Prostaglandin E\(_2\) Inhibits p53 in Human Breast Adipose Stromal Cells: A Novel Mechanism for the Regulation of Aromatase in Obesity and Breast Cancer

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Abstract

Obesity is a risk factor for postmenopausal breast cancer and the majority of these cancers are estrogen dependent. Aromatase converts androgens into estrogens and its increased expression in breast adipose stromal cells (ASC) is a major driver of estrogen receptor–positive breast cancer. In particular, obesity-associated and tumor-derived factors, such as prostaglandin E\(_2\) (PGE\(_2\)), have been shown to drive the expression of aromatase by stimulating the activity of the proximal promoter II (PII). The tumor-suppressor p53 is a key regulator of cell-cycle arrest and apoptosis and is frequently mutated in breast cancers. Mutations in p53 are rare in tumor-associated ASCs. Therefore, it was hypothesized that p53 is regulated by PGE\(_2\) and involved in the PGE\(_2\)-mediated regulation of aromatase. Results demonstrate that PGE\(_2\) causes a significant decrease in p53 transcript and nuclear protein expression, as well as phosphorylation at Ser15 in primary human breast ASCs. Stabilization of p53 with RITA leads to a significant decrease in the PGE\(_2\)-stimulated aromatase mRNA expression and activity, and PII activity. Interaction of p53 with PII was demonstrated and this interaction is decreased in the presence of PGE\(_2\). Moreover, mutation of the identified p53 response element leads to an increase in the activity of the promoter. Immunofluorescence on clinical samples demonstrates that p53 is decreased in tumor-associated ASCs compared with ASCs from normal breast tissue, and that there is a positive association between perinuclear (inactive) p53 and aromatase expression in these cells. Furthermore, aromatase expression is increased in breast ASCs from Li–Fraumeni patients (germline TP53 mutations) compared with non-Li–Fraumeni breast tissue. Overall, our results demonstrate that p53 is a negative regulator of aromatase in the breast and its inhibition by PGE\(_2\) provides a novel mechanism for aromatase regulation in obesity and breast cancer. Cancer Res; 75(4); 645–55. ©2015 AACR.

Introduction

Breast cancer is one of the most common cancers in women and the risk of breast cancer increases with increasing age beyond menopause (1). Breast cancer growth in postmenopausal women is largely dependent on the estragonadal production of estrogens in breast adipose stromal cells (ASC), which requires the aromatase enzyme (reviewed in ref. 2). Indeed, aromatase inhibitors are first-line therapy for the treatment of hormone receptor–positive breast cancer in postmenopausal women (3). There is also strong evidence to indicate that the risk of breast cancer increases with increasing body mass index (BMI; reviewed in ref. 4) and excess adiposity is associated with the increased expression of aromatase in the breast of postmenopausal women (5). Animal studies have provided evidence that overexpression of aromatase has a direct effect on estrogen-dependent breast tumor growth via the increased biosynthesis of estrogens leading to mammary gland hyperplasia, dysplasia, and other premalignant changes (6).

The aromatase gene CYP19A1 is composed of nine coding exons II–X and is regulated by tissue-specific promoters (reviewed in ref. 7). In normal breast adipose tissue, aromatase gene expression is mainly under the control of the distal promoter PI.4 and expression levels are maintained low. In obesity, however, the coordinated activation of the proximal promoters I.3 and promoter II (PII) causes a significant increase in aromatase expression and has been shown to be positively associated with the presence of crown-like structures (CLS) within the breast (8). These CLS form around necrotic adipocytes and are composed of macrophages shown to release a number of inflammatory factors, including prostaglandin E\(_2\) (PGE\(_2\)). Similarly, the ASCs of
tumorigenic breast tissue have increased expression of PI3/P1I–specific transcripts, hypothesized to occur as a consequence of the increased production of PGE2 from the tumor (9). The stimulation of promoters I3/P1I is mediated by the increased expression and/or activity of various transcription factors and coregulators, including cAMP response element binding protein 1A (CREB1A), CREB-regulated transcription coactivator 2 (CRTC2), liver receptor homolog-1 (LRH-1), and hypoxia-inducible factor-1α (HIF-1α; refs. 10–13).

The tumor-suppressor p53 is encoded by the TP53 gene and plays an important role in stimulating cell-cycle arrest and apoptosis in response to DNA damage (reviewed in ref. 14). Recently, p53 has also been shown to be involved in regulating energy homeostasis by suppressing glycolysis and promoting oxidative phosphorylation (reviewed in ref. 15). Wild-type p53 is expressed at low steady-state levels within cells and is phosphorylated at Ser15 by several upstream kinases, including AMP-activated protein kinase (AMPK), which acts as a central cellular energy sensor and a key negative regulator of aromatase (11). Phosphorylation at this site leads to the stabilization of p53 and its increased activity (16). Wild-type p53 can also be stabilized using the compound RITA that prevents the interaction of p53 with HDM2, and hence prevents proaeseal degradation and leads to the activation of p53-target genes (17). Germline mutations in TP53 lead to Li–Fraumeni Syndrome (LFS) with affected individuals having a 49% incidence of cancer by the age of 30 years (18). Among women with LFS, the most common disease is breast cancer with 84% of cases being hormone receptor–positive (18, 19). Furthermore, 30% of breast cancer cases have sporadic mutations in p53 (20). However, such mutations are rare in tumor-associated ASCs (21). We have identified three putative p53 response elements on aromatase promoter PI I suggesting that p53 may be involved in the regulation of aromatase within breast ASCs. We therefore hypothesized that p53 is a negative regulator of aromatase and that tumor-derived factor PGE2 regulates the expression and localization of p53 in breast adipose tissue of women.

Materials and Methods

Plasmids

The CYP19A1 PI–516 luciferase reporter construct, which contains 302 bp (−516 to −14) of the proximal promoter PI I was generated as previously described (22). Mutated CYP19A1 PI–516 luciferase constructs were generated according to the manufacturer’s instruction using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) and primers PI I p53-binding mutant F: 5′-AACGATGGAGGCTCGAGAAGCCAATCT- CACCCAGAAAATGAGTCTCAACCTGACATC-3′; and PI I p53-binding mutant R: 5′-GTAACAGCCAGGGCCCTCAGGAA-3′; and PII 0.1% bovine serum albumin before treatment. The study has been approved by Monash Health Human Research Ethics Committee B (#B00109).

Reverse transcription and quantitative PCR

Serum-starved ASCs were treated for 6 hours with PGE2 (1 μmol/L; Sigma-Aldrich) and/or RITA (10 μmol/L; Cayman Chemical). Total RNA was extracted using the RNasy Mini Kit (Qiagen) and DNA was digested using the DNA-free DNase Treatment and Removal Kit (Ambion). RNA (0.3–0.5 μg) was reverse transcribed using the AMV RT Kit (Promega) or SuperScript III First-Strand Synthesis System (Invitrogen) as directed by the manufacturer. Quantitative PCR (qPCR) was performed on the LightCycler using LightCycler FastStart DNA Master SYBR Green Kit (Roche). Primers used were: hArom F (RT7): 5′- TTGGAATTCTGACCGAT-3′; hArom R (RT8): 5′-CAGGAACTC- TCGCCGTCGAGA-3′; aromatase PI I F: 5′-GCAACACGAGACT- TAGATGAC-3′; aromatase PII (ExII)R: 5′-CAGCCGGTTGAGT- TAGTTCGACCTGC-3′; p53 F: 5′-CCCTCTGACTCA- AACA-3′; and p53 R: 5′-TCACTGAGCCTGATGTT- C-3′. All the samples were quantified using standards of known concentrations and corrected for abundance with the housekeeping gene β-actin.

Protein extraction and Western blot analysis

Serum-starved ASCs were treated for 6 hours with PGE2 (1 μmol/L; Sigma-Aldrich). Nuclear extracts and whole-cell extracts were obtained as previously described (11, 13). Protein amount was quantified using BCA protein assay (Thermo Scientific) according to the manufacturer’s instructions. Fifteen micrograms of protein was loaded onto a 10% denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. p53 protein abundance was examined from nuclear extracts using p53-specific antibody (#9282; Cell Signaling Technology) and detected using Alexa Fluor 700–tagged secondary antibody (goat anti-rabbit Alexa Fluor 700; Life Technologies). Nuclear protein amount was normalized to histone H3 (ab1791; Abcam). Phosphorylation of p53 at Ser15 was assessed using a phospho-specific antibody (p-p53 Ser15 #9284; Cell Signaling Technology) and normalized to total p53 (Pab 1801, #sc-98; Santa Cruz Biotechnology), aromatase protein expression was detected by mouse monoclonal primary antibody 677 and protein amount was normalized to β-tubulin (rabbit, #sc-9104; Santa Cruz Biotechnology). Primary antibodies were detected using secondary antibodies tagged with Alexa Fluor 700 and IRDye 800. Membranes were scanned using the Odyssey infrared imaging system (LI-COR Biosciences), and the intensity of the bands was quantified using densitometric analysis.

Immunofluorescence on isolated primary human breast ASCs

The expression and localization of p53 in primary breast ASCs before and after PGE2 treatment (1 μmol/L; Sigma-Aldrich) were observed using immunofluorescence followed by confocal microscopy. ASCs were plated onto UV-sterilized coverslips and cultured until they reached approximately 50% confluency. Cells were then

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cells were serum-starved overnight and treated for 24 hours. Cells were prepared for immunofluorescence as previously described (11). The p53 antibody CM1 (VP-P955; Vector Laboratories) and nucleolar marker anti-lamin B1(+B2) (#ab4825; Abcam) were added to coverslips and incubated overnight. Then, coverslips were washed with PBS and anti-rabbit Alexa Fluor 546 (red) and anti-mouse Alexa Fluor 488 (green) from Invitrogen were added and incubated for 1.5 hours. Confocal microscopy (1024 x 1024 pixels; Olympus Optical Co. Ltd.) was used to obtain images. Images presented are representative of the majority of cells examined.

**Reported assays**

3T3-L1 cells (authenticated mouse preadipocyte cell line, purchased from ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) with 10% fetal calf serum. Cells were transfected with wild-type or mutated CYP19A1 PII-516 luciferase constructs using FuGene HD (Promega), according to the manufacturer's instructions. β-Galactosidase was cotransfected and used as a transfection control. After transfection, cells were serum-starved for 24 hours in phenol-red-free DMEM containing 0.1% bovine serum albumin. Cells were then treated for 24 hours with FSK/PMA [25 μmol/L forskolin/4 nmol/L phorbol ester; to mimic PGE2 (11)] and/or RITA (10 μmol/L; Cayman Chemical). Luciferase reporter assays were carried out using the Luciferase Assay System (Promega) according to the manufacturer's protocol and all data were normalized to β-galactosidase activity.

**Aromatase activity assays**

Serum-starved ASCs and breast tissue explants were treated with PGE2 (1 μmol/L; Sigma-Aldrich) and/or RITA (1 μmol/L; Cayman Chemical) for 24 hours. Aromatase activity was measured using the titrified water-release assay using androst-4-ene-3,17-dione (NET926001MC; PerkinElmer) as a substrate, as previously described (24). Specific activity was normalized to total protein amount.

**Chromatin immunoprecipitation**

To examine endogenous binding of p53 to aromatase PII, primary human breast ASCs were treated with PGE2 (1 μmol/L; Sigma-Aldrich) for 45 minutes. Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express Kit (Active Motif). Some modifications were made from the manufacturer's protocol as previously described (11). Briefly, cells were fixed using 1% formaldehyde and sheared into small fragments using sonication at 20% amplitude, seven times for 30-second pulses. Specific protein–DNA complexes were immunoprecipitated using anti-p53 antibody (Pab 1801, sc-98; Santa Cruz Biotechnology), IgG, or water as controls. qPCR was then performed using primer sets that are upstream of the promoter PII transcription start site: PII F1R1 –453 to –353bp, F: 5'-AAGACCTCAGACGACCCCA-3', R: 5'-GGCAATCTCCTCCCTC-3'; PII F2R2 –371 to –278bp, F: 5'-GCTCATTCCAGGTCGAGCTC-3', R: 5'-CATGTCGCCCGTGAGGTGC-3'; PII F3R3 –278 to –178bp, F: 5'-GACCTCCACCTCTGGATTAGAC-3', R: 5'-CATGTCGCCCGTGAGGTGC-3'; PII F4R4 primer –212 to –112bp, F: 5'-ATGCAGCCATCTCCAAG-3', R: 5'-GGGAATCCTGGGTGAAG-3'; and PII F5R5 –164 to –128bp, F: 5'-GACCAAGCGTCTAAAGGGAC-3', R: 5'-TGTGATCATAGATTGCGGAC-3'.

**Immunofluorescence and confocal imaging of clinical breast tissue**

Sections of formalin-fixed and paraffin-embedded breast tissue were obtained from 10 cancer-free women and 10 patients with breast cancer [estrogen receptor–positive (ER+); invasive ductal carcinoma, from Tohoku Hospital, Sendai, Japan]. Li–Fraumeni patient samples were obtained from kConFab (Melbourne, VIC, Australia). The studies have been approved by Monash Health Human Research Ethics Committee B, The Peter MacCallum Cancer Centre (Melbourne, VIC, Australia), and by the ethics committee at Tohoku University Hospital (Sendai, Japan). Patient details including age and tumor receptor status (in the case of cancer samples) are included in Supplementary Table S1. Immunofluorescence on clinical samples was performed after antigen retrieval in a microwave-heated citrate buffer bath for 2 × 5 minutes at high power. Sections were blocked with 10% horse serum in CAS block (Invitrogen) for 30 minutes. Aromatase mouse monoclonal primary antibody 677 (Prof. Dean Edwards, Baylor College of Medicine, Houston, TX; ref. 25) and/or rabbit p53 CM1 antibody were added to the slides and incubated overnight at 4°C. Hoechst 33342, anti-mouse Alexa Fluor 546, and anti-rabbit Alexa Fluor 488 (Invitrogen) were added to the slides and incubated for 1.5 hours. Finally, the sections were mounted with fluorosave reagent (Calbiochem). Imaging was done using the Nikon inverted confocal microscope (1,024 x 1,024 pixels, sequential scanned images). To limit intraexperimentional variability, samples were analyzed using identical settings. Images presented are representative of the majority of sections examined.

**Image analysis**

Localization of p53 and aromatase was evaluated from confocal images using Metamorph Software (Molecular Devices). Briefly, blue nuclear staining was binalized and then processed with "Fill Holes" to create a nuclear mask. The nuclear mask was then eroded once to remove small nonspecific staining then, dilated to include the perinuclear area. The nucleus was then subtracted from the dilated mask to obtain the perinuclear mask. Images were then analyzed using these masks. Breast ASCs in patient tissue sections were selected on the basis of shape factor (0.3–0.8 for normal ASCs and 0.25–0.65 for tumor-associated ASCs), elliptical form factor (>1.5 for normal ASCs and >1.7 for tumor-associated ASCs), and area (30 < x < 120 pixels). Staining intensity of perinuclear p53 relative to aromatase in tissue sections was plotted for each stromal cell in a representative area (>150 cells/patient). To compare nuclear intensity of p53 in normal ASCs from tumor-free and tumor-bearing breast tissue and tumor-associated ASCs from patients with breast tumor, average nuclear intensity of p53 for each patient was calculated. Average aromatase intensity in tumor-associated ASCs from the 14 Li–Fraumeni and 10 non-Li–Fraumeni breast cancer patients were also analyzed using the described method. To compare data from multiple experiments, samples were normalized to common controls (Figs. 4B–E).
there was an association between p53 and aromatase staining in clinical samples, Spearman correlation analysis was performed. All data analysis was performed using GraphPad Prism version 6.

Results

p53 is downregulated by tumor-derived factor PGE2 in primary human breast ASCs

To examine the effect of PGE2 on p53 mRNA and protein expression in ASCs, qPCR and Western blotting were performed, respectively. qPCR showed that PGE2 causes a significant decrease in p53 mRNA expression (Fig. 1A; 72.5% of vc; \( P < 0.005 \)) and nuclear p53 protein expression in ASCs (Fig. 1B; 73.4% of vc; \( P < 0.05 \)). Immunofluorescence performed on isolated primary human ASCs demonstrates reduced nuclear p53 intensity after PGE2 or FSK/PMA (to mimic PGE2) treatment compared with vehicle control (Fig. 1C). An increase in cytoplasmic p53 staining was also observed in these samples. In comparison, RITA-treated ASCs had increased nuclear expression of p53, which confirmed the stabilization effect of RITA on p53 (Fig. 1C). Consistent with
these results, quantification of average fluorescence intensity demonstrates that both PGE₂ and FSK/PMA cause a significant decrease in nuclear p53 staining, whereas RITA treatment leads to a significant increase in nuclear p53 staining in ASCs (Fig. 1D). Analysis also showed that the perinuclear:nuclear ratio of p53 is increased after PGE₂ treatment (Fig. 1E). The effect of PGE₂ on p53 phosphorylation at Ser15 was also examined by Western blot analysis (Fig. 1E). Results demonstrate that phosphorylation of Ser15 is significantly decreased after PGE₂ treatment in relation to total p53 expression.

p53 is a negative regulator of aromatase in human breast ASCs via direct interactions with promoter PII

The effect of p53 on aromatase transcript expression and enzymatic activity was examined. qPCR was performed to determine the effect of RITA-stabilized p53 on aromatase mRNA expression in primary ASCs. Results demonstrate that RITA causes a significant decrease in the PGE₂-stimulated expression of aromatase mRNA (Fig. 2A) and protein (Fig. 2B). The effect of p53 on PII activity was examined by performing qPCR using primer pairs that amplify PII-specific transcripts and by luciferase reporter assays in 3T3-L1 cells. Similar to effects on total aromatase mRNA, RITA significantly inhibited the PGE₂-mediated expression of PII-specific transcripts (Fig. 2C). As 3T3-L1 cells do not respond well to PGE₂, we used the PGE₂ mimetics FSK/PMA to stimulate PII activity in these cells. Results demonstrate that RITA-stabilized p53 significantly inhibits the basal and FSK/PMA-stimulated aromatase PII activity (Fig. 2D). Aromatase activity assays in both primary human breast tissue explants and isolated ASCs also demonstrate that stabilization of p53 using RITA causes a significant decrease in the PGE₂-mediated induction of aromatase activity (Fig. 2E and F, respectively).

![Graphs showing the effect of RITA on aromatase expression and activity](image)

**Figure 2.**

p53 is a negative regulator of aromatase in ASCs. Stabilization of p53 using RITA leads to the inhibition of PGE₂-stimulated aromatase transcript (A) and protein (B) expression, as well as promoter PII-specific transcript expression (C) in primary breast ASCs. D, RITA-stabilized p53 inhibits the basal and FSK/PMA-stimulated promoter PII activity in 3T3-L1 cells. Similar to effects observed at the transcript and promoter level, RITA treatment suppresses the PGE₂-mediated activity of aromatase in breast tissue explants (E) and isolated ASCs (F). Asterisks denote effects that are significantly different from control. VC, vehicle control; P+R, PGE₂ + RITA; FSK/PMA, forskolin/phorbol ester; A4, androstenedione; β-gal, β-galactosidase. n = 3, experiment repeated twice. Data presented as mean ± SEM.
p53 interacts with and represses aromatase promoter PII. A, three putative binding sites for p53 were identified on aromatase PII. B, region-specific qPCR was performed on ChIP samples to characterize p53-binding sites. p53 bound with highest affinity to a region that contains the distal p53 response element in ASCs and this interaction was decreased with PGE2 treatment (C). D, aromatase promoter PII reporter assay demonstrating that mutation of the distal p53 response element leads to an increase in promoter activity. VC, vehicle control. Asterisks denote effects that are significantly different from control. n = 3, experiment repeated twice. Data presented as mean ± SEM.

Discussion

This study provides evidence for a novel mechanism for the regulation of aromatase in obesity and breast cancer, namely, via the downregulation of p53 that we found to be a negative regulator of aromatase in human breast ASCs.

The activity of p53 is often suppressed in breast tumors, which leads to uncontrolled cell proliferation. This suppression can occur as a consequence of sporadic TP53 mutations, which are found in more than 30% of breast cancers (27), or due to the downregulation of p53 (reviewed in ref. 28). Nevertheless, TP53 mutations are rare in ASCs (21), suggesting that alternative mechanisms are involved in the regulation of p53 in these cells. Tumor-associated ASCs have an increased rate of proliferation that allows them to encapsulate and infiltrate tumors, a process termed desmoplasia (29). PGE2 is a key inflammatory mediator produced in the adipose tissue in the context of obesity and breast cancer (8, 30, 31), and stimulates the proliferation and inhibits the differentiation of preadipocytes or ASCs (Supplementary Fig. S1A and 32). In the case of obesity, a
Figure 4.
p53 is decreased in tumor-associated ASCs, and perinuclear p53 and aromatase are positively correlated in breast adipose stroma in vivo. A, confocal images demonstrating p53 (green) staining in ASCs from cancer-free breast tissue, normal regions of tumor-bearing breast, and tumor-associated ASCs [representative images (TF9 and non-LF6; Supplementary Table S2); scale bars, 100 μm; n = 10/group]. B, average p53 nuclear intensity in breast ASCs from patients who were tumor-free and from patients with breast cancer. C, perinuclear:nuclear ratio of p53 in ASCs from normal areas of the breast and tumor-associated ASCs. D, data demonstrating a positive correlation between aromatase intensity and perinuclear (inactive) p53 intensity in breast ASCs from normal areas of the breast and tumor-associated ASCs [r = 0.8376; P < 0.0001; representative data (non-LF10; Supplementary Table S2), n = 10]. E, average aromatase fluorescence intensity is higher in tumor-associated ASCs from Li-Fraumeni (n = 14) compared with non-Li-Fraumeni breast tissue (n = 10). Asterisks denote effects that are significantly different between groups.
We and others have previously demonstrated that aromatase transcript expression is lost during the differentiation of ASCs into mature adipocytes (44–46). The bulk of adipose tissue is composed of mature lipid-laden adipocytes and studies examining the effect of PGE2 on aromatase in adipocytes are lacking. We therefore examined the expression and regulation of aromatase in ASCs differentiated in culture, as well as in adipocytes isolated from collagenase digestion of breast tissue (Supplementary Fig. S1B). Consistent with previous reports, aromatase mRNA was significantly lower in differentiated ASCs (3% of ASCs) and undetectable in mature adipocytes. Treatment with PGE2 significantly increased the expression of aromatase in differentiated ASCs, but not to the same extent as in ASCs. Comparatively, aromatase transcript expression under conditions examined was negligible compared with ASCs, and we believe that much of the residual expression may come from cells that have not properly differentiated in culture. These findings are also consistent with immunohistochemistry results, demonstrating that aromatase staining is highest in ASCs of the breast (47).

We also examined the relationship between p53 and aromatase in vivo using breast tissue from women with LFS, with 11 of the 14 having identified germline mutations in TP53. We demonstrate that aromatase staining intensity is higher in ASCs from Li–Fraumeni breast cancer samples compared with non–Li–Fraumeni ASCs, further supporting the hypothesis that p53 is a negative regulator of aromatase. Women with LFS have a dramatically increased risk of developing premenopausal breast cancer (48), with 84% of Li–Fraumeni-related breast tumors being positive for estrogen and/or progesterone receptors (18). Our findings therefore suggest that the increased local expression of aromatase may be a key driver of breast cancer growth in these women.

To confirm the hypothesized inhibitory effect of p53 on aromatase in vitro, we used the compound RITA, which is known to inhibit the interaction of p53 with HDMD2, and hence prevent the proteosomal degradation of p53 (47). Results demonstrate that stabilization of p53 inhibits the PGE2-mediated expression and activity of aromatase, as well as the activity of the aromatase promoter PII. Because we wanted to examine the effect of a fine-tuned regulation of p53, similar to what is observed during the maintenance of energy homeostasis, we used a concentration of RITA that stimulates SCO2 expression (Supplementary Fig. S3). Consistent with this, Weilbacher and colleagues (49) demonstrated that fibroblasts (a.k.a. ASCs) are relatively insensitive to RITA compared with cancer cell lines when examining the effect on cell-cycle arrest and apoptosis. Therefore, the inhibitory effect of RITA on aromatase is likely not to be due to stimulation of ASC cell death. Two mouse studies have previously examined the relationship between p53 and aromatase. Choi and colleagues (50) found that aromatase was increased in p53-inactivated mammary epithelial cells. The increased phosphorylation and nuclear accumulation of CREB was proposed as a mechanism for the increased expression of aromatase. Indeed, CREB and its coactivators (CRTC) are required for the PGE2–dependent upregulation of aromatase (11, 51, 52). It is difficult to know, however, whether stimulation of CREB was a direct result of the loss of p53 or whether it occurred as a consequence of tumor formation, associated with an increase in PGE2. Aromatase has been reported to be expressed in some breast tumor cells, albeit at lower levels than in tumor-associated ASCs (53). It will be important, therefore, to determine whether p53 can regulate aromatase in the human tumorous epithelium and higher number of CLS in the breast adipose is associated with an increase in PGE2 and aromatase (5, 8). In the case of breast cancer, PGE2 is produced by COX-2-expressing tumor cells (33). This study demonstrates that PGE2 significantly down-regulates p53 expression at the transcript and protein level in ASCs that may account for the increased proliferation of these cells in breast cancer. Consistent with this hypothesis, our data demonstrate that p53 expression is decreased in tumor-associated ASCs compared with normal ASCs in tumor-bearing and tumor-free breast tissue. Previous studies have demonstrated that overexpression of COX-2, the rate-limiting enzyme in the biosynthesis of prostaglandins, is associated with the repression of p53-target genes in normal human mammary epithelial cells (34). Interestingly, an inverse association has also been described whereby p53 inhibits COX-2 expression and PGE2 biosynthesis in mouse embryonic fibroblasts (MEF; ref. 35).

We have previously demonstrated that PGE2 can suppress LKB1/AMPK signaling in primary human breast ASCs (11). LKB1/AMPK are master regulators of energy homeostasis that are activated in response to depleted ATP levels. In this study, we demonstrate that phosphorylation of p53 at Ser15 is inhibited by PGE2 treatment. AMPK has been shown to stimulate the phosphorylation of p53 at that site, as well as cause the dissociation of p53 from HDMD2 (human homolog of MDM2), leading to binding to and activation of p53 target genes (16, 36). The implication that p53 may be involved in maintaining energy homeostasis is well supported in the literature. Previous studies have demonstrated that p53 can induce oxidative phosphorylation (37); however, it also inhibits aerobic glycolysis and as a consequence, inhibits the Warburg effect, a phenomenon that refers to the shift in energy production that occurs in rapidly dividing cells. One mechanism whereby this occurs is via the stimulation of TIGAR (38) and SCO2 (synthesis of cytochrome c oxidase 2; ref. 39), and the inhibition of the expression of glucose transporters (40). We have also previously shown that HIF1α, which is known to stimulate the Warburg effect, is stimulated by PGE2 in primary human breast ASCs (13). Taken together, these findings suggest that PGE2 leads to the dysregulation of ASC metabolism, similar to what occurs in tumor cells and that is now regarded as a hallmark of cancer.

Promoter PII-driven aromatase expression is significantly increased in human breast ASCs in obesity and breast cancer (8, 9), and is believed to be responsible for stimulating the growth of estrogen-dependent breast cancer. PGE2 has been shown to be the major driver of aromatase PII-specific expression in ASCs (41). The identification of putative p53-response elements on PII suggested that p53 may be involved in regulating aromatase in ASCs via direct interactions with the promoter. Our findings demonstrate that p53 does indeed interact with aromatase PII in the region that encompasses the distal p53-response element. This interaction is decreased in the presence of PGE2, which may be due to the decreased expression and nuclear localization of p53 in response to PGE2 described in this study. We also demonstrate that mutation of this site is sufficient to cause an increase in the basal activity of PII, supporting the hypothesis that p53 is a repressor of aromatase expression. Transcriptional repression by p53 has previously been shown for several genes, including c-fos, via direct interaction with canonical p53 response elements or indirectly via interactions with other transcription factors or the transcriptional machinery (reviewed in ref. 42, 43).

We and others have previously demonstrated that aromatase...
whether mutations in p53, which occur frequently in these cells, have an impact on the p53-mediated transcriptional repression of aromatase. Another animal study demonstrated that global p53 deficiency in male mice leads to the downregulation of aromatase and results in enhanced lipid accumulation in the liver (54). Contrary to our study and that of Choi and colleagues, their results suggest that p53 stimulates aromatase. Specifically, it was demonstrated that p53 deficiency leads to elevated testosterone levels and an increased ratio of testosterone:estradiol in male mice fed a high-fat diet. Consistent with this animal model, a number of studies in humans and animals have demonstrated that an elevated androgen:estrogen ratio is associated with nonalcoholic fatty liver and increased adiposity (55, 56). However, this group also demonstrates that p53 binds to the mouse aromatase promoter and reporter assays performed in male MEFs demonstrate that p53 causes the increased activity of the mouse aromatase promoter. Opposing results between our study and theirs may be due to a number of factors, including inter-species Cyp19a1 differences (human vs. mouse), sexually dimorphic effects (female vs. male), and cell-specific effects (ASCs vs. MEFs).

Our in vitro studies are also supported by results demonstrating reduced nuclear p53 intensity and increased perinuclear nuclear p53 staining in tumor-associated ASCs, as well as a significant positive correlation between perinuclear or inactive p53 and aromatase in clinical samples. Moll and colleagues (57) previously found that wild-type p53 can accumulate in the cytoplasm of tumor cells in inflammatory breast cancers, leading to functional inactivation of p53. Interestingly, Mollari and colleagues (58) demonstrated that estradiol caused the perinuclear accumulation of wild-type p53 in MCF7 breast cancer cells leading to the G1–S transition. These findings therefore suggest that there is an active mechanism for the decreased expression and nuclear exclusion of p53 in tumor-associated ASCs that consequently leads to the increased expression of aromatase (Fig. 5).

Conclusions

The results presented herein demonstrate that in addition to its conventional role in regulating cell-cycle arrest and apoptosis, p53 may prevent mammary gland hyperplasia and dysplasia of estrogen-dependent breast cancer by inhibiting aromatase expression in ASCs. p53 expression and localization in ASCs is therefore an important indicator of the local production of estrogens in the breast. The role of p53 in the regulation of tumor formation and growth is therefore multidimensional and our study provides evidence for a novel role for p53 in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Wang, H. Sasano, E.R. Simpson, K.A. Brown
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, M.M. Docanto, Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer, K.A. Brown
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