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P53 genotype as a determinant of ER expression and tamoxifen response in the MMTV-Wnt-1 model of mammary carcinogenesis

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Abstract

Clinical studies show that estrogen receptor- α (ER) expressing tumors tend to have better prognosis, respond to antiestrogen therapy and have wild-type p53. Conversely, tumors with inactivating mutations in p53 tend to have worse outcomes and to be ER-negative and unresponsive to antihormone treatment. Previous studies from our laboratory have shown that p53 regulates ER expression transcriptionally, by binding the ER promoter and forming a complex with CARM1, CBP, c-Jun, RNA polymerase II and Sp1. In this study, the MMTV-Wnt-1 transgenic mouse model was used to demonstrate that p53 regulation of ER expression and function is not solely an in vitro phenomenon, but it is also operational in mammary tumorigenesis in vivo. The expression of ER and the ability to respond to tamoxifen were determined in mammary tumors arising in p53 wild type (WT) or p53 heterozygous (HT) animals carrying the Wnt-1 transgene. In p53 WT mice, development of ER-positive tumors was delayed by tamoxifen treatment, while tumors arising in p53 HT mice had significantly reduced levels of ER and were not affected by tamoxifen. P53 null tumors were also found in the p53 HT mice and these tumors were ER-negative. ER expression was upregulated in mouse mammary tumor cell lines following transfection with WT p53 or treatment with doxorubicin. These data demonstrate that p53 regulates ER expression in vivo, and affects response to tamoxifen. Results also provide an explanation for the concordant relationship between these prognostic proteins in human breast tumors.

Keywords

p53; Estrogen receptor; MMTV-Wnt-1; Tamoxifen; Breast cancer; Mammary cancer

Introduction

Two important prognostic indicators assessed routinely in breast cancer patients are the estrogen receptor- α (ER) and the progesterone receptor (PR). Increased expression of ER and PR are correlated with better prognosis and increased disease-free survival in response to hormone therapy such as tamoxifen [1,2]. Conversely, loss of functional p53, which occurs in approximately 30–40% of human breast tumors, is an additional indicator of poor prognosis and tends to correlate with ER-negativity, axillary node involvement and poor response to therapy [3,4].

The effect of estrogen on p53 expression is well documented and reports show that ER regulates p53 by two independent mechanisms: transcriptional regulation and protein stabilization [5-7]. In contrast, until recently there has been limited information about the regulation of ER by p53 and the possible impact on tumor development, progression and prognosis. Only two studies have previously demonstrated a direct influence of the p53

pathway on the ER status of the tumors [8,9]. A third study by Lin et al. [10] showed that mammary tumors have variable levels of ER expression in conditional p53 KO mice, depending on the type of Cre promoter and when p53 is lost.

Recently, we have reported a new mechanism by which ER expression is regulated that provides insight into the relationships between p53 and the ER α status in breast cancers. We showed that p53 regulates ER expression in breast cancer cell lines by binding to the proximal promoter in conjunction with other transcriptional cofactors, including CARM1, CBP, c-Jun, RNA polymerase II and Sp1. These results suggest the presence of a regulatory loop, in which ER and p53 regulate each other's expression, establishing a mechanism to balance apoptotic and proliferative signals in mammary cells and tumors [11].

In the current study, we used an animal model to investigate the influence of p53 genotype on the ER expression in mammary tumors in vivo, as well as the role of p53 in mediating response to tamoxifen. For these studies, we chose a bigenic animal model generated by crossing p53 $^{+/-}$ (heterozygotes, HTs) with transgenic (Tg) mice overexpressing Wnt-1 under the control of the mouse mammary tumor virus (MMTV) promoter [12,13]. Although some variability in tumor latency has been reported, most studies show that mammary glands of MMTV-Wnt-1 mice display hyperplasia, that 100% of female mice develop mammary tumors by 65 weeks [12,14] and that approximately 40% of males develop tumors within 1 year [12,13]. Previous studies showed that the loss of p53 accelerates tumorigenesis in Wnt-1 Tg mice, decreasing average tumor latency [15]. These authors also showed that about half of the mammary tumors arising in the p53 HT mice become p53 null due to loss of heterozygosity (LOH) [15].

This report of recent studies in MMTV-Wnt-1 mice shows that the p53 genotype had profound effect on tumor latency, ER α expression and response to tamoxifen, further supporting the concept of a functional link between p53 and response to hormonal therapy in breast cancer.

Materials and methods

Animals and dosing

MMTV-Wnt-1 transgenic mice (C57BL/6 X SJL mixed background) were generated with p53 WT or HT (from 129/sv background) mice for this study and genotyped as previously described [15]. Mice were housed in an AA-ALAC-accredited facility, weaned at 21 days and provided chow and water ad libitum.

Beginning at 7–9 weeks of age, MMTV-Wnt-1 Tg mice were treated by oral gavage, daily (7 days/week) for 75 days with 7.65 mg/kg tamoxifen citrate (Sigma Chemical Co., St. Louis, MO) dissolved in aqueous cyclodextran. Animals were monitored daily for morbidity and/or tumor development and upon detection of either, animals were killed by CO₂ asphyxiation and tissues harvested and portions snap frozen in liquid N₂ for analysis. All manipulations were carried out in strict accordance with IACUC-approved protocols.

RNA extraction

Tumor tissues were homogenized and extracted with Absolutely RNATM RT-PCR Miniprep kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. Whole mammary lysates were incubated on ice for an hour for fat separation. Five micrograms of total RNA were reverse transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers (Promega, Madison, WI). The resulting cDNA was diluted to 1 ng/ μ l working solutions. One arbitrarily chosen wild-type WT tumor from a vehicle-treated animal, as well as whole mammary tissue from a young, untreated Wnt-1 transgenic mouse,

were extracted, reverse transcribed and diluted to 5, 1, and 0.2 ng/μl working solutions. These extracts were stored at -70°C in single-use aliquots and used as calibrators in standard curve determinations.

Real-time quantitative RT-PCR (QPCR)

QPCR was performed using the ABI Prizm[®] 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). ER, TATA box binding protein (TBP), and Wnt-1 primers and probes were designed with Primer Express[®] software (Applied Biosystems) and cross-evaluated with Oligo 4.0[®]. The ER primers and probe used were: forward primer (5'-CCATGACCCTTCACACCAAAG-3'); reverse primer (5'-CCAGCTCGTTCCCTTGGAT-3') and TaqMan[®] probe (5'-FAM-CTCGGAATGGCCTTGCTGCAC-TAMRA-3'). The Wnt-1 primers and probe used were: forward primer (5'-CTGCCTCCTCATCACTGTGTAAA-3'); reverse primer (5'-TGGAACCCAGCACAATAAATAGTTT-3') and TaqMan[®] probe (5'-FAM-CGCAGAGCCACGCGTTCGTTAT-TAMRA-3'). The TBP (reference genes) primers and probe used were: forward primer (5'-GGTGGCA GCATGAAGTGACA-3'); reverse primer (5'-GCACAGAGCAAGCAACTCACA-3') and TaqMan[®] probe (5'-FAM-CCTCTGCACTGAAATCACCTGCAGCA-TAMRA-3'). Both target genes, ER and Wnt-1, were run in duplicate on the same plate, along with the reference gene, TBP. The relative standard curve for ER was generated from the randomly chosen wild-type tumor sample; the standard curve for the Wnt-1 transgene was generated from whole mammary tissue of a young, untreated Wnt-1 transgenic mouse. Three points consisting of 25, 5, and 1 ng sample size per well were added to the assay for a linear standard curve. The average C_t (threshold cycle) of each sample was plotted against the log of the ng cDNA added to the wells of the standard curve, and the reverse log of that number was normalized to its corresponding TBP value. The result is a unitless number that reflects the amount of target gene in each sample relative to the tissue-specific calibrator, and normalized to the reference gene.

Analysis of LOH by southern blot

DNA was extracted using a simplified genomic DNA isolation procedure. The tumors were placed in 600 μl of lysis buffer, digested overnight with proteinase K, isopropanol precipitated, washed with 70% ethanol, and resuspended in 10 mM Tris, pH 8, 1 mM EDTA. The DNA was digested with BamHI, resolved electrophoretically, and probed as previously described [16].

Immunohistochemistry

ER protein expression was evaluated in paraffin-embedded tumor sections by immunohistochemistry using the avidin-biotin-complex method. Endogenous peroxidase activity was inhibited by incubation in 3% H₂O₂ for 10 min and non-specific binding was blocked by incubation with the Blocking Reagent (cat no. BS966M, Biocare Medical, Concord CA), also for 10 min. Antigen retrieval was performed by incubating slides in 10 mM Citrate Buffer, pH 6.0, for 15 min in a microwave oven. Sections were then incubated with the ERα (MC-20) rabbit polyclonal antibody (Stanta Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:500. Anti-rabbit-HRP (DAKO EnVision plus labeled polymer, Hamburg, Germany) was used as secondary antibody, followed by development with the DAKO DAB Enhancer (Dako, Hamburg, Germany). Slides were counterstained with hematoxylin.

Frozen sections of selected tumors were also used for the IHC detection of ERα using similar techniques. For the frozen sections two rabbit polyclonal ER antibodies were used, one from Zymed (South San Francisco, CA), and the other was a generous gift from Dr. Geoffrey Greene (ER21) [17]. Both antibodies gave the same results.

Uterine sections were used as positive controls. Negative controls consisted of either substituting preimmune rabbit serum for the primary antibody or preincubation of the primary antibody with the immunizing ER peptide. In the case of the paraffin sections, we also used adjacent normal mammary gland as an internal positive control to rule out possible fixation artifacts.

Slides were evaluated by two pathologists (CJC and IBGC) in a blinded fashion using the Allred scoring method, which takes into account the intensity of the stain and the proportion of positive cells [18,19]. Representative microphotographs of tumors from mice with different genotypes were taken with an Olympus photomicroscope (Olympus America Inc, Center Valley, PA).

Cell lines, transfections, treatments, and western analysis

The MMTV-Wnt-1 D4 and G4 cell lines were originally derived at the National Cancer Institute by Dr. Lyuba Varticovski. The initial cell lines (W1204 (G4)) and W1308 (D4)) were isolated from a brie of tumors arising in MMTV-Wnt-1, p53 WT (+/+) mice, as previously described [20]. Both cell lines are ER+/PR+ and readily form tumors when transplanted into the mammary fat pad or subcutaneously in C57BL/6 mice. 4T1 and NMuMG cells were obtained from ATCC (Manassas, VA). Cell cultures were grown in RPMI medium with 10% FBS. For transfection and doxorubicin experiments, cells were transferred to serum-free medium with 1% BSA overnight. Cells were then transfected with a WT p53 expression vector [21] (gift of Dr. Bert Vogelstein), using Lipofectamine 2000 (Invitrogen). Controls included cells that were mock transfected (no DNA) or transfected with a control vector (pGL2basic; Promega). Twelve hours after transfection, cells were lysed in RIPA buffer and protein concentrations were determined by BCA assay (Pierce; Rockford, IL). Three separate transfection experiments were conducted. For treatments, cells were plated and serum-deprived, as described above, and treated with 50 nM doxorubicin (dox; Tocris Bioscience) for the times indicated in the figure. Treatments were repeated three times and representative blots are shown.

Fifty micrograms aliquots of protein were electrophoresed on 10% acrylamide/SDS gels (BioRad; Hercules, CA) and electroblotted onto polyvinylidene difluoride membranes (Pierce). Blots were probed with primary antibodies against ER (1:1000; Zymed), p53 (1:5000; Santa Cruz Biotechnology; Santa Cruz, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Abcam; Cambridge, MA) in 5% nonfat dry milk in PBS with 0.1% Tween-20. Blots were then incubated with HRP-linked secondary antibodies, either goat anti-rabbit IgG (1:2000 to 1:10,000; Cell Signaling; Danvers, MA) or goat anti-mouse IgG (1:5000; Santa Cruz), and bands visualized by enhanced chemiluminescence (Pierce).

Statistical analysis

The ER results were analyzed using both t-tests and two factor (treatment and genotype group) analysis of variance. Because the standard deviations were proportional to the means, both the raw data and log transformed (base 10) data were analyzed. The *t* test results are given in the figure legends.

The two-factor analysis of variance was used to assess treatment effect and interaction between treatment and genotype. ER expression in tumors with LOH was analyzed using the Tukey HSD post hoc test for the raw data, and the log-transformed data was used to assess ER expression in LOH tumors compared to p53 HT and WT tumors.

Results

We initially examined spontaneous mammary tumor development in female MMTV-Wnt-1 transgenic mice with either WT or HT p53 genotype. As shown in Fig. 1, development of mammary tumors in Wnt-1 Tg mice was affected by the p53 genotype, as seen previously in MMTV-Wnt-1/p53 null mice but not in p53+/- mice [15]. However, since the power used in the current study was greater than in the original study conducted by Donehower et al. [15] (16 WT and 32 HT mice were used in the original study while 115 WT and 83 HT were used in the experiments described here) we were able to show that the proportion of tumor-free animals was significantly reduced in the p53 HT mice compared to the p53 WT mice ($P < 0.001$). In our hands, approximately 50% of the Wnt-1 Tg mice with WT p53 remained tumor-free for ≥ 55 weeks, whereas the median tumor-free survival for p53 HT animals was 30 weeks. These results show that loss of just one allele of p53 accelerated mammary tumorigenesis driven by the Wnt-1 transgene.

We then used this animal model to investigate whether the regulatory effect of p53 on ER previously described by our laboratory in cell lines was operational in vivo. This was tested by measuring ER expression by QPCR in tumors from MMTV-Wnt-1 Tg mice with either WT or HT p53 genotype. Since Donehower et al. [15] showed that both p53 null and p53 HT tumors develop in p53 HT (+/-) mice, we first confirmed LOH in study tumors by Southern blot (Fig. 2a). These results were used to stratify 38 tumors into three groups: p53+/+ (WT), p53+/- (HT), and p53-/- (null). Quantification of ER expression by QPCR in these tumors showed that the p53 genotype had a profound influence on the expression of ER (Fig. 2b). ER expression was significantly greater in tumors arising in p53 WT than in p53 HT mice ($P = 0.03$). In tumors with LOH at the p53 locus (p53-/-), ER levels were reduced by over 6-fold compared to tumors with WT p53 ($P = 0.004$), and over 3-fold compared to p53 HT tumors ($P < 0.001$), indicating that the level of ER α expression was closely associated with p53 genotype.

We also analyzed the expression of ER protein by immunohistochemistry in frozen sections and paraffin embedded tissues. In the evaluation of paraffin sections, positive tissue controls (mouse uterus) as well as internal controls (areas of normal mammary gland) were used to evaluate possible fixation artifacts. Both paraffin and frozen section gave almost identical results and Fig. 3 shows representative fields of the immunohistochemical staining of tissues and tumors in paraffin-embedded sections. All the tumors analyzed contained an internal control consisting of normal mammary ducts (panel A). As indicated in panel B, the strongest nuclear staining of ER protein was seen in p53 WT tumors. Reduced ER staining was seen in p53 HT tumors (panel C), and a further reduction was noted in p53 null tumors that have undergone LOH (panel D). In sections shown in panels C and D, reduced staining as well as more irregular areas and negative foci were observed.

A more detailed evaluation using the Allred scoring system was carried out in the paraffin-embedded sections. The WT p53 tumors gave an average score of 5.4 ± 0.24 ($n = 5$), while the HT p53 tumors that retain one p53 allele had an average Allred score of 3.3 ± 0.66 ($n = 3$). For those tumors from HTs that had demonstrated LOH, the score was 2.5 ± 0.5 ($n = 8$). The differences between WT and HT tumors were highly significant ($P < 0.01$). Overall, these results were consistent with the quantification of ER mRNA shown in Fig. 2.

To demonstrate a causal relationship between expression of p53 and ER, we transfected either WT or noncoding p53 expression vectors into three mouse mammary tumor cell lines and one untransformed mammary epithelial cell line. Two of the cell lines, MMTV-Wnt-1 D4 and MMTV-Wnt-1 G4, were derived from tumors arising in MMTV-Wnt-1/p53 WT mice [20] and the third line, 4T1, is an established cell line derived from a spontaneous

Balb/c mammary adenocarcinoma [22]. A WT p53 expression vector [21] was transiently transfected into the three cell lines and levels of ER protein expression was determined 12 h later by Western blot analysis. As shown in Fig. 4, overexpression of WT p53 in all three mammary tumor cell lines resulted in up-regulation of ER expression. Transfection of p53 into NMuMG, a ER-negative “normal” mouse mammary cell line derived from Swiss mice [23], was unable to induce ER expression (data not shown), suggesting that in these cells the ER gene is permanently silenced, possibly by promoter methylation [24]. Additionally, D4 and G4 cells were treated with DNA damaging agent doxorubicin, to upregulate endogenous p53, which also resulted in increased ER expression (Fig. 4d, e).

In order to determine if the effect of the p53 genotype on ER expression is biologically relevant, we investigated the effect of tamoxifen on the development of mammary tumors in MMTV-Wnt-1 mice with two p53 genotypes. P53 WT and p53 HT mice were treated daily with either tamoxifen (7.65 mg/kg) or vehicle for 75 days. Treatment was initiated when animals were sexually mature, i.e., 7–9 weeks of age, allowing for the normal development of mammary glands in an endogenous hormonal milieu. As shown in Fig. 5, 10 weeks of daily administration of tamoxifen significantly affected tumor incidence and latency in p53 WT mice ($P = 0.035$), but not in p53 HT mice ($P = 0.669$). These data showed that tumors arising in p53 HT mice not only developed more rapidly, confirming our first experiment, but also that they were more refractory to antiestrogen inhibition.

To rule out the possibility that tamoxifen treatment affected transgene expression, tumor-free mammary glands from tamoxifen- and vehicle-treated animals were evaluated for Wnt-1 mRNA expression by real-time quantitative RT-PCR (QPCR). There was no significant change in normalized transgene expression in p53 WT or HT mice treated with tamoxifen (data not shown). We also investigated whether the treatment with tamoxifen had an effect on the expression of ER in either WT or HT mice. Although results showed a small reduction of ER levels in tumors from tamoxifen-treated WT mice, the difference was not statistically significant ($P = 0.41$). In the tumors from HT animals, ER levels were virtually identical in vehicle- and tamoxifen-treated mice (Fig. 5b).

Discussion

We have recently provided evidence demonstrating that p53 binds to the proximal promoter of the ER gene in conjunction with other transcriptional cofactors, including CARM1, CBP, c-Jun, RNA polymerase II and Sp1, and has a strong effect on ER transcription [11]. In the present study, we investigated whether this regulatory effect was fully functional in an animal model that closely resembles many features of human breast cancer.

The mechanisms leading to ER-negative breast tumors are still controversial. An attractive new hypothesis, which has gained support of many investigators in the last few years and is supported by solid data, suggests that the development of ER-negative carcinomas results from transformation of stem or progenitor cells (reviewed in [25]). On the other hand, there is a large body of literature accumulated over the last 30 years showing that changes in signal transduction pathways that result from mutations in critical regulatory genes can affect the ER status of breast tumors (reviewed in [26,27]). It is important to note that the different hypotheses that have been formulated to explain the development of ER-negative tumors are not mutually exclusive and the extensive research suggests that the control of ER expression in breast cancer is likely to be both very complex and governed by multiple factors.

The data presented here fully support our previous in vitro observations [11] by showing that the response of mammary tumorigenesis in the MMTV-Wnt-1 model to hormonal

manipulation is dependent on the p53 genotype. Results also demonstrate a tight correlation between the p53 genotype (WT, p53^{+/-}, and p53^{-/-}) and ER expression at both the mRNA and protein levels.

Crosstalk between ER and p53 is an important feature of tissue development, homeostasis and tumorigenesis [5,6,28,29]. E₂ has been shown to up-regulate p53 expression and promoter activity in breast cancer cell lines [5,28]. Likewise, treatment of female mice with E₂ plus progesterone increases mammary gland expression and nuclear localization of p53 [29]. Although the p53 promoter does not contain a consensus estrogen-responsive element, CCAAT-binding transcription factor-1, and nuclear factor- κ B binding sites in the promoter are necessary for E₂ responsiveness [28], indicating that ER may regulate p53 expression indirectly as part of a multi-protein complex. ER has also been shown to inhibit p53 degradation by HDM2 via direct binding to p53 and HDM2 [6].

In contrast, until recently, data supporting a role for p53 in regulating ER have been scarce and indirect. Agents that induce p53 have been shown to stabilize ER protein levels and abolish E₂-dependent ER turnover [8]. One report showed that p53 up-regulates the expression of ER mRNA in MCF-7 breast cancer cells [9]. Another study demonstrated that spontaneous tumors in p53 conditional knockout mice can be either ER-positive or negative [10]. These and other authors speculate that the variation in ER status in mammary tumors may be attributed to differences in the cell of origin [10,12].

Although mouse mammary cancer models have traditionally been considered hormonally unresponsive, recent evidence indicates sex steroids can impact tumorigenesis. Medina et al. [30] demonstrated the dependence of premalignant mammary lesions on ovarian steroids. In addition, studies using Tg mice with polyoma middle T antigen under the control of the MMTV promoter demonstrated not only hormonal dependence of tumor development, but also the presence of ER protein in resulting mammary tumors [31]. In other genetic models, ER expression is detectable in normal ductal epithelium and in early in situ lesions, even though mammary tumors from these models are primarily ER-negative [30,32-34].

Hormonal influence on the tumorigenesis in Wnt-1 mice has also been demonstrated by crossing them with ER knockout (ERKO) mice to generate Wnt-1/ER null mice [14]. Tumor development in the Wnt-1/ERKO females is significantly delayed compared to Tg animals with functional receptor [14]. Likewise, ovariectomy of prepubertal Wnt-1 Tg females similarly delays tumor development [14,35]. Both of these results suggest that ER-mediated signaling contributes to Wnt-1-induced tumorigenesis. However, in both ERKO and prepubertally ovariectomized females, mammary gland development is significantly impacted, which likely contributes to delayed tumorigenesis [14]. Zhang et al. [35] also reported the development of ER⁺ tumors in p53 WT, MMTV-Wnt-1 mice, but found that growth of transplanted tumors is not inhibited by ovariectomy or tamoxifen treatment. However, in these studies, ablative treatments were not initiated until after the transplanted tumors reached 0.7 cm in diameter. Similarly, in MMTV-Wnt-1 Tg mice, if ovariectomies are performed after mammary tumors have developed, reports indicate that there is little effect on tumor growth [14].

In the current study, tamoxifen treatment was initiated postpubertally, but before tumors were detectable, and results showed that tumorigenesis was responsive to antiestrogens in the early stages of development. Since about 50% of tumors arising in p53 HTs undergo LOH, we also demonstrated a relationship between p53 gene dosage and ER expression in vivo. Our data are consistent with the interpretation that tamoxifen treatment inhibits tumorigenesis in p53 WT (+/+) animals but is not effective in inhibiting tumors in animals with reduced levels of ER α . Consistent with our findings, clinical studies carried out in

patients with high breast cancer risk have shown that tamoxifen [36] or raloxifene [37] prevent the development of ER α -positive tumors but have no effect on development of ER α -negative tumors. Interestingly, authors of a recent study by Konduri et al. [38] show that ER antagonists disrupt ER α -p53 complexes, releasing active p53 and inhibiting cellular proliferation. Thus, the effect of tamoxifen appears to be dependent on both ER and p53 levels. Unfortunately, there have been no studies to date focused on the effects of tamoxifen in patients with increased breast cancer risk as a result of the presence of germ line mutations in p53 (Li-Fraumeni patients). Our results predict that SERMs will be ineffective in preventing the development of breast cancer in these patients.

A better appreciation of the relationship between p53 and ER pathways maybe important in understanding how different p53 mutations, acquired during tumorigenesis, affect ER expression and hormonal responsiveness. Our results in this model indicate that acquisition of p53 mutations during tumorigenesis that cause loss of p53 expression or function, will result in downregulation of ER and lack of tamoxifen response. Further, our results show that activation of endogenous p53 by treatment with doxorubicin or transfection of WT p53 induces ER expression, demonstrating that these cells retained the ability to upregulate receptor expression if p53 is WT.

Taken together, these results indicate that ER and p53 are interactively controlled, possibly as participants in a functional regulatory loop that maintains the balance between ER and p53 activity. Our results suggest that the regulation of expression is positive in each direction, i.e., ER action upregulates p53 expression and loss of p53 is associated with down-regulation of ER expression. Since ER activity stimulates proliferation while p53 inhibits it, this loop may be part of a developmental program, retained in some tumors, that controls proliferation and organogenesis (as diagrammed in Fig. 6). Mammatrophic hormones may activate such a program during development or at other phases of robust proliferative growth.

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Abbreviations

MMTV	Mouse mammary tumor virus
dox	Doxorubicin
E₂	17 β -estradiol
ER	Estrogen receptor- α
HT	Heterozygous
LOH	Loss of heterozygosity
QPCR	Real-time quantitative RT-PCR
Tg	Transgenic
WT	Wild-type

Fig. 1. Kaplan-Meier curves of MMTV-Wnt-1 transgenic mice with different p53 genotypes. Wnt-1 transgenic mice with WT p53 ($n = 115$) had a significantly longer tumor free interval (increased latency) compared to p53 HT mice ($n = 83$), $P < 0.0001$

Fig. 2.

Estrogen receptor expression in tumors arising in Wnt-1 transgenic animals varies with p53 genotype. **a** Southern analysis of tumors arising in Wnt-1 transgenics on a p53 HT background, showing tumors were either HT or null (LOH) for p53. Arrows denote bands from the *p53* pseudogene (10 Kb), the *p53* knockout (KO) allele (6.5 Kb), the *p53* WT allele (~5 Kb) and effects of vehicle (HT Veh; $n = 37$), $P = 0.67$. **b** Estrogen receptor levels correlate with p53 status. ER mRNA levels were determined by QPCR, as described in the “Materials and methods” section. Compared to tumors from transgenic mice with WT p53, ER mRNA levels were reduced about 2-fold in tumors from p53 HT mice (* $P = 0.03$) and further reduced by 6-fold in tumors with LOH (HT with LOH) (* $P = 0.004$). Mammary tumors with LOH at the p53 locus had a 3-fold reduction in ER message levels compared to tumors arising in p53 HT mice that did not undergo LOH (** $P < 0.001$)

Fig. 3. Immunolocalization of ER α in paraffin sections of mouse mammary tumors arising in MMTV-Wnt-1 transgenics. ER protein expression paralleled the level of ER message as determined by QPCR (Fig. 2). **a** Normal mammary gland adjacent to a p53 WT tumor (internal positive control), **b** p53 WT tumor, **c** p53 HT tumor, and **d** p53 null tumor

Fig. 4.

Increased expression of WT p53 in mammary tumor cells upregulates ER. **a–c** Mammary tumor cell lines 4T1, MMTV-Wnt-1 D4, and MMTV-Wnt-1 G4 were either mock transfected (no vector) or transiently transfected with 0.5 or 2.0 μg of WT p53 or a control, noncoding vector. Cell lines, G4 (**d**) and D4 (**e**), were treated with DNA-damaging agent doxorubicin (50 nM) for the time periods indicated in the figure. Results presented are representative Western blots of whole cell lysates and were sequentially probed with antibodies against ER, p53, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) as described in the “Materials and methods” section

Fig. 5.

The effect of tamoxifen treatment on tumor development depends on p53 genotype. **a** Ten weeks of tamoxifen treatment significantly delayed tumorigenesis in Wnt-1 transgenics on a WT p53 background (WT Tam; $n = 58$) compared to vehicle (WT Veh; $n = 57$), $P = 0.035$. Conversely, tamoxifen treatment did not alter tumor development in Wnt-1 transgenics on a p53 HT background (HT Tam; $n = 46$) compared to vehicle (HT Veh; $n = 37$), $P = 0.669$. **b** Treatment with tamoxifen did not affect ER expression in tumors from Wnt-1 Tg mice with p53 WT ($P = 0.41$) or p53 HT ($P = 0.38$). Numbers at the base of each column refer to the number of tumors of each genotype/treatment group, which were analyzed by QPCR. Data are presented as ER mRNA expression relative to TBP (reference gene)

Fig. 6. Hypothetical regulatory loop involving p53 and ER in the mammary gland. P53 up-regulates ER expression and ER upregulates p53 expression. While p53 leads to cell cycle arrest or apoptosis, activation of ER leads to proliferation, differentiation, and mammary gland development. This loop maintains a balance between proliferation and growth arrest in the mammary gland