Enhanced expression of aromatase in p53-inactivated mammary epithelial cells

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Abstract
Both the functional loss of p53 and the overexpression of aromatase are important for the progression of breast cancer in postmenopausal women. Here, we found that aromatase expression was up-regulated in primary cultures of mammary epithelial cells (p53<sup>Δ5,6 MEC</sup>) isolated from mice with a defect in exons 5 and 6 of the p53 gene. Aromatase basal activity and expression levels were significantly increased in p53<sup>Δ5,6 MEC</sup> when compared with wild-type MEC. Reporter gene activity in p53<sup>Δ5,6 MEC</sup> transfected with the aromatase promoter or the cAMP-responsive element (CRE) minimal promoter was higher than wild-type MEC. p53 inactivation increased both Ser133-phosphorylated CRE-binding protein (CREB) and the nuclear accumulation of CREB. Inhibition of extracellular signal-regulated kinase (ERK) or Src tyrosine kinase blocked aromatase gene transactivation and CREB activation in the p53<sup>Δ5,6 MEC</sup>. These results support the hypothesis that a genetic defect in the function of p53 enhances the expression of aromatase via ERK or Src activation in MEC, which suggests that aromatase expression is closely related to the p53 status in MEC.

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Introduction
Breast cancer is one of the most common malignancies in Western women. Human breast tumors arise from normal cells after the accumulation of multiple mutations of tumor suppressor genes, such breast cancer susceptibility gene 1/2 (BRCA1/2) and p53 (Mann & Borgen 1998). Approximately, 30–50% of breast cancers carry a mutation in the p53 gene (Sjogren et al. 1996). Germ-line mutations in the DNA-binding domain of p53 gene are present in 70% of patients with Li–Fraumeni syndrome, which shows development of early-onset breast cancer (Malkin et al. 1990, Srivastava et al. 1990).

Breast cancer is also characterized by hormone-dependent proliferation. Ovarian steroid hormones, including estrogen, are critical in both mammary gland development and breast carcinogenesis (Petrageli et al. 1994, Mueller et al. 2002). Clinical application of aromatase (CYP19) inhibitors in adjuvant therapy for hormone-sensitive breast cancer has grown rapidly over the past few years. Unlike tamoxifen that antagonizes estrogen receptors (ER) and subsequently inhibits transcription of estrogen-responsive genes, aromatase inhibitors block synthesis of estrogens by blocking the catalysis of C<sub>19</sub> androgens to estrogens. Although tamoxifen has been approved for the chemoprevention of breast cancer and is the most widely used anti-estrogen in ER-positive breast cancer patients, serious side effects can occur, including endometrial cancer, thrombosis, and embolism (Mourits et al. 2001, Cuzick et al. 2003). Additionally, tamoxifen resistance is a serious problem for long-term tamoxifen treatment (Clemons et al. 2002). Hence, there is a shift toward treatment with aromatase inhibitors, especially after third-generation inhibitors (anastrozole, letrozole, and exemestane) have shown superiority to tamoxifen (Smith & Dowsett 2003, Koberle & Thurlimann 2005).

Two mouse lines have been established with conditional knockouts of the p53 genes in mammary glands using the Cre–loxP recombination system (Lin et al. 2004). The system requires two mouse lines: one
carrying the floxed p53 alleles (p53<sup>fpfp</sup>) and the other expressing Cre recombinase under the regulation of the whey acidic protein (WAP) promoter (WAP-Cre). Cre expression leads to recombination within the loxP sequences in introns 4 and 6 of the p53 gene, which deletes part of the DNA-binding domain (exons 5 and 6) of p53 and inactivates the gene (p53<sup>Δ5,6</sup>). Using the p53<sup>fpfp</sup> and p53<sup>Δ5,6</sup> mammary epithelial cells (MEC), we recently showed that ErbB2 expression level is higher in the p53-inactivated MEC through AP-2 activation (Yang et al. 2006).

The expression of aromatase is stimulated by diverse pathophysiological factors such as prostaglandin E<sub>2</sub> and leptin (Catalano et al. 2003, Richards & Brueggemeier 2003). Recent studies also revealed that the transcription of aromatase gene is affected by a tumor suppressor, BRCA1 (Hu et al. 2005). Although both p53 mutation and aromatase expression are important for the progression of breast cancer in postmenopausal women, the interaction of aromatase expression with p53 inactivation is unknown. Here, we show for the first time that p53 inactivation enhances aromatase expression and the enzyme activity in primary cultured MEC. We also show that Src- or extracellular signal-regulated kinase (ERK)-dependent cAMP-responsive element-binding protein (CREB) activation is required for the aromatase induction in p53-inactivated MEC.

### Materials and methods

#### Materials

The anti-CREB and aromatase antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and Abcam (Cambridge, UK) 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, USA). The 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium and phRL-SV40 plasmid were purchased from Promega. PP2 was supplied from Calbiochem (La Jolla, CA, USA). The anti-actin antibody and the other reagents in the molecular studies were obtained from Sigma Chemical. Glucocorticoid response element (pGRE-Luc) plasmid was a gift from Dr Lee KY (Chonnam National University, Gwangju, South Korea). pCRE-Luc was purchased from Stratagene (La Jolla, CA, USA). The aromatase-Luc was kindly provided by Dr Jameson (Northwestern University Medical School, Chicago, IL, USA; Ito et al. 2000).

#### Generation of WAP-Cre p53<sup>fpfp</sup> mice and MEC culture

WAP-Cre mice were mated with p53<sup>fpfp</sup> mice, and Cre-mediated recombination and p53 deletion were confirmed by PCR analysis and x-gal staining, as published previously (Lin et al. 2004). MEC were isolated from the number 4 mammary gland from 6-month-old p53<sup>fpfp</sup> or WAP-Cre p53<sup>fpfp</sup> mice using a slight modification of a previously published method (Deome et al. 1959). 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 37°C. The cells were 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 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were used for experiments up to ten passages.

#### Preparation of the nuclear fraction

Cells in dishes were washed with ice-cold PBS, scraped, transferred to microtubes, and allowed to swell after adding 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 phenylmethylsulfonylflouride. Cell membranes were disrupted by vortexing, and the lysates were incubated for 10 min on ice and centrifuged at 7200 g for 5 min. Pellets containing 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 phenylmethylsulfonylfuoride, 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

SDS-PAGE and immunoblot analysis were performed according to the procedures reported in the literature (Kang et al. 2003). 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 β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfuoride, and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10 000 g for 10 min to remove debris. The proteins were fractionated using a 10%
separating gel and then transferred electrophoretically to nitrocellulose paper for immunoblotting. 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 (enhanced chemiluminescence) chemiluminescence system.

**Determination of aromatase activity**

Aromatase activity was measured in p53fpfp MEC and p53Δ5,6 MEC with a tritiated water release assay, described previously (Kinoshita & Chen 2003). Experiments were conducted when cells reached 80% confluence. p53fpfp MEC and p53Δ5,6 MEC were incubated in serum-free medium for 36 h and the cells were treated with [1β-³H] androst-4-ene-3,17-dione (100 nM) for an additional 1 h. The medium was then mixed thoroughly with 5% charcoal/0.5% dextran for 12 h and centrifuged at 10 000 g for 30 min at 4 °C to remove any residual androst-4-ene-3,17-dione. A 1 ml volume of the supernatant was added in a scintillation vial containing 3 ml scintillation cocktail, and ³H was measured as disintegrations per minute using a liquid scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA, USA).

**Reporter gene assay**

A dual-luciferase reporter assay system (Promega) was used to determine promoter activity. Briefly, cells were transiently transfected with 0.5 μg aromatase-Luc, pGRE-Luc, pCRE-Luc, or pERE-Tk-Luc plasmid and 0.2 μg pCMV-β-gal plasmid (β-galactosidase expression for normalization) using the LipofectAMINE Plus Reagent. The luciferase and β-galactosidase activities were determined as described previously (Choi et al. 2001). The reporter activity was calculated by normalizing the reporter-driven firefly luciferase activity to that of β-galactosidase activity.

**cAMP determination**

A commercial chemiluminescent ELISA kit (Promega) was used to determine 3', 5'-cyclic AMP levels in cell lysates, according to the manufacturer’s protocol.

**Gel shift assay**

A double-stranded DNA probe (1.75 pmole/μl) for the consensus sequences of CRE (5'-CACGTGATTTCCGT-CACATGGG-3') was obtained from Promega and used for gel shift analyses after end-labeling the probe with [γ-³P]ATP and thyroxine polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer with 20% glycerol, 5 mM MgCl₂, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dl-dC, 50 mM Tris–Cl (pH 7.5), 15 μg nuclear extracts, and sterile water to a total volume of 10 μl. Incubations were performed at room temperature for 20 min by adding 1 μl probe (10⁶ c.p.m.) after a 10-min preincubation. The specificity of DNA/protein binding was determined through competition reactions using a 20-fold molar excess of unlabeled CRE oligonucleotide. For the immunodepletion assay, the antibody against CREB (1 μg) was added to the reaction mixture. Samples were loaded onto 5% polyacrylamide gels at 100 V. After electrophoresis, the gels were removed, dried, and imaged using autoradiography.

**Data analysis**

One-way 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 effect of treatment. Statistical significance was set at either P < 0.05 or P < 0.01.

**Results**

**Enhanced activity and expression of aromatase in p53-inactivated MEC**

MEC were isolated from 6-month-old p53fpfp or WAP-Cre p53fpfp mice. We previously showed that the presence of loxP sites in introns 4 and 6 did not interfere with the transcription of p53, and full-length p53 protein was detected in the doxorubicin-treated p53fpfp MECs (Yang et al. 2006). In contrast, a smaller protein product with the expected mass of 39 kDa, which was designated p53Δ5,6 by Cre recombination, was found in the WAP-Cre p53fpfp MECs, and p53-dependent transcription was inhibited (Lin et al. 2004, Yang et al. 2006).

We first tested whether aromatase levels were changed in p53fpfp MEC and p53Δ5,6 MEC when they were compared. The basal expression of aromatase in the p53Δ5,6 MEC was significantly higher than that of p53fpfp MEC (Fig. 1A). Consistent with differences in expression, the conversion of [³H]androstenedione to [³H] estrogen in the p53Δ5,6 MEC was 3.2-fold higher than wild-type MEC (Fig. 1B). These results demonstrate that aromatase activity is enhanced through the induction of aromatase protein in p53-inactivated MEC.
This enhanced aromatase expression in p53<sup>5,6</sup> MEC could result from additional genetic mutations in the mice. An adenosine over-expressing Cre recombinase (Ad-Cre) was introduced to the p53<sup>fp/fp</sup> MECs to directly remove exons 5 and 6 out of the p53 gene. The truncated 39 kDa p53 band was found in the Ad-Cre-treated p53<sup>fp/fp</sup> MEC, but not in β-galactosidase expressing adenovirus (Ad-gal)-treated p53<sup>fp/fp</sup> MECs (Fig. 1C), which shows that Ad-Cre infection to p53<sup>fp/fp</sup> MECs efficiently removes the transactivation domains of p53 (Fig. 1C). We then determined the expression levels of aromatase in Ad-Cre-treated MECs. Exposing the p53<sup>fp/fp</sup> MECs to Ad-Cre for 24 h resulted in a concentration-dependent increase in aromatase protein levels (Fig. 1C). In contrast, the ectopic expression of the p53 gene by p53-overexpressing adenovirus (Ad-p53) decreased the aromatase protein levels in p53<sup>Δ5,6</sup> MEC (Fig. 1D). These results suggest that aromatase expression is directly coupled with p53 activity in MECs.

**CRE activation is required for aromatase overexpression in p53-inactivated MECs**

Aromatase gene expression is regulated by multiple tissue-specific promoters (e.g., promoters I.1 and I.2 in the placenta; promoter I.4 in skin fibroblasts and adipocytes; promoters I.3 and I.11 in the ovary; Means et al. 1991). The promoter I.4 and I.11 are predominantly used in normal mammary epithelial tissues and breast cancer tissues respectively (Utsumi et al. 1996). Both CREB binding to CRE or glucocorticoids binding to GRE can regulate the transcription of the aromatase gene (Simpson & Zhao 1996, Young & MacPhaul 1998), so we examined whether the activation of GR and CREB mediated p53 activation.

We first compared reporter activity in both the p53<sup>fp/fp</sup> MEC and p53<sup>Δ5,6</sup> MEC transfected with an aromatase-Luc plasmid containing a −294/+20 bp promoter region of rat aromatase gene (Ito et al. 2000). The aromatase-Luc reporter activity in p53<sup>Δ5,6</sup> MEC was higher than that of p53<sup>fp/fp</sup> MEC (Fig. 2A), which suggests that the enhanced aromatase levels are mainly due to the transcriptional activation of the aromatase gene. In addition, the basal CRE reporter activity in p53<sup>Δ5,6</sup> MEC was 5.6-fold higher than that of p53<sup>fp/fp</sup> MEC (Fig. 2B, left panel). Moreover, cAMP levels in p53<sup>Δ5,6</sup> MEC were also 2.6-fold higher than that of p53<sup>fp/fp</sup> MEC (Fig. 2B, right panel). These results suggest that the transactivation of aromatase gene is associated with aromatase overexpression in p53-inactivated MEC.

Glucocorticoid receptor binding to the putative GRE binding site in the I.4 promoter region is involved in the expression of human aromatase gene (Shozu et al. 2000). However, GRE-luciferase reporter activity was not significantly altered in p53<sup>Δ5,6</sup> MEC (Fig. 2C). The data suggest that GR activation is not involved in transactivation of aromatase gene in p53-inactivated MEC.
Increases in CRE reporter activity might result from changes in the nuclear migration of CREB. Nuclear levels of CREB were significantly higher in p53Δ5,6 MEC compared with p53fp/fp MEC (Fig. 3A). Moreover, levels of serine 133-phosphorylated CREB in total cell lysates from p53Δ5,6 MEC were higher than that from the p53fp/fp MEC (Fig. 3A). To determine whether the nuclear CREB binding to CRE was increased in the p53-inactivated MEC, we isolated nuclear extracts from p53Δ5,6 MEC or p53fp/fp MEC and probed them with a radiolabeled CRE binding sequence. The band of the slow migrating complex was higher in samples from the p53Δ5,6 MEC than in the wild-type MEC (Fig. 3B). Competition experiments using an excess (20×) of the unlabeled CRE oligonucleotides or immunodepletion experiment using 1 µg anti-CREB antibody confirmed that the increased band resulted from enhanced binding of the nuclear CREB protein (Fig. 3B). Thus, CREB phosphorylation and nuclear translocation in p53-inactivated MEC are essential for CRE binding in the promoter region of the aromatase gene.

Role of Src and ERK in CREB-mediated aromatase expression in p53-inactivated MEC

The activities or expression levels of most transcription factors are regulated by members of the kinase family, which are triggered in response to a variety of stimuli (Treisman 1996). Two mitogen-activated protein (MAP) kinases, ERK and p38 kinase, are involved in Ser-133 phosphorylation (and activation) of CREB (Cammarota et al. 2001, Hokari et al. 2005, Gelain et al. 2006). The incubation of cells with PD98059 (20 µM), a specific inhibitor of MAPK/ERK kinase 1/2 (MEK1/2, upstream kinase of ERK), for 24 h significantly suppressed both aromatase expression and the

Figure 2 Role of CRE activation in aromatase expression.
(A) Induction of luciferase activity in p53Δ5,6 MEC (p53− MEC) transiently transfected with aromatase-Luc, which contained the 294 bp promoter region of the rat aromatase gene and luciferase cDNA. Activation of the reporter gene was calculated as a relative change to β-galactosidase activity. The data represent the mean ± s.d. with three different samples (significant when compared with the p53fp/fp MEC (p53+ MEC), **P < 0.01). (B) Upper panel; increase of CRE reporter activity in p53− MEC. The data represent the mean ± s.d. with three different samples (significant when compared with the p53+ MEC, **P < 0.01). Lower panel; increase of cAMP in p53− MEC. The cell lysates were obtained from both the p53+ and the p53− MEC incubated in serum-free medium for 18 h. cAMP levels were determined using ELISA kit. The data represent the mean ± s.d. with three different samples (significant when compared with the p53+ MEC, **P < 0.01). (C) No increase of GRE reporter activity in p53− MEC. The data represent the mean ± s.d. with three different samples.
nuclear CREB levels in the p53<sup>5,6</sup> MEC (Fig. 4A). In contrast, p38 kinase inhibition by SB203580 (10 μM, a p38 kinase inhibitor) did not affect the levels of aromatase and nuclear CREB (Fig. 4A).

Src tyrosine kinase is coupled with p53 status (Pal et al. 2001) and Src can phosphorylate CREB (Kawasaki et al. 2004). Pretreatment of cells with PP2 (10 μM), a Src inhibitor, completely suppressed aromatase expression by p53 inactivation (Fig. 4B) and blocked nuclear CREB localization (Fig. 4B). These results indicate that Src tyrosine kinase is also involved in CREB-dependent aromatase expression in p53<sup>5,6</sup> MEC. In addition, the enhanced reporter activity in p53<sup>5,6</sup> MEC was 85 and 76% inhibited by PP2 and PD98059 respectively (Fig. 4C). These results suggest that Src and ERK regulate CREB activation and subsequent aromatase gene expression in p53-inactivated MEC.
Discussion

p53 is a key tumor suppressor gene that is mutated or lost in ~50% of all human cancer cases (Levine et al. 1991). Because p53 target genes are involved in mediating cell-cycle arrest and apoptosis, p53 dysfunction results in uncontrolled cell proliferation and ultimately carcinogenesis (Voussen & Lu 2002). In postmenopausal women, locally produced estrogen through aromatase promotes proliferation of ER-positive breast cancer (Simpson 2003). Estrogens produced by aromatase in breast carcinomas or MECs may be sufficient to stimulate cell proliferation (Brodie et al. 1997). Although the expression of the tumor suppressor genes, p53 or retinoblastoma, was decreased in aromatase transgenic mammary glands (Kirma et al. 2001), the interaction of aromatase expression and p53 status is still unknown. We therefore focused on testing whether aromatase expression was up-regulated in p53-inactivated MEC and exploring the controlling signal transduction pathways. Aromatase expression was enhanced in p53 inactivated MEC, demonstrating p53 dysfunction could cause overproduction of estrogens in mammary glands. A representative tumor suppressor gene, BRCA1, inhibits transcription of the aromatase gene (Hu et al. 2005, Lu et al. 2006), and aromatase expression is inversely correlated with BRCA1 abundance in human adipose stromal cells (Ghosh et al. 2007). Since both p53 and BRCA1 inhibit aromatase gene expression, their inactivation in MEC could lead to a local increase in estrogen production, which subsequently may cause estrogen-dependent hyperproliferation of mammary glands or breast carcinomas.

Because there are no putative binding site(s) for p53 in the aromatase gene promoter, the stimulatory effect of aromatase gene transcription by p53 inactivation may result from the activation of other transcription factor(s). In particular, CRE and GRE binding sites can regulate aromatase expression in MEC and breast cancer cells (Simpson & Zhao 1996, Young & MacPhaul 1998, Kijima et al. 2006). We found that CRE, but not GRE, activity was enhanced in p53-inactivated MEC. CREB phosphorylation and nuclear translocation was also increased in p535,6 MEC, which strongly supports the essential role of CREB activation in aromatase overexpression in p53-inactivated MEC.

Ser-133 phosphorylation of CREB, which can be mediated by p38 kinase or ERK, is important for binding to CRE in the promoter region of the GSTP1 gene (Lo & Ali-Osman 2002). Although a p38 kinase inhibitor did not suppress aromatase expression, ERK inhibition by PD98059 significantly reduced the enzyme expression and nuclear levels of CREB in p53-inactivated MEC. Src-mediated tyrosine phosphorylation also causes CREB activation (Wu et al. 2005). Here, Src inhibition significantly suppressed CREB activation and blocked aromatase expression. How is CREB-dependent aromatase expression simultaneously regulated by ERK and the Src pathway? Since ERK activity depends on upstream Src tyrosine kinase (Barthet et al. 2007), p53 inactivation may persistently activate Src tyrosine signaling to stimulate ERK-dependent CREB phosphorylation and subsequent aromatase induction. PP2- and PD98059-mediated inhibitory activities in aromatase-reporter gene assay (Fig. 4C) are higher than those in western blot analyses (Fig. 4A and B). The discrepancy of inhibition intensity may result from the assay system difference between western blot and reporter gene analysis. Reporter gene assay results directly reflect newly synthesized luciferase activity. In contrast, western blot results represent the endogenous aromatase levels.

We previously showed that Her-2/ErbB2 was over-expressed in p53-inactivated MEC and suggested that exaggerated EGF receptor signaling through ErbB2 induction is responsible for the uncontrolled proliferation of MEC in response to EGF (Yang et al. 2006). Our current data indicate that p53 inactivation in MEC enhances aromatase activity through the induction of the aromatase gene through Src/ERK-dependent CREB activation. Thus, the increased de novo synthesis of estrogen via aromatase overexpression may be associated with the uncontrolled estrogen-dependent growth of p53-inactivated mammary glands.

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