activator protein (AP)-2α activation in skin tissues of hairless mice. (a) cAMP levels in the tissue lysates from hairless mice exposed to UVA (3000 mJ/cm^2). The skin tissue lysates were obtained at 30 min after UVA exposure. cAMP levels were determined using an enzyme-linked immunosorbent assay kit (significant compared with control group, $^{**}P < 0.01$). (b) Immunohistochemical analysis of AP-2α in the skin tissues (×200). Hairless mice skin tissues were removed 6 h after exposure to UVA (3000 mJ/cm^2). The brown-colored staining represents nuclear ErbB2 expression.

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AP-2γ are more active than AP-2β in transcriptional activation of the *erbB2* gene.[15] Thus, increased AP-2α in the nucleus may be associated with the induction of ErbB2 in UVA-irradiated cells. Moreover, reporter gene assays using the human *erbB2* promoter and the AP-2 binding site-mutated promoter strongly suggest a key role for AP-2 activation in ErbB2 overexpression in UVA-exposed HaCaT cells. It has been also suggested that PEA3, a transcription factor of the Ets family, negatively regulates transcription of the *erbB2* gene. PEA3 specifically targets a DNA sequence near the TATA box in the *erbB2* gene promoter and downregulates the promoter activity.[18] However, nuclear levels of PEA3 were not altered by UVA in this study, which suggests that changes in PEA3 activity are not related to UVA-mediated ErbB2 induction.

A number of transcription factors are activated by distinct kinases that are triggered in response to a variety of signals.[27,28] Two distinct MAP kinases, ERK and p38 kinase, are reported to be involved in AP-2 activation.[22,29] Our recent study also revealed that cAMP–PKA is actively involved in the induction of ErbB2.[13] Here, we showed that UVA transiently enhanced cellular cAMP levels, whereas ERK or p38 kinase was not distinctly activated by UVA in our experimental conditions. We further demonstrated that the inhibition of ERK or p38 kinase did not affect AP-2-mediated ErbB2 expression in response to UVA. In contrast, PKA inhibition by H-89 led to simultaneous blocking of AP-2α accumulation in the nucleus and ErbB2 induction. Hence, activation of the cAMP–PKA signaling pathway by UVA irradiation may serve as an essential mechanism for induction of the *erbB2* gene.

One of the leading causes of skin tumors is sun exposure, specifically wavelengths in the UV region of the solar spectrum.[3] Our results revealed that single exposure to UVA enhanced the colony formation of HaCaT cells. Although multiple doses of UVA and UVB have been reported to cause malignant transformation of HaCaT cells,[30] we report for the first time that single exposure of HaCaT cells to 900 mJ/cm^2 UVA was sufficient to promote malignant transformation. Tissue-specific overexpression of wild-type ErbB2 in the basal layer of the epidermis using the keratin-5 promoter led to hyperplasia of the interfollicular epidermis and even squamous cell carcinomas.[5] We also demonstrated that stable overexpression of ErbB2 in HaCaT cells enhanced the transformation potential. In the present study, UVA-mediated ErbB2 overexpression seems to be related to an increase in the malignant transformation of HaCaT cells as AG825, a specific inhibitor of ErbB2 tyrosine kinase, significantly suppresses the colony formation induced by UVA. Although colony formation incidence was suppressed marginally by the ErbB2 inhibitor, the chemical inhibitor potently diminished the sizes of colonies. Considering clinical situations, it does not seem possible that transient ErbB2 induction from a single exposure to UVA is enough to cause malignant transformation of keratinocytes. The major difference between normal and HaCaT keratinocytes is that HaCaT cells express a mutated form of p53, the gene for which carries mutations that are characteristic of the UV signature.[31] Thus, UVA-stimulated p53 activation may not be functional in HaCaT cells, which may result in exaggerated malignant transformation in response to ErbB2 induction.

In summary, the present study provides evidence that ErbB2 expression is enhanced by UVA irradiation in both hairless mice and HaCaT keratinocytes, and this expression is mediated through the cAMP–PKA-dependent activation of AP-2α. Moreover, UVA-induced ErbB2 overexpression is involved in the malignant transformation of HaCaT cells. The results suggest that AP-2 and ErbB2 could serve as therapeutic targets against UVA-mediated skin tumorigenesis.

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References

1 Matsui MS, DeLeo VA. Longwave ultraviolet radiation and promotion of skin cancer. *Cancer Cells* 1991; **3**: 8–12.
2 Valencia A, Kochevar IE. Ultraviolet A induces apoptosis via reactive oxygen species in a model for Smith–Lemli–Opitz syndrome. *Free Radic Biol Med* 2006; **40**: 641–50.
3 Bachelor MA, Bowden GT. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Semin Cancer Biol* 2004; **14**: 131–8.
4 He YY, Huang JL, Chignell CF. Delayed and sustained activation of extracellular signal-regulated kinase in human keratinocytes by UVA: implications in carcinogenesis. *J Biol Chem* 2004; **279**: 53 867–74.
5 Kiguchi K, Bol D, Carbajal S *et al*. Constitutive expression of erbB2 in epidermis of transgenic mice results in epidermal hyperproliferation and spontaneous skin tumor development. *Oncogene* 2000; **19**: 4243–54.
6 Ahmed NU, Ueda M, Ichihashi M. Increased level of c-erbB-2/neu/HER-2 protein in cutaneous squamous cell carcinoma. *Br J Dermatol* 1997; **136**: 908–12.
7 Xian W, Rosenberg MP, DiGiovanni J. Activation of erbB2 and c-src in phorbol ester-treated mouse epidermis: possible role in mouse skin tumor promotion. *Oncogene* 1997; **14**: 1435–44.
8 Madson JG, Lynch DT, Tinkum KL, Putta SK, Hansen LA. ErbB2 regulates inflammation and proliferation in the skin after ultraviolet irradiation. *Am J Pathol* 2006; **169**: 1402–14.
9 El-Abaseri TB, Putta S, Hansen LA. Ultraviolet irradiation induces keratinocyte proliferation and epidermal hyperplasia through the activation of the epidermal growth factor receptor. *Carcinogenesis* 2006; **27**: 225–31.
10 El-Abaseri TB, Fuhrman J, Trempus C, Shendrik I, Tennant RW, Hansen LA. Chemoprevention of UV light-induced skin tumorigenesis by inhibition of the epidermal growth factor receptor. *Cancer Res* 2005; **65**: 3958–65.
11 von Montfort C, Fernau NS, Beier JI, Sies H, Klotz LO. Extracellular generation of hydrogen peroxide is responsible for activation of EGF receptor by ultraviolet A radiation. *Free Radic Biol Med* 2006; **41**: 1478–87.
12 Vernimmen D, Begon D, Salvador C, Gofflot S, Grooteclaes M, Winkler R. Identification of HTF (HER2 transcription factor) as an AP-2 (activator protein-2) transcription factor and contribution of the HTF binding site to ERBB2 gene overexpression. *Biochem J* 2003; **370**: 323–9.
13 Yang JW, Lee EY, Kang KW. ErbB2 overexpression in p53-inactivated mammary epithelial cells. *FEBS Lett* 2006; **580**: 6501–8.
14 Perissi V, Menini N, Cottone E *et al*. AP-2 transcription factors in the regulation of ERBB2 gene transcription by oestrogen. *Oncogene* 2000; **19**: 280–8.
15 Bosher JM, Totty NF, Hsuan JJ, Williams T, Hurst HC. A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* 1996; **13**: 1701–7.
16 Choi HK, Yang JW, Kang KW. Bifunctional effect of resveratrol on the expression of ErbB2 in human breast cancer cells. *Cancer Lett* 2006; **242**: 198–206.
17 Hurst HC. Update on HER-2 as a target for cancer therapy: the ERBB2 promoter and its exploitation for cancer treatment. *Breast Cancer Res* 2001; **3**: 395–8.
18 Xing X, Wang SC, Xia W *et al*. The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. *Nat Med* 2000; **6**: 189–95.
19 Treisman R. Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol* 1996; **8**: 205–15.
20 Guccione M, Silbiger S, Lei J, Neugarten J. Estradiol upregulates mesangial cell MMP-2 activity via the transcription factor AP-2. *Am J Physiol Renal Physiol* 2002; **282**: 164–9.
21 Salim S, Standifer KM, Eikenburg DC. ERK1/2 mediated transcriptional regulation of GRK3 expression in neuronal cells. *J Pharmacol Exp Ther* 2007; **321**: 51–9.
22 Tuli R, Seghatoleslami MR, Tuli S, Howard MS, Danielson KG, Tuan RS. p38 MAP kinase regulation of AP-2 binding in TGF-β1-stimulated chondrogenesis of human trabecular bone-derived cells. *Ann N Y Acad Sci* 2002; **961**: 172–7.
23 Garcia MA, Campillos M, Marina A, Valdivieso F, Vazquez J. Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation. *FEBS Lett* 1999; **444**: 27–31.
24 Maziere C, Floret S, Santus R, Morliere P, Marcheux V, Maziere JC. Impairment of the EGF signaling pathway by the oxidative stress generated with UVA. *Free Radic Biol Med* 2003; **34**: 629–36.

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25 Li M, Wang Y, Hung MC, Kannan P. Inefficient proteasomal-degradation pathway stabilizes AP-2α and activates HER-2/neu gene in breast cancer. *Int J Cancer* 2006; **118**: 802–11.
26 Pellikainen J, Naukkarinen A, Ropponen K *et al*. Expression of HER2 and its association with AP-2 in breast cancer. *Eur J Cancer* 2004; **40**: 1485–95.
27 Gupta S, Campbell D, Derijard B, Davis RJ. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 1995; **267**: 389–93.
28 Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J* 1996; **15**: 4629–42.
29 Milanini J, Viñals F, Pouysségur J, Pagès G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem* 1998; **273**: 18 165–72.
30 Gupta N, Chakrobarty A, Raman G, Banerjee G. Cloning and identification of EDD gene from ultraviolet-irradiated HaCaT cells. *Photodermatol Photoimmunol Photomed* 2006; **22**: 278–84.
31 Chouinard N, Valerie K, Rouabhia M, Huot J. UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochem J* 2002; **365**: 133–45.

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