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碩士學位論文

ErbB2 overexpression in p53-inactivated mammary epithelial cells

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ABSTRACT

ErbB2 overexpression in p53-inactivated mammary epithelial cells

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Abstract

Functional loss of p53 and ErbB2 overexpression are the frequent genetic alterations in human breast carcinomas. Here, we found that ErbB2 expression was up-regulated in primary cultured mammary epithelial cells (MECs) isolated from mice with a defect in exons 5 and 6 of the p53 gene ($p53^{\triangle 5,6}$). The reporter gene activity in the $p53^{\triangle 5,6}$ MECs transfected with the 756 bp flanking region of the *hErbB2* gene was higher than the wild type MECs. p53 inactivation selectively increased the level of AP-2α, but not AP-2β and AP-2γ and a mutation of the two AP-2 binding sites completely inhibited the reporter activity.

Key words: p53; ErbB2; AP-2α mammary epithelial cell protein kinase A

1. Introduction

Breast cancer is one of the most common malignancies in Western women. Human breast tumors arise from normal cells as a result of the accumulation of multiple mutations of tumor suppressor genes such *BRCA and p53* [1].p53 is a transcription factor that controls the cellular stress response[2,3]. A defect in the function of p53 is the most frequent genetic alteration in human tumors. Germ-line mutations of p53, which are predominantly amino acid substitutions within the DNA binding domain, have been linked to 70% of families with Li-Fraumeni syndrome. These patients have a high risk of early-onset breast cancers as well as other tumors [4,5]. Indeed, a mutation of the *p53* gene is frequently found in human breast carcinomas. Approximately30-50% of breast cancers carry a mutation in the *p53* gene and showp53 protein expression by immunohistochemistry [6].

Activational mutations of oncogenes also play a key role in breast carcinogenesis. One of the most important activational mutations involves the *ErbB2/HER-2/neu* gene, which is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors. The overexpression of ErbB2 is a common event in breast cancer[7]. An inverse relationship between the estrogen response and EGFR/ErbB2 expression in clinical breast cancer has been reported, with ErbB2 overexpression being associated with a decreased sensitivity to anti-estrogen (tamoxifen) therapy and a poor prognosis [8,9]. Herceptin (Trastuzumab®), which is anti-ErbB2 monoclonal antibody, has been shown to be active as either a single agent or in combination with chemotherapy for ErbB2-positive metastatic breast cancer [10]. Moreover, it is also useful in diminishing the level of tamoxifen resistance of breast cancer [11,12]. Hence, the expression levels of the

ErbB2 gene are closely related to successful chemotherapy against breast cancer.

Previously, two lines of mouse models of breast cancer were established by the conditional knockout of p53 genes through the CreloxP recombination system in the mammary glands of mice [13]. In this system, two lines of mice are required: one carrying the floxed p53 alleles ($p53^{fp/fp}$) and the other expressing Cre recombinase under the regulation of the whey acidic protein (WAP) promoter (WAP-Cre). Cre expression leads to a recombination within the lox P sequences placed within introns 4 and 6 of the *p53* gene, which results in a deletion of a part of the DNA binding domain (exon 5 and 6) of p53 that inactivates the gene ($p53^{\triangle 5,6}$). It was found that many characteristics of these mouse models resemble human breast cancer. In particular, the expression levels of the ErbB2 gene were obviously increased in the mammary tumor lysates obtained from the $p53^{\triangle 5,6}$ mice.[13] In clinical studies, ErbB2 overexpression in breast cancer tissues, which is associated with increased proliferation, is often observed in tumors with p53 alterations [14].

Despite both p53 and ErbB2 being crucial factors for the pathological progress of breast cancer, the precise role of p53 in ErbB2 expression during mammary tumorigenesis is unclear. This paper shows for the first time that ErbB2 expression is up-regulated in mammary epithelial cells (MEC) from $p53^{\triangle 5,6}$ mice, in which the induction of AP-2α through the sustained activation of protein kinase A (PKA) is a key step.

2. Materials and methods

2.1. Materials

U0126 was supplied by Cell Signaling Technology (Beverly, MA). The anti-AP-2β and AP-2γ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against AP-2α, p53 and ErbB2 were obtained from Upstate Inc. (Charlottesville, VA), Novocastra Laboratory Ltd (Newcastle, UK) and DakoCytomation (Glostrup, Denmark), respectively. The alkaline phosphatase and horseradish peroxidase-conjugated donkey anti-mouse, anti-rabbit and anti-goat IgGs were acquired from Jackson ImmunoResearch (West Grove, PA). The 5-Bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium and pRL-SV40 plasmid were purchased from Promega (Madison, WI). The H-89 was supplied by Calbiochem (La Jolla, CA). The anti-actin antibody and the other reagents in the molecular studies were obtained from Sigma Chemical (St. Louis, MO). The plasmids, p756-Luc construct containing 756 bp in the hErbB2-promoter region and the AP-2 binding site mutated p756dm-Luc construct were kindly provided by Dr. Winkler R. (University of Liege, Belgium) [15].

2.2. Generation of WAP-Cre $p53^{fp/fp}$ mice and mammary epithelial cell (MEC) culture

WAP-Cre mice were mated with $p53^{fp/fp}$ mice, and the Cre mediated recombination and p53 deletion were confirmed by PCR analysis and x-gal staining, as published in previous report [13]. The MECs were isolated from the number 4 mammary gland from the 6-month-old $p53^{fp/fp}$ or WAP-Cre $p53^{fp/fp}$ mice using a slight modification of a previously published method [16]. After washing with

sterile PBS, the mammary gland was minced gently with two knives and dissociated in the presence of 0.15% collagenase for 12 h at 37C. The cells was collected and embedded in a fetuin-coated plastic dish and maintained in F-12/DMEM containing 10 ng/ml epidermal growth factor (EGF), 1 μg/ml insulin, 15% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37C in a humidified atmosphere containing 5% CO_2. The cells used for the experiments were from up to 10 passages .

2.3. Preparation of nuclear fraction

The nuclear extracts were prepared according to the method reported by Schreiber et al [17]. Briefly, the cells in dishes were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after adding 100 μl of a 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 for 10 min on ice and centrifuged at 7,200*g* for 5 min. The pellets containing the crude nuclei were resuspended in 100 μl of an 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 in order to obtain the supernatants containing the nuclear extracts. The nuclear extracts were stored at -80C until needed.

2.4. Immunoblot analysis

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to the procedures

reported in the literature [18]. The cells were lysed in a 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 -glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000*g* for 10 min to remove the debris. The proteins were fractionated using a 10% separating gel. Briefly, the fractionated proteins were transferred electrophoretically to nitrocellulose paper. The proteins were immunoblotted with each specific antibody. The secondary antibodies used were horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibody. The nitrocellulose paper was developed using 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium chloride or developed using an ECL chemiluminescence system.

2.5. Reporter gene assay

A dual-luciferase reporter assay system (Promega, Madison, WI) was usedto determine the promoter activity. Briefly, the cells were transiently transfected with 1 μg of p756-Luc, p756m-Luc or p53-Luc (Stratagene, La Jolla, CA) plasmid and 20 ng of pRL-SV plasmid (*Renilla* luciferase expression for normalization) (Promega, Madison, WI) using the Genejuice® Reagent (Novagen, Madison, WI). The firefly and *Renilla* luciferase activities in the cell lysates were measured using a luminometer (Turner Designs; TD-20, CA). The relative luciferase activity was calculated by normalizing the ErbB2 promoter-driven firefly luciferase activity to that of *Renilla* luciferase.

2.6. Gel Shift Analysis

A double stranded oligonucleotide containing the putative AP-2 binding site was used for gel shift analysis after end-labeling the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The promoter region of the *ErbB2* gene contains a putative AP-2 binding site, which induces the strong activation of *ErbB2* gene transcription in BT-474 cells.[15] The sequences of the AP-2 consensus oligonucleotide was 5'-GAGAACGGCTGCAGGCAACCCAGG-3'. The reaction mixtures contained 4 μl of a 5 ×binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC and 50 mM Tris-Cl (pH 7.5), 10 g of nuclear extracts and sterile water in a total volume of 20 l. The reaction mixtures were preincubated for 10 min. The DNA-binding reactions were carried out at room temperature for 20 min after adding a 1 μl probe (10^6 cpm). The specificity of binding was determined using competition experiments, which were carried out by adding a 20-fold excess of the unlabeled AP-2 oligonucleotide to the reaction mixture before the DNA-binding reaction. For the immuno-inhibition assay, the antibodies against AP-2α or AP-2γ (1 μg each) were added to the reaction mixture. The samples were loaded onto 4 % polyacrylamide gels at 100 V. The gels were removed and dried, and imaged by autoradiography.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated using total RNA isolation kit (RNAgents®, Promega, Madison, WI). The total RNA (1.0 μg) obtained from the cells was reverse-transcribed using an oligo(dT) 18mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for the mouse AP-2α and S16 ribosomal protein (S16r) genes. The PCRs

were carried out for 35 cycles using the following conditions: denaturation at 98C for 10 sec, annealing at 50C for 0.5 min, and elongation at 72C for 1 min. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

2.8. Data analysis

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

3. **Results**

3.1. Enhanced expression of ErbB2 in p53-inactivated MEC

The MEC used were isolated from the 6 months old $p53^{fp/fp}$ or WAP-Cre $p53^{fp/fp}$ mice. The presence of loxP sites in introns 4 and 6 did not interfere with the transcription of p53, and the full-length p53 protein was detected in thedoxorubicin (3 or 10 μM)-treated $p53^{fp/fp}$ MECs (Fig. 1A). A smaller protein product with theexpected mass of 39 kDa, which was designated $p53^{5,6}$ by Cre recombination was detected in the WAP-Cre $p53^{fp/fp}$ MECs (Fig. 1A)[13]. After exposing the cells to doxorubicin (1 or 3 μM) for 18 h, the p53-dependent reporter transcriptionwas concentration-dependently increased in the wild type $p53^{fp/fp}$ MECs transiently transfected with the p53-Luc reporter plasmid (Fig. 1B). In contrast, $p53^{\triangle 5,6}$ is transcriptionally inactive and the p53-reporter activity was lower in the $p53^{\triangle 5,6}$MECs. This shows that p53 in the $p53^{\triangle 5,6}$ MECs was truncated at the DNA binding site of p53 (exon 5 and 6) and was transcriptionally inactive.

The ErbB2 expression levels in both $p53^{fp/fp}$ MEC and $p53^{\triangle 5,6}$ MEC were then compared. The basal expression level of ErbB2 in the $p53^{\triangle 5,6}$ MEC was 3.5 times higher than that of the $p53^{fp/fp}$ MEC (Fig. 1C). The basal tyrosine phosphorylation intensity of ErbB2 in both cells was compared in order to further understand the functional activity of ErbB2 overexpression in the p53-inactivated MEC. The tyrosine 1248 or 877 residue of ErbB2 is an important ErbB2 autophosphorylation site [19,20] and there is a correlation between the extent of phosphorylation and the ErbB2 tyrosine kinase activity. As shown in Fig. 1D, the phosphorylation of the tyrosine 877 and 1248 residues in ErbB2 was also higher in the $p53^{\triangle 5,6}$ MEC compared with those in the $p53^{fp/fp}$

MEC. These results show that ErbB2 expression is induced in the p53-inactivated MEC, which might be involved in the increased activity of ErbB2.

Because primary cultured MECs isolated from p53-inactivated mice was used in this study, it is possible that the enhanced ErbB2 expression in $p53^{\triangle 5,6}$ MEC is the result of additional genetic mutations during the growth period of the mice. The adenovirus overexpressing Cre recombinase gene (Ad-Cre) was introduced to the $p53^{fp/fp}$ MECsin order to directly remove exons 5 and 6 out of the *p53* gene. The truncated p53 band was found in the Ad-Cre-treated $p53^{fp/fp}$ MEC, but not in β-galactosidase expressing adenovirus (Ad-gal)-treated $p53^{fp/fp}$ MECs. This result reveals that infecting the cells with Ad-Cre efficiently blocks the transcriptional function of p53 (Fig. 2A).

Further experiments were performed to determine if *in vitro* p53 inactivation affected the expression of ErbB2 in MECs. Exposing the $p53^{fp/fp}$ MECs to Ad-Cre for 24 or 48 h resulted in a significant increase in the ErbB2 protein level (Fig. 2B). In contrast, the ectopic expression of the p53 gene by Ad-p53 caused a concentration-dependent decrease in the ErbB2 protein levels in $p53^{\triangle 5,6}$ MEC (Fig. 2C). This suggests that ErbB2 expression is directly associated with p53 activity in MECs.

3.2. Requirement of AP-2α induction for the overexpression of ErbB2 in p53-inactivated MEC

We then focused on how p53 inactivation causes ErbB2 induction in MECs. A family of AP-2 transcription factors plays a key role in transducing theErbB2 induction signal [21,22]. In the *ErbB2* gene, Vernimmen et al. [15] reported that two putative AP-2 binding sites regulate ErbB2 gene expression at the level of the potent enhancer elements. Therefore, it was hypothesized that the p53 status is associated

with the activation or expression of the AP-2 transcription factor(s). The nuclear extracts isolated from the $p53^{\triangle 5,6}$ MEC or $p53^{fp/fp}$ MEC were probed with the radiolabeled AP-2 putative binding sequence to determine if the nuclear AP-2 binding proteins are activated in the p53-inactivated MEC. The band of the slow migrating complex was higher in the samples from the $p53^{\triangle 5,6}$ MEC than in thewild-type MEC (Fig. 3A, left panel). Competition experiments using an excess (20X) of the unlabeled AP-2 oligonucleotides confirmed that the increased band resulted from the enhanced binding of the nuclear AP-2 proteins (Fig. 3A, left panel). Among the AP-2 family of transcription factors, AP-2α and AP-2γ can bind to the ErbB2 promoter as either homo- or heterodimers. In addition, both AP-2α and AP-2γare expressed at higher levels in most ErbB2 overexpressing mammary tumor cells [21]. Immunodepletion experiments were carried out with the nuclear extracts isolated from the $p53^{\triangle 5,6}$ MEC in order to clarify the component(s) responsible for the enhanced binding. The addition of anti-AP-2α IgG to the reaction mixture depleted the enhanced DNA binding and caused a supershifted band (Fig. 3A, right panel). However, the band intensity was minimally affected by the addition of antibodies against AP-2γ(Fig. 3A, right panel). This suggests that AP-2α interactswith the AP-2 binding sequence in the ErbB2 promoter region.

The increases in the band intensity obtained from the gel shift analyses might be the result of the nuclear migration or the enhanced expression of the AP-2 transcription factors. Therefore, the AP-2α, AP-2β and AP-2γ expression levels in cell lysates from both $p53^{\triangle 5,6}$ MEC and $p53^{fp/fp}$ MEC were determined. The levels of AP-2α, but not AP-2β or AP-2γ in thecell lysates were significantly higher in the $p53^{\triangle 5,6}$ MEC compared with $p53^{fp/fp}$MEC (Fig. 3B). Moreover, the nuclear and mRNA levels of AP-2α in $p53^{\triangle 5,6}$ MEC were also higher than that

in the $p53^{fp/fp}$ MEC (Fig. 3B). In order to confirm these results, the AP-2α levels in thecell lysates were determined after infecting $p53^{fp/fp}$MEC with Ad-Cre. The AP-2α expression level was increased by *in vitro* p53 inactivation (Fig. 3C). This shows that the selective induction of AP-2α and the subsequent nuclear accumulation of AP-2α in the p53-inactivated MEC might be an essential component for the specific binding complex in the promoter region of the *ErbB2* gene.

Given the role of AP-2 in ErbB2 expression and the activation of AP-2α in the p53 inactivated MEC, this study next evaluated whether or not the *ErbB2* gene was transcriptionally activated via the AP-2 binding elements in the $p53^{\triangle 5,6}$ MEC. Reporter gene assays were performed using the MEC transfected with p756-Luc, which contain the luciferase cDNA and 756 bp ErbB2 promoter. The luciferase activity in the $p53^{\triangle 5,6}$ MEC was approximately 7 times higher than that in the $p53^{fp/fp}$ MEC (Fig. 3D, left panel). pm756-Luc, which contain two mutated AP-2 sites in the p756-Luc reporter vector (pm756-Luc), was used to confirm the contribution of AP-2 activation to ErbB2 transactivation in p53-inactivated MEC [15]. After transfection with pm756-Luc, the enhanced reporter activity in $p53^{\triangle 5,6}$ MEC was significantly diminished (Fig. 3D, left panel). Moreover, infection of $p53^{fp/fp}$ MEC with Ad-Cre significantly increased p756 reporter activity (Fig. 3D, right panel). These results confirmedthat the selective induction of AP-2α in the $p53^{\triangle 5,6}$ MEC was essential for the upregulation of the *ErbB2* gene.

3.3. Role of PKA in AP-2α-mediated ErbB2 induction in p53-inactivated MEC

The activities or expression levels of most transcription factors are regulated by distinct members of the kinase family, which aretriggered in response to a variety of stimuli [23]. Extracellular signal-regulated

kinase (ERK), p38 kinase and protein kinase A (PKA) are involved in the activation of AP-2 [24-26]. Therefore, experiments were carried out to determine if the ERK, p38 kinase or PKA pathway is associated with the induction of ErbB2 in p53-inactivated MEC. The incubation of cells with H-89 (10 μM), a specific PKA inhibitor for 24 h completely suppressed ErbB2 expression in the $p53^{\triangle 5,6}$ MEC (Fig. 4A). On the other hand, treatment of the cells with U0126 (10 μM, ERK inhibitor) or SB203580 (10 μM, a p38 kinase inhibitor) had no affect on the levels of the ErbB2 protein (Fig. 4A).

The AP-2α levels were monitored in the $p53^{\triangle 5,6}$ MEC exposed to each kinase inhibitor in order to identify the possible relationship between the cellular kinase cascade and the activation of AP-2α as a transcription factor(s)required for the induction of ErbB2 in the p53-inactivated MEC. Treatment of the $p53^{\triangle 5,6}$ MEC with H-89 inhibited the expression of AP-2α, while U0126 or SB203580 did not affect the expression of AP-2α (Fig. 4B). Stimulation of the cAMP/PKA pathway caused an increase in the AP-2 mRNA levels in the hippocampus [27], which suggests that the increased AP-2α transcription in $p53^{\triangle 5,6}$ MEC results from PKA activation. These results show that the PKA pathway plays a key role in the activation of AP-2α-dependent *ErbB2* gene expression in the p53-inactivated MEC.

It is unclear if p53 inactivation affects the PKA activity in MEC. The activity of PKA was assessed using Profluor® PKA assay kit (Promega, Madison, WI). The basal activity of PKA was 5 times higher in the $p53^{\triangle 5,6}$ MEC than in the $p53^{fp/fp}$ MEC (Fig. 4C). This shows that PKA is continuously activated in p53 inactivated MEC.

4. Discussion

p53 is a key tumor-suppressor gene that is mutated or lost in approximately 50% of all human cancer cases worldwide[28]. A variety of studies have revealed that p53 exerts its various functions mainly by regulating gene expression of its target genes through a consensus DNA-binding site. Most p53 target genes are involved in mediating cell-cycle arrest and apoptosis. Thus, dysfunction of p53 results in uncontrlolled cell proliferation and ultimately stimulates carcinogenesis [29]. ErbB2 as a member of the EGFR family of tyrosine kinases, causes the proliferation, angiogenesis and metastasis of cancer cells via the EGF-related pathways [30]. ErbB2 is over-expressed in 2530% of primary breast cancers and is associated with a shorter survival, higher recurrence rate and a lower response to chemotherapy and hormone therapy [31]. In the immunostaining analyses using clinical breast cancer tissues, ErbB2 protein overexpression was frequently found with p53 altered expression [32,33]. In addition, double immunohistochemical staining results showed p53 and ErbB2 immunoreactivity in the same cells [33]. Hence, it would be plausible to hypothesize that p53 function regulates the expression of ErbB2.

We previously generated two different mammary gland-specific p53 knock-out mice and about two-thirds of tumors from *p53-inactivated* mice showed ErbB2 overexpression by immunostaining and Western blot analysis using anti-ErbB2 and phosphotyrosine antibodies [13]. In the present study, we showed that ErbB2 was up-regulated in p53-inactivated MECs. The data defines a novel regulatory loop between p53 as a tumor suppressor and ErbB2 as a oncogene in MEC. Considering pathophysiological condition during

breast carcinogenesis, p53 inactivation leads to induction of ErbB2, which subsequently may cause estrogen-independent growth of mammary epithelial tissue through exaggerated EGF pathway activation.

Because there are no putative binding site(s) for p53 in the promoter region of *ErbB2* gene, p53 seems not to directly control transactivation of *ErbB2* gene. The transcription factor, AP-2, plays a keyrole in regulating *erbB2* gene transcription [22,34]. In the human *ErbB2* gene, two putative AP-2 binding sites are involved in the expression of the *ErbB2* gene at the level of the potent enhancer elements [15]. Therefore, the role of AP-2 in ErbB2 expression in $p53^{5,6}$ MEC was examined. The present gel-shift assay data support the notion that p53 truncation in MEC activated the AP-2 binding proteins consisting AP-2α, and transcriptionally stimulated *ErbB2* gene expression. The expression levels of AP-2α, but not AP-2β and AP-2γ were selectively enhanced in the p53-inactivated MEC, which combined with the luciferase-reporter gene and promoter mutation assays strongly supports the essential role of AP-2α in the overexpression of ErbB2 in $p53^{5,6}$ MEC. It has been also known that PEA3, a transcription factor in Ets family, negatively regulates transcription of *ErbB2* gene. It specifically targets a DNA sequence near the TATA box on the *ErbB2* gene promoter and down-regulatesthe promoter activity [35]. The protein levels of PEA3 in total cell lysates were not diminished, rather increased in p53-inactivated MEC (data not shown). These results suggest that PEA3 is not involved in the ErbB2 inductionin p53-inactivated MEC.

Despite the identification of the essential transcriptional factor, AP-2 which binds to the ErbB2 promoter, the cellular signaling pathway for the induction of ErbB2 has not been clearly defined. It has

beenreported that the activation of two distinct MAP kinases, ERK and p38 kinase is involved in the process of AP-2 activation [24,25]. Inhibition of ERK or p38 kinase did not reduce the AP-2α-mediated ErbB2 expression in $p53^{\triangle 5,6}$ MEC. Hence, these kinases are unlikely to be responsible for the induction of ErbB2 by p53 inactivation. PKA has also been shown to increase both the activity and the expression of AP-2 [26,27]. Whether p53 inactivation led to activation of PKA was assessed in the present study to address the physiological significance of this kinase. Here, we showed that basal PKA activity was persistently enhanced in $p53^{\triangle 5,6}$MEC. This was further supported by the observation that inhibition of PKA by H-89 led to simultaneous blocking of AP-2αand ErbB2 protein increase. Thus, signaling cascades involving the PKA activation in p53-inactivated MEC may serve as an essential mechanism for the induction of *ErbB2* gene.

In summary, thepresent study provides strong evidence that the ErbB2 expression level is higher in the p53-inactivated MEC through the selective upregulation of AP-2α, and that the activation of PKA may be a crucial pathway for AP-2α-dependent ErbB2 expression.

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5. Figure Legends

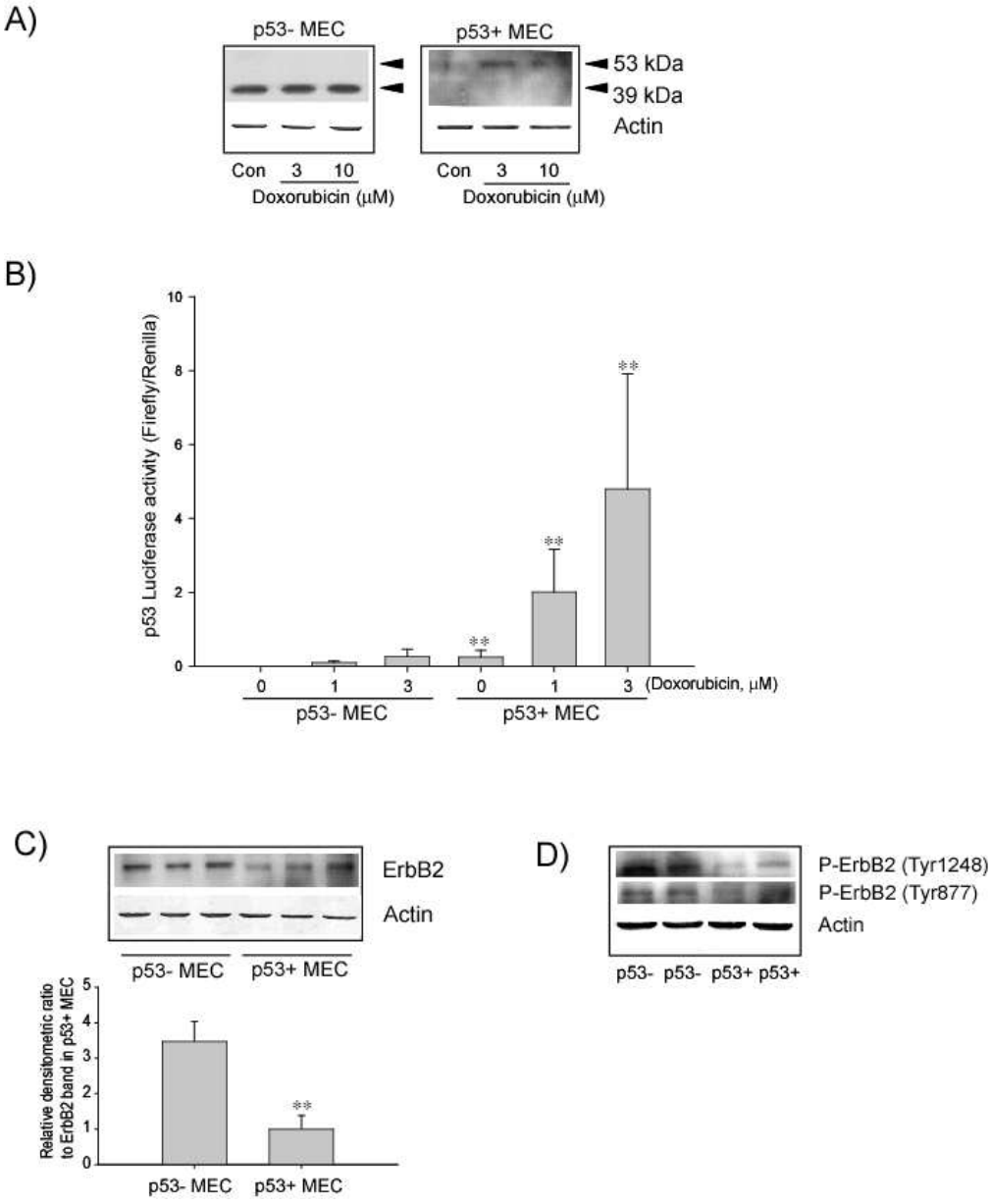

Fig. 1. Effect of p53 inactivation on the expression of ErbB2 in mammary epithelial cells (MEC). (A) Immunoblot analysis of p53. A representative immunoblot shows the p53 protein in both p53■5,6 MEC (p53- MEC) and $p53^{fp/fp}$ MEC (p53+ MEC) incubated with doxorubicin (3 and 10 μM) for 18 h. Each lane was loaded with 20 g of the cell lysates. An equal loading of the proteins was verified by actin immunoblot. Scanning densitometry was used to assess the relative change in ErbB2. (B) p53 reporter gene analysis. Luciferase activities in both p53- MEC and p53+ MEC transiently transfected with the p53-Luc plasmid, which contains repeated p53 binding sequences, was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on the lysed cells that had been co-transfected with the p53-Luc (firefly luciferase) and pRL-SV (*Renilla* luciferase)(a ratio of 100:1) after exposure to doxorubicin (1 or 3 μM) for 18 h. The activation of the reporter gene was calculated as a relative change in the *Renilla* luciferase activity. The data represents the mean SD of 3-4 separate experiments (significant compared with the p53- MEC, **$p<0.01$). (C) Immunoblot analysis of ErbB2. Each lane was loaded with 10 g of the cell lysates. Scanning densitometry was used to assess the relative change in ErbB2. The data represents the mean SD of 3 separate samples (significant compared with the p53- MEC, **$p<0.01$). (D) Immunoblot analysis of phosphorylated ErbB2 (Tyr-1248 and Tyr-877 phosphorylation).

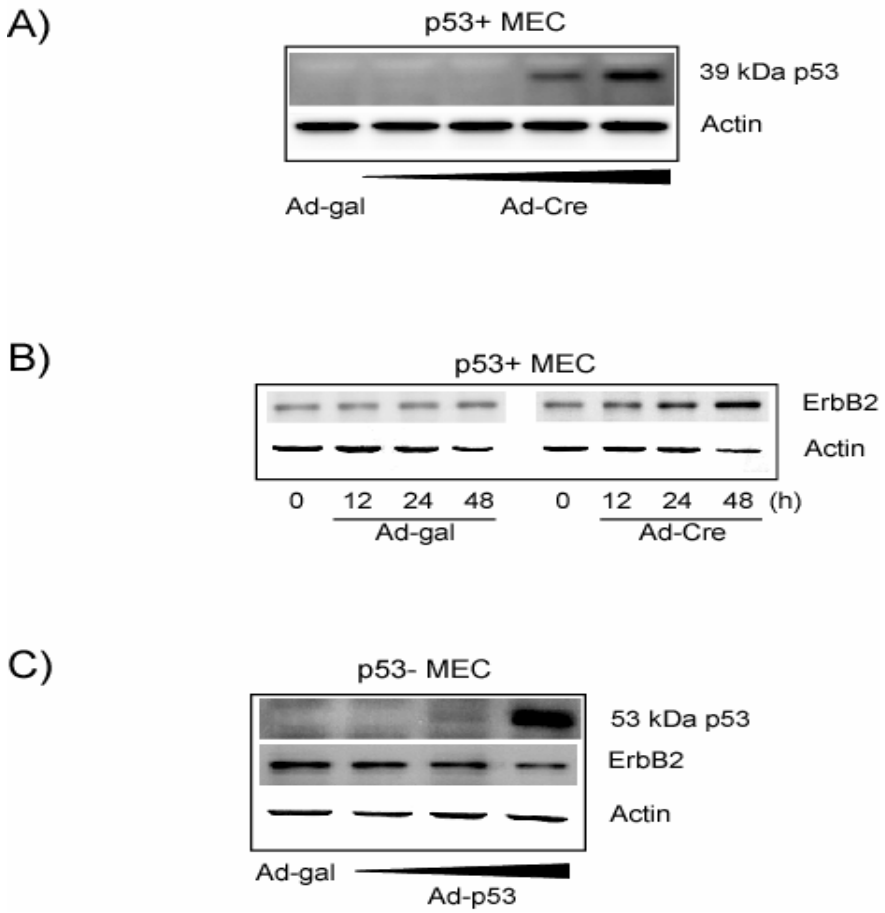

Fig. 2. Induction of ErbB2 in MEC by *in vitro* p53 inactivation. (A) Immunoblot analysis of p53 after Cre expressing adenovirus (Ad-Cre) exposure to $p53^{fp/fp}$ MEC (p53+ MEC) (24 h). β-galactisidase expressing adenovirus (Ad-gal) was used for a mock infection purpose. (B) Immunoblot analysis of ErbB2 after Ad-Cre or Ad-gal exposure to p53+ MEC for 12-48 h. (C) Immunoblot analyses of p53 and ErbB2 after Ad-Cre or Ad-gal exposure to p53+ MEC (24 h).

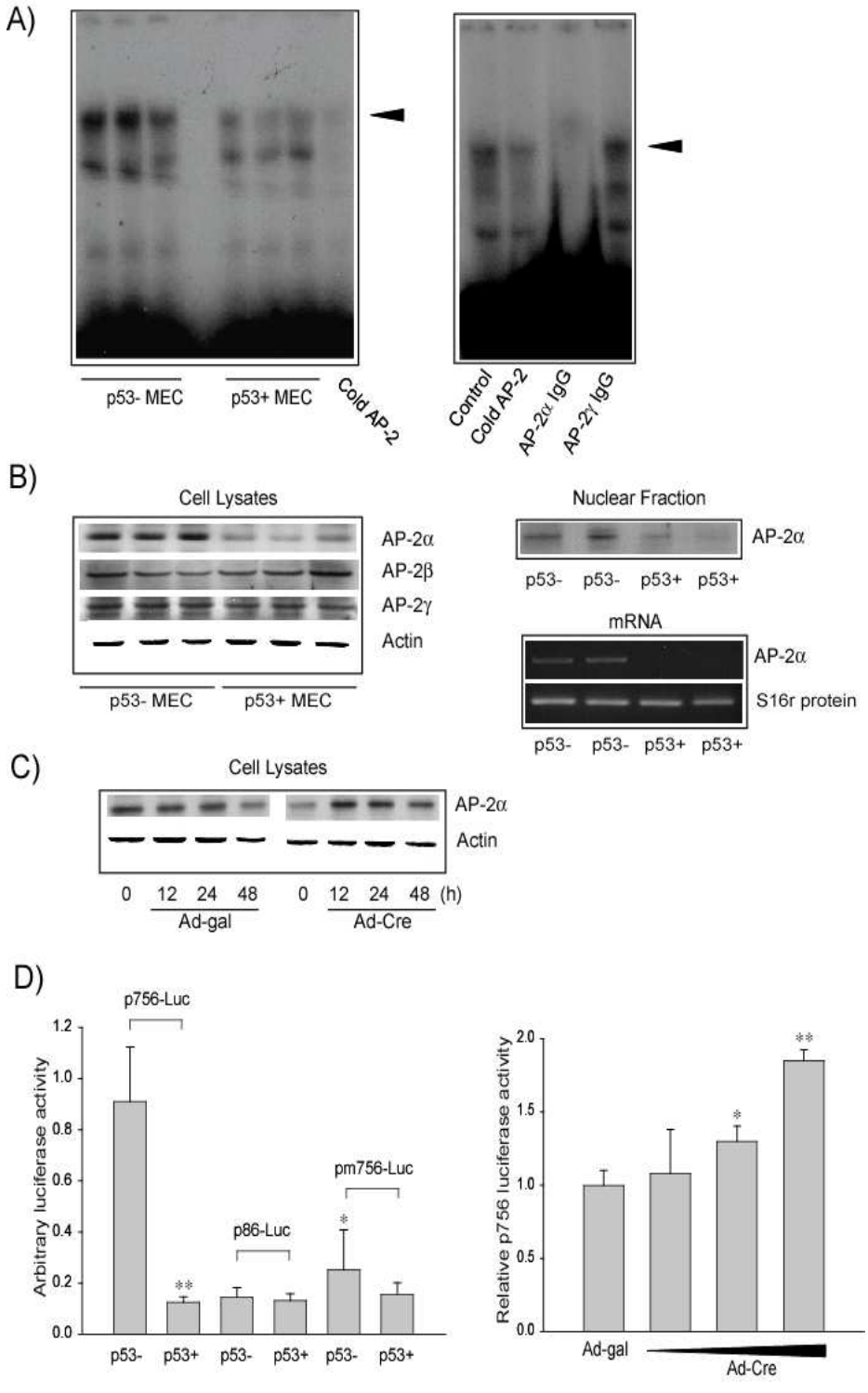
A)
p53- MEC
p53+ MEC
Cold AP-2
Control
Cold AP-2
AP-2α IgG
AP-2γ IgG
B)
Cell Lysates
AP-2α
AP-2β
AP-2γ
Actin
p53- MEC
p53+ MEC
Nuclear Fraction
AP-2α
p53- p53- p53+ p53+
mRNA
AP-2α
S16r protein
p53- p53- p53+ p53+
C)
Cell Lysates
AP-2α
Actin
0 12 24 48 0 12 24 48 (h)
Ad-gal
Ad-Cre
D)
p756-Luc
p86-Luc
pm756-Luc
Arbitrary luciferase activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
p53- p53+ p53- p53+ p53- p53+
Relative p756 luciferase activity
2.0
1.5
1.0
0.5
0.0
*
**
Ad-gal
Ad-Cre

Fig. 3. Role of AP-2α activation in ErbB2 expression. (A) Left panel; Gel shift analysis of the AP-2 transcription complex. Nuclear extracts were prepared from both p53$^{\blacksquare 5,6}$ MEC (p53- MEC) and p53$^{fp/fp}$ MEC (p53+ MEC). All lanes contained 8 μg of nuclear extracts and 5 ng of the labeled AP-2 consensus sequence. Competition studies were carried out by adding a 20-fold excess of unlabeled AP-2 consensus sequence. Right panel; Immunodepletion studies were carried out by adding an AP-2α or AP-2γ antibody (1 μg) to the nuclear extracts of p53- MEC. (B) AP-2 expression changes in both p53- MEC and p53+ MEC. Left panel; Immunoblot analysis of AP-2α, AP-2β or AP-2γ in both p53- MEC and p53+ MEC. Right panel; Nuclear levels and mRNA levels of AP-2α in both p53- MEC and p53+ MEC. Nuclear AP-2α was detected immunochemically with specific antibody. The AP-2αmRNA expression levels were determined by RT-PCR analysis. The mRNA expression of the S16 ribosomal protein was comparable among the samples. (C) Immunoblot analysis of AP-2α after Ad-Cre or Ad-gal exposure to p53+ MEC for 12-48 h. (D) Analysis of AP-2-responsive element in the ErbB2 gene promoter. Left panel; Induction of luciferase activity in p53- MEC transiently transfected with the ErbB2 chimeric gene construct, p756-Luc, which contained the 756 bp promoter region of the *ErbB2* gene and luciferase cDNA. A dual luciferase reporter assay was performed as in panel (B) of Fig. 1. p86-Luc was used as a control vector containing the minimal promoter region. pm756-Luc contains mutated two AP-2 sites in the p756-Luc reporter vector. The data represents the mean S.D. of 4 separate experiments (significant compared with the value from p53- MEC transiently transfected with p756-Luc plasmid, *$p<0.05$; **$p<0.01$). Right panel: p756-Luc reporter activity in p53+ MEC infected with Ad-Cre. (significant compared with the Ad-gal treated group, *$p<0.05$; **$p<0.01$).

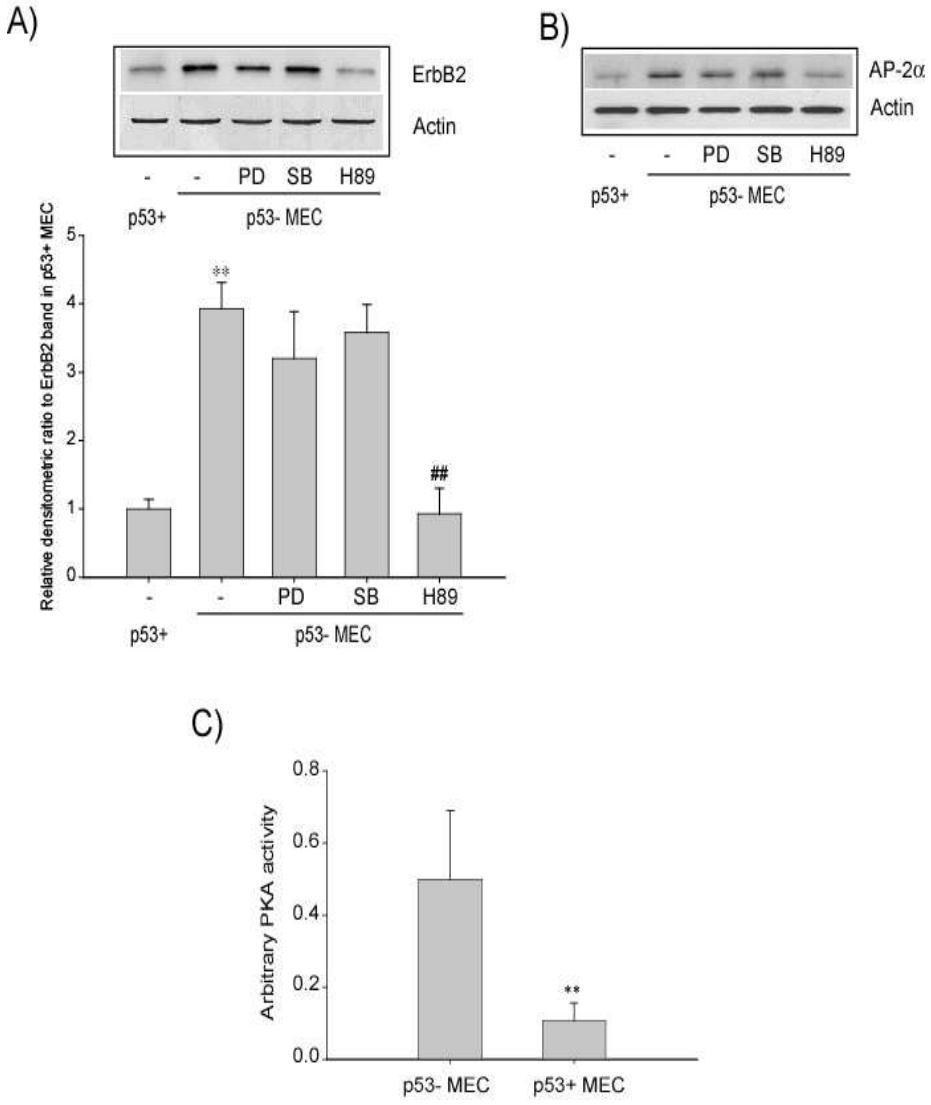
A)
ErbB2
Actin
- - PD SB H89
p53+
p53- MEC
Relative densitometric ratio to ErbB2 band in p53+ MEC
0
1
2
3
4
5
**
##
B)
AP-2α
Actin
C)
Arbitrary PKA activity
0.0
0.2
0.4
0.6
0.8
p53- MEC
p53+ MEC

Fig. 4. Effects of thechemical inhibitors of ERK, p38 kinase and PKA on the induction of ErbB2 in p53-inactivated MEC. (A) The effects of ERK, p38 kinase and PKA inhibitors on the increase in the ErbB2 level. Both the p53■5,6 MEC (p53- MEC) and p53fp/fp MEC (p53+ MEC) were incubated with PD98059 (PD, 50? μM), SB203580 (SB, 10 μM) or H-89 (H89, 10 μM) for 24 h and the levels of ErbB2 were immunochemically assessed. Scanning densitometry was used to assess the relative change in ErbB2. The data represents the mean SD of 3 separate experiments (significant compared with the p53+ MEC, **p<0.01 significant compared with the p53- MEC, ##p<0.01). (B) The effects of ERK, p38 kinase and PKA inhibitors on the increase in the AP-2α level. (C) PKA enzyme activity in both p53■5,6 MEC (p53- MEC) and p53fp/fp MEC (p53+ MEC). The levels of PKA activity were assessed using Profluor® PKA assay kit (Promega, Madison, WI).

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