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 cancer. 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 basal 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 extragonadal production of estrogens.
tumorigenic breast tissue have increased expression of PI.3/PII-specific transcripts, hypothesized to occur as a consequence of the increased production of PGE2 from the tumor (9). The stimulation of promoters I.3/PII 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 (CRT2C), liver receptor homolog-1 (LRH-1), and hypoxia-inducible factor-1α (HIF1α; 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 it 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 proapoptotic 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, 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 PII-516 luciferase reporter construct, which contains 502 bp (−516 to −14) of the proximal promoter PII was generated as previously described (22). Mutated CYP19A1 PII-516 luciferase constructs were generated according to the manufacturer’s instruction using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) and primers PII p53-binding mutant F: 5′-AACCTGAGGACCTGAGAAGACTCAT-3′; and PII p53-binding mutant R: 5′-GTAAGGTCACTGAGAAGACCTTT-CATCTGAGGACCTGAGAAGACTCAT-3′; and PII p53-binding mutant R: 5′-GTAAGGTCACTGAGAAGACCTTT-CATCTGAGGACCTGAGAAGACTCAT-3′.

**Human primary breast adipose tissue, ASC isolation, and culture**

Primary human breast adipose tissue was obtained from women undergoing reduction mammoplasty. For explant aromatase activity assays, 1-cm³ pieces of tissue were cultured in serum-free Waymouth medium in 12-well plates. ASCs were isolated following digestion by collagenase and hyaluronidase, as previously described (23). Cells were cultured in Waymouth media containing 15% fetal calf serum and serum starved for 24 hours in Waymouth medium containing 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 RNeasy 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 were used: hArom F (RT7): 5′-TTGAAATGCTGAGCCGTAT-3′, hArom R (RT8): 5′-CAGGAATCTGCCTGGGAGA-3′; aromatase FII F: 5′-GCCAACAGCCTGATGATTGAAC-3′; aromatase PII (ExIIIR): 5′-CACCCGGTTGTGAGTTTGCAGGCACTGCC-3′; p53 F: 5′-CCGTTCTCTAGATTAGGAAAG-3′; and β-actin F: 5′-TCGCCATACATTAGAGGAAG-3′, β-actin R: 5′-GTCTGAGTTTCTCTACCACTCCA-3′. Cycling conditions were one cycle at 95°C for 10 minutes followed by a variable number of cycles of 95°C for 10 seconds, 60°C for 5 seconds and 72°C for 10 seconds for aromatase, or 95°C for 10 seconds, 59°C for 5 seconds and 72°C for 10 seconds for p53 and β-actin. 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
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 nucleomarker 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 × 1024 pixels; Olympus Optical Co. Ltd.) was used to obtain images. Images presented are representative of the majority of cells examined.

**Reporter assays**

3T3-L1 cells (authenticated mouse preadipocyte cell line, purchased from the ATCC) were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) with 10% fetal calf serum. Cells were transfected with wild-type or mutated p53 expression vectors. The p53 antibody CM1 (VP-P955; Vector Laboratories) and nucleotide sequencing was performed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The p53 reporter assay was performed using the p53-Luciferase reporter construct (Promega) according to the manufacturer’s instructions. Aromatase activity was measured using the Chl-IT Express Kit (Active Motif). Some modifications were made from the manufacturer’s protocol as previously described (24).

**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. Hoechst33342, 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 × 1,024 pixels, sequential scanned images). To limit intraexperiment 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 binarized 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 area was then subtracted from the dilated mask to obtain the perinuclear mask. Images were then analyzed using these masks. BreastASCs 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).

**Statistical analysis**

All data are expressed as mean ± standard error mean (SEM). For experiments with two groups, statistical analysis was performed using the Student t test. For experiments with multiple comparisons, statistical analysis was performed using oneway ANOVA. Statistical significance was defined as *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.0001. To determine whether
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 A

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Figure 1. p53 is downregulated by tumor-derived factor PGE2 in primary human breast ASCs. PGE2 treatment results in a significant decrease in p53 transcript (A) and nuclear protein expression (B). C, nuclear p53 immunofluorescence (red) is decreased in ASCs in response to PGE2 (top right) and FSK/PMA (bottom left) compared to vehicle control (VC; top left), and is increased in response to RITA (bottom right). D, quantification of average p53 nuclear staining intensity from C. E, the ratio of perinuclear:nuclear p53 is increased in isolated ASCs in response to PGE2. F, PGE2 causes a significant decrease in phosphorylation of p53 at Ser15. Asterisks denote effects that are significantly different from vehicle control. VC, vehicle control; FSK/PMA, forskolin/phorbol ester. \( n = 3 \), experiment repeated twice. Data presented as mean ± SEM.
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).

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.
To determine whether effects of p53 on aromatase expression are mediated via direct effects on promoter activity, a search for putative p53 response elements was performed using PROMO3.0 (26). Three putative p53 binding sites were identified (Fig. 3A). Binding of p53 to each site was examined using ChIP and specific primer pairs (Fig. 3B). Our results demonstrate that under basal conditions, p53 binds to PII with highest affinity within a region located −453 to −353 bp upstream of the transcription start site (F1R1; Fig. 3B). Treatment with PGE2 leads to a significant decrease in p53 binding to this region (Fig. 3C). Mutation of the distal p53 response element leads to a significant increase in PII reporter activity under basal conditions when compared with wild-type PII (Fig. 3D).

p53 is decreased in tumor-associated ASCs, and perinuclear p53 and aromatase are positively correlated in breast adipose stroma in vivo

Immunofluorescence for p53 was performed on breast tumor and normal breast tissue sections (Fig. 4A; representative image; n = 10/group). Staining for p53 was detectable in the nuclei of normal ASCs from tumor-free and tumor-bearing breast tissue. Conversely, p53 staining was low/undetectable in the nuclei of breast tumor–associated ASCs. Consistent with the qualitative assessment, quantification of the average nuclear p53 intensity in ASCs using Metamorph software revealed a significant decrease in staining intensity in tumor-associated ASCs compared with normal ASCs from cancer-free and tumor-bearing breast tissue (Fig. 4B). Analysis of the subcellular localization of p53 revealed that the ratio of perinuclear:nuclear p53 staining intensity is higher in tumor-associated ASCs compared with ASCs from normal areas of the breast (Fig. 4C).

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.
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 HDM2 (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 PI1-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 PI1-specific expression in ASCs (41). The identification of putative p53-response elements on PI1 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 PI1 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 PI1, 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 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 HDM2, and hence prevent the proteosomal degradation of p53 (17). Results demonstrate that stabilization of p53 inhibits the PGE₂-mediated expression and activity of aromatase, as well as the activity of the aromatase promoter PI1. Because we wanted to examine the effect of a finely-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...
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, Moll 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
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, H. Sasano, Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer, K.A. Brown
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, M.M. Docanto, Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer, C. Lo, K.A. Brown
Writing, review, and/or revision of the manuscript: X. Wang, C. Lo, E.R. Simpson, K.A. Brown
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