

Hormone-Induced Chromosomal Instability in p53-Null Mammary Epithelium

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ABSTRACT

The absence of p53 function increases risk for spontaneous tumorigenesis in the mammary gland. Hormonal stimulation enhances tumor risk in p53-null mammary epithelial cells as well as the incidence of aneuploidy. Aneuploidy appears in normal p53-null mammary epithelial cells within 5 weeks of hormone stimulation. Experiments reported herein assessed a possible mechanism of hormone-induced aneuploidy. Hormones increased DNA synthesis equally between wild-type (WT) and p53-null mammary epithelial cells. There were two distinct responses in p53-null cells to hormone exposure. First, Western blot analysis demonstrated that the levels of two proteins involved in regulating sister chromatid separation and the spindle checkpoint, Mad2 and separase (*ESPL1*) were increased in null compared with WT cells. In contrast, the levels of securin and Rad21 proteins were not increased in hormone-stimulated p53-null compared with WT cells. *ESPL1* RNA was also increased in p53-null mouse mammary cells *in vivo* by 18 h of hormone stimulation and in human breast MCF7 cells in monolayer culture by 8 h of hormone stimulation. Furthermore, both promoters contained p53 and steroid hormone response elements. Mad2 protein was increased as a consequence of the absence of p53 function. The increase in Mad2 protein was observed also at the cellular level by immunohistochemistry. Second, hormones increased gene amplification in the distal arm of chromosome 2, as shown by comparative genomic hybridization. These results support the hypothesis that hormone stimulation acts to increase aneuploidy by several mechanisms. First, by increasing mitogenesis in the absence of the p53 checkpoint in G₂, hormones allow the accumulation of cells that have experienced chromosome missegregation. Second, the absolute rate of chromosome missegregation may be increased by alterations in the levels of two proteins, separase and Mad2, which are important for maintaining chromosomal segregation and the normal spindle checkpoint during mitosis.

INTRODUCTION

Genetic instability is a frequently reported event in p53 gene-deleted cells. The genetic instability observed in both normal and tumor cells of p53 knockout mice is evidenced by high incidences of aneuploidy (1, 2), centrosome amplification (2, 3), and loss of heterozygosity (4, 5). The frequency of these events is dependent on cell type and on specific cell stimuli. For example, inactivation of p53 in the diploid human colorectal cancer cell line HCT116 generates neither aneuploidy, chromosomal instabilities nor increased sister chromatid exchange (6). In a mouse transgenic model for choroid plexus tumors, absence of p53 does not generate chromosomal imbalances as measured by comparative genomic hybridization or flow cytometry (7). In the absence of p53, aneuploidy and centrosome amplification are common occurrences in cell cultures of fibroblasts

but infrequent in cell cultures of mammary epithelial cells (1, 8). However, a short-term exposure (5 weeks) to steroid hormones is sufficient to generate a high frequency of aneuploidy in these same mammary cells. Interestingly, centrosome amplification is not observed concomitantly with the aneuploidy (8).

Aneuploidy is a common characteristic of tumors (9–11) and has also been proposed as a necessary event for tumorigenesis (11, 12). Aneuploidy occurs not only in p53-null mouse mammary cells but also in estradiol-induced mammary tumors in the August Copenhagen Irish rat (13, 14). There is considerable controversy as to the importance of aneuploidy in tumorigenesis and tumor progression, although there is general agreement that it plays some role in the generation of the malignant phenotype (15, 16). The molecular mechanisms for generating aneuploidy are thought to be diverse, but all involve deregulation of some aspect of chromosomal replication and segregation (17). The mechanisms may be very specific, as in alterations of centrosome replication (18) and fidelity of the spindle apparatus, or in regulation of sister chromatid separation during mitosis (17). Additionally, the mechanisms may be passive, such as increased mitogenesis that leads to increased frequency of cells with missegregated chromosomes in the absence of normal cellular checkpoints (17, 19, 20). Finally, the mechanism might be nonspecific and generated as a result of random DNA damage. Estrogens are genotoxic in certain cell types, such as the rodent kidney cell (21, 22) and produce DNA adducts and mutations.

Both p53 heterozygous and p53-null mouse mammary epithelial cells are at increased risk for tumorigenesis (23, 24). The tumors are aneuploid and metastatic, and the pathogenesis mimics that observed in human breast cancer (25). The absence of p53 gene function does not significantly disturb normal mammary development, measured either morphologically (8, 26) or functionally (27). However, hormone stimulation of the gland increases not only tumorigenic risk but also the frequency of aneuploid cells (8). The mechanism of this hormone effect has not been established. In the experiments reported herein, we examine the possible cellular mechanisms for hormone-induced aneuploidy and conclude that the effect is both indirect because of increased mitogenesis and direct because of increased expression of two proteins involved in regulating chromosome segregation, separase (*ESPL1*) and Mad2.

MATERIALS AND METHODS

Transplantation

All of the mice were bred and maintained in a conventional mouse facility at Baylor College of Medicine with food and water provided *ad libitum* and the room temperature set at 70°F. The animal facility is American Association of Laboratory Animal Care accredited. Samples of mammary ducts were isolated from 7 8-week-old p53-null and p53 wild-type (WT) BALB/c mice and transplanted into the cleared mammary fat pads of 3-week-old WT BALB/c mice (23). The transplanted duct samples grew and filled the fat pads in 6–8 weeks. In each experiment, at least one fat pad was processed as a whole mount at 8 weeks to examine the growth and morphology of the outgrowth

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(23). The remaining transplanted samples were examined as described below for the individual experiments.

In experiment 1, the proliferative and apoptotic indices of WT and p53-null normal mammary glands in the presence and absence of hormonal stimulation were examined using two different protocols. In both approaches, samples of the p53 WT normal duct and the p53-null normal duct were transplanted into the contralateral fat pads of each mouse. The first approach was a modification of an earlier experiment where the transplanted epithelium was subjected to a 5-week period of hormone stimulation starting two weeks after transplantation (8). In this modification, a silastic tubing containing estrogen (50 μg) plus progesterone (20 mg) was used instead of a pituitary isograft as in the original experiment. A pituitary isograft results in marked increases in the circulating levels of prolactin and progesterone (28). Five transplants were collected from the hormone-treated and untreated control mice for BrdUrd-labeling index. In the second approach, the mice were untreated until 8 weeks after transplantation, when the transplanted ducts had filled the mammary fat pad and were entering into a steady growth state. The animals were divided into three groups: untreated, received a silastic tubing containing estrogen (50 μg) and progesterone (20 mg), or untreated. Two-three transplants from each group were collected at 3 and 4 weeks thereafter and examined for morphological development, BrdUrd-labeling index, and apoptotic index.

Cell Culture

MCF-7 cells contain WT p53. The MCF-7 cells were seeded in improved minimal essential medium (IMEM) supplemented with 5 $\mu\text{g}/\text{ml}$ bovine insulin and with 5% charcoal stripped serum for 24 h then in serum-free IMEM for another 48 h. Cells were exposed to hormones or left untreated in fresh serum-free media and harvested at 0, 0.5, 2, 8, 20, and 48 h posttreatment.

Immunohistochemistry

Samples of the transplants were evaluated for BrdUrd index by standard methods as described in Ref. 29. For BrdUrd immunohistochemistry, the samples were fixed in cold 4% paraformaldehyde for 1 h before being processed for paraffin-embedded sections. The antibody to BrdUrd was BD PharMingen BrdUrd *In Situ* Detection Kit (BD Biosciences, San Diego, CA). Animals were injected with BrdUrd (50 mg/kg body weight) 2 h before sacrifice. At least five separate samples were examined for each outgrowth line for each assay.

Apoptotic Index

Samples of transplant generations 7–8 were fixed in 10% neutral buffered formalin, embedded and stained with H&E, and evaluated for apoptotic indices by the terminal deoxynucleotidyl transferase-mediated nick end labeling method (30). For this assay, at least 3 samples were read per outgrowth and 500 cells counted per slide.

Genetic Analysis

Cytogenetic Analysis. Samples of the p53-null and p53 WT normal mammary cells, both untreated and hormone-stimulated, as well as p53-null primary tumors were evaluated for chromosome number and cytogenetic changes using conventional cytogenetic techniques as described in Ref. 8. For each preparation, 50 metaphases were counted. The types of cytogenetic changes examined included breaks, dicentrics, translocations, and aneuploidy.

Comparative Genomic Hybridization. Comparative genomic hybridization was performed on the hormone-stimulated mouse mammary epithelial cells of p53 WT and p53-null genotype. Each individual sample was composed of epithelial cell pellets isolated from three mammary fat pads. Comparative genomic hybridization was performed as described previously (31, 32). Normal control DNA was prepared from spleen tissue of normal mice, and test DNA was prepared from p53^{-/-} and p53^{+/+} mammary cells, using standard DNA extraction protocols. Test DNA was labeled with biotin-16-dUTP and control DNA with digoxigenin-11-dUTP (Boehringer Mannheim Corporation, Indianapolis, IN), using nick translation. Five hundred ng of each labeled genome (control DNA and test DNA) were hybridized in the presence of excess mouse Cot-1-DNA (Invitrogen, Carlsbad, CA), to metaphase chromosomes prepared from a karyotypically normal mouse. The biotin-labeled test genome was visualized with avidin conjugated to FITC (Vector Labora-

tories, Burlingame, CA), and the digoxigenin-labeled control DNA was visualized with a mouse antidigoxigenin antibody (Sigma, St. Louis, MO), followed by a goat antimouse antibody conjugated to tetramethylrhodamine isothiocyanate (Sigma). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole and embedded in antifading agent to reduce photobleaching. Gray scale images of the FITC-labeled test DNA, the tetramethylrhodamine isothiocyanate-labeled control DNA, and the 4',6-diamidino-2-phenylindole counterstain for at least 15 metaphases/sample were captured with a cooled charge-coupled device camera (CH250; Photometrics, Tucson, AZ) connected to a Leica DMRBE microscope equipped with fluorochrome-specific optical filters TR1, TR2, and TR3 (Chroma Technology, Brattleboro, VT). Quantitative evaluation of the hybridization was performed using a commercially available comparative genomic hybridization analysis software (CW4000CGH). Average ratio profiles were computed as the mean value of at least 8 ratio images and were used to identify changes in chromosome copy number.

Western Blots

Western blot analysis of the effect of hormone stimulation on the expression of mitotic proteins involved in sister chromatid cohesion and separation was performed as described in Ref. 33. Briefly, after five weeks of hormone stimulation, the mammary glands containing transplants of both genotypes were collected, the epithelial cells were isolated and concentrated by gentle enzymatic treatment as described in Ref. 34, and the epithelial cell pellet processed for protein evaluation. The protein lysate was prepared as described previously (33). Cell pellets or tissue samples were lysed in radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate) or PBS containing 1% Triton X-100 (v/v), 0.5% (w/v) sodium deoxycholate, and 1% SDS (w/v) containing protease and phosphatase inhibitors (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg pepstatin/ml, 30 μl aprotinin/ml, 0.5 μg leupeptin/ml, 100 mM sodium orthovanadate, and 100 mM sodium fluoride; all from Sigma-Aldrich, St. Louis, MO) for 10–15 min on ice, followed by passage through a 21G needle. When appropriate, additional phosphatase inhibitors cocktail I and II (Sigma-Aldrich) were added to the lysis buffer at a dilution of 1:100. Lysates were then centrifuged at 1000 $\times g$ for 20 min, and the supernatants were aliquoted and frozen at -80°C until use. After protein quantification (using detergent compatible protein dye and BSA standards from Bio-Rad; Hercules, CA) and normalization, 30–40 μg of protein extracts were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The filters were initially blocked with 5% nonfat-dry milk in Tris buffer saline containing 0.1% Tween 20 for 1–2 h at room temperature and then probed with hseparase mAb(1:500), p53CDC(1:500), Cdh1(1:500), mouse securin(1:250), hBub1(1:500), Mad2(1:500), hRad21(1:100), cyclin B1(1:500), Cdc2(1:1000), cyclin E(1:5000), Rad51(1:500), and β -actin antibodies(1:100,000). All of the antibodies except hRad21, hCdh1 (Neumarkers, Fremont, CA), and hseparase (a gift from Jan-Michael Peters, Research Institute of Molecular Pathology, Vienna, Austria) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The bound antibodies were visualized by the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, England) in combination with the horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies as appropriate, and intensity of the specific bands in the exposed films was quantified. In some of the later studies, bound primary antibodies were detected with IRD800 dye-labeled appropriate species-specific secondary antisera, and signal was visualized on a Li-Cor Odyssey IR scanner (Lincoln, NE).

Northern Blots

Total RNA was isolated using RNeasy mini kit from Qiagen. Fifty micrograms of total RNA were diluted in $\times 2$ RNA sampling buffer [20% formaldehyde; 1.65% Na_2HPO_4 (pH 6.8); 63.5% formamide; and 1 \times loading buffer] and separated on 1.0% agarose gel with 1 M formaldehyde in 1 \times running buffer [0.2 M 3-*N*-(morpholino) propanesulfonic acid, 50 mM sodium acetate, and 10 mM EDTA (pH 7.0)]. After transfer onto Nytran super charge membrane (Schleicher & Schuell), prehybridization, and hybridization in Clontech ExpressHyb solution at 75°C without/with [^{32}P]CTP-labeled *ESPL1* cDNA probe for 2 h, the membrane was washed 15 min twice with 1 \times washing buffer containing 300 mM NaCl, 30 mM sodium citrate, and 0.05% SDS at room

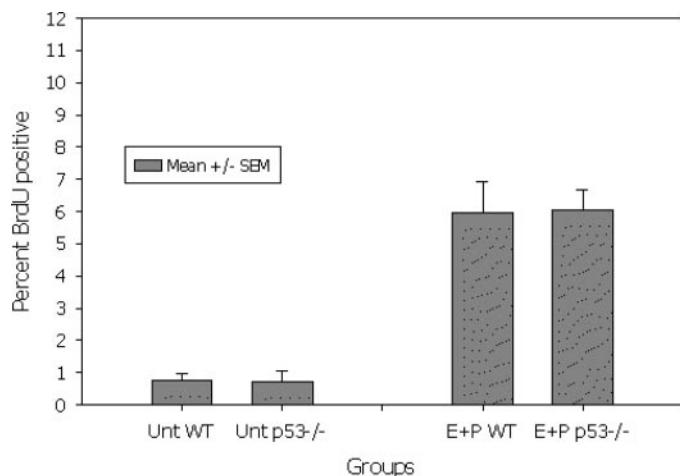


Fig. 1. Proliferation indices in p53-null and p53 WT normal epithelium exposed to estrogen (E) and progesterone (P) for 5 weeks. Mice received a silastic tubing of estrogen and progesterone at 2 weeks after transplantation. Bars, \pm SEM; $n = 5$ /group. (Unt, untreated).

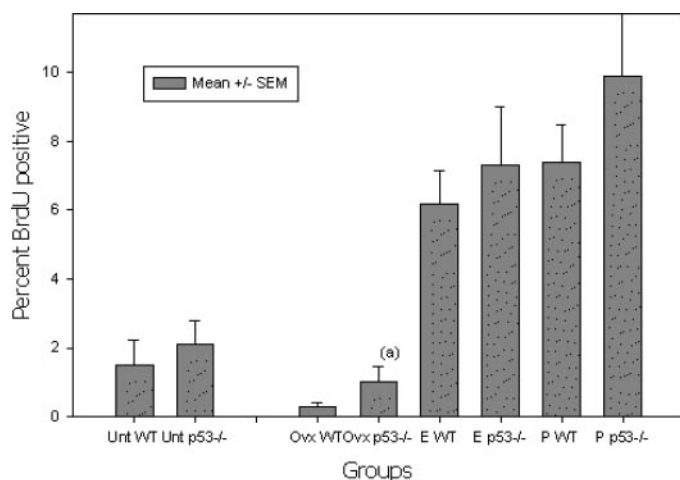


Fig. 2. Proliferation indices in p53-null and p53 WT normal epithelium exposed to estrogen (E) alone, progesterone (P) alone, or absence of estrogen and progesterone. Treatments were for 3–4 weeks starting at 8 weeks after transplantation. Bars, \pm SEM; $n = 5$ /group. (a, significantly different from WT; OVX, ovariectomized).

temperature followed by two washes with wash solution containing 15 mM NaCl, 1.5 mM sodium citrate, and 0.1% SDS at 50°C for 20 min each. Blots were visualized on a phosphor screen, and bands were quantitated on a STORM Imager (Molecular Dynamics). The membrane was then stripped (0.1 \times saline sodium citrate and 1% SDS) and rehybridized with keratin 18 or β -actin probes as a control. The absorbance of the bands was quantified using ImageQuant software, and the values were normalized relative to the control.

Promoter Analysis

The sequence of the 5' upstream region of both mouse and human *ESPL1* (separase) and *Mad2* genes were extracted using the Ensembl database⁸ and Stanford Source database⁹ and analyzed for potential transcription start sites using Genomatix Suite and a neural network promoter prediction program. Potential transcriptional factor binding sites were analyzed using the Transcription Element Search Software and MatInspector/TRANSFAC programs (35).¹⁰

⁸ Web address: www.ensembl.org.

⁹ Web address: genome-www5.stanford.edu/cgi-bin/SMD/source/.

¹⁰ See the following Web sites: <http://genome.ucsc.edu/>, <http://www.ensembl.org>, <http://www.genomatix.de/>, <http://www.cbil.upenn.edu/tess/index.html>, <http://www.cbrc.jp/research/db/TFSEARCH.html>, and http://www.fruitfly.org/seq_tools/promoter.html.

RESULTS

Effect of Hormones on Cell Proliferation. It is well established that both estrogen and progesterone induce cell proliferation in the normal mammary gland although to different extents and in different cell compartments. Therefore, the induction of aneuploidy in the p53-null cells by only low doses of progesterone was unexpected (8). We examined the effects of estrogen and/or progesterone on cell proliferation as a function of time of exposure. Examination of the whole mounts indicated that the two cell genotypes responded in a similar manner to the hormones. In the untreated mice, the mammary cells were organized as well-spaced primary and secondary ducts. The absence of hormones (ovariectomy) resulted in ducts with very narrow lumina, thus giving the appearance of atrophic ducts. The effect of estrogen was to increase slightly ductal density and to increase the size of the duct lumina. The effect of progesterone was distinct from estrogen because it led to an increase in small duct branching but only occasional alveolar buds. Estrogen and progesterone together resulted in development of alveoli.

The proliferation results are shown in Figs. 1 and 2. Three results are of interest. First, it is evident that the p53-null cells had a similar proliferative activity as the p53 WT cells at 7 weeks (Fig. 1) and at 11–12 weeks (Fig. 2) posttransplantation. The former time period represents cells approaching a steady state (80% fat pad filled) and the latter time period a maintained steady state with respect to proliferation. All of the three hormone treatments increased the proliferative activities of both the WT and null cells. The absolute percentage increases were the same for the WT and null cell phenotypes and were 4.1- and 3.5-fold, respectively, in estrogen-treated cells (Fig. 2), 4.9- and 4.7-fold, respectively, in progesterone-treated cells (Fig. 2), and 7.8- and 8.3-fold, respectively, in estrogen-progesterone treated cells (Fig. 1). Thus, there was little evidence of increased susceptibility of the null cells to hormone-induced regulation of proliferation. The deletion of hormones reduced the proliferative activities of the WT and null cells by 81% and 52%, respectively, at 4 weeks after ovariectomy (Fig. 2), confirming earlier experiments that the null cells, like WT cells, were ovarian hormone-dependent. The slightly higher proliferative activity of null cells in ovariectomized mice is statistically significant ($P < 0.05$) and probably has some significance over the lifetime of the mouse, because some tumors do develop in ovariectomized mice (36), but not in the short term.

The apoptotic indices were evaluated at 3 weeks after hormone stimulation. Apoptotic activity was low ($\leq 1\%$) and was not different with respect to hormone treatment or p53 status (data not shown).

The results of the cytogenetic analyses are shown in Table 1 and Fig. 3. Wild-type normal mammary cells were not examined, because such cells are uniformly diploid and rarely show aberrations (8). The

Table 1. Cytogenetic analysis of p53 null mammary epithelium

Group	No. aneuploid (%)	\bar{X} Chromosome number	Total aberrations	Type aberrations*
Untreated	0/50 (0)	39.6	6	1/5
Untreated	2/50 (4)	39.4	3	3/0
Untreated	9/50 (18)	49.2	16	6/10
Pituitary isograft	7/50 (14)	41.4	14	7/7
Pituitary isograft	23/50 (46)	56.7	17	3/14
Estrogen	3/50 (6)	41.8	12	4/8
Progesterone	14/50 (28)	50.6	7	3/4
Progesterone	17/50 (34)	49.2	14	10/4
Tumors (irrad.)	37/50 (74)	67.2	25	19/6
Tumors (unt.)	45/50 (90)	65.9	27	25/2
Tumors (irrad.)	49/50 (98)	59.7	26	8/18
Tumors (irrad.)	49/50 (98)	68.0	10	5/5
Tumors (irrad.)	49/50 (98)	75.8	5	2/3

Abbreviations: irrad, irradiation; unt, untreated.

* chromosomes/chromatid.

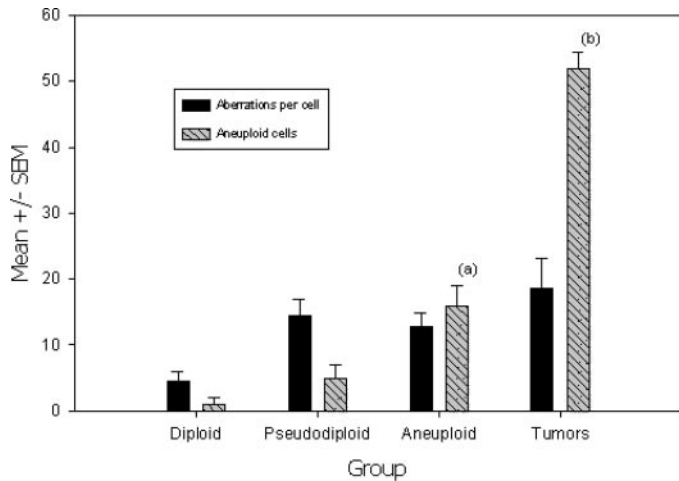


Fig. 3. Cytogenetic analysis of p53-null mammary epithelium. The groups analyzed were untreated p53-null normal ($n = 3$), pituitary isograft-stimulated p53-null normal ($n = 2$), progesterone- or estrogen-treated p53-null normal ($n = 3$), and randomly selected primary tumors ($n = 5$). The p53-null normal cells were collected from treatments described in experiment 1 (Fig. 1). Wild-type normal mammary were not examined because such cells were uniformly diploid. Bars, \pm SD. (a, $P < 0.05$ compared with diploid and pseudodiploid; b, $P < 0.05$ compared with aneuploid).

pseudodiploid cell populations were those defined as 41–42 chromosome number. As expected, the number of aneuploid cells increased with hormone stimulation and was greatest in the tumors. The frequency of chromosome aberrations increased slightly in the pseudodiploid cells but did not accumulate to larger numbers in the aneuploid “normal” cells or tumors. This difference was not statistically significant when analyzed by ANOVA. Thus, it appeared that chromatid and chromosome aberrations reflected the underlying state of genetic instability in the p53-null cells but were not correlated directly with aneuploidy. We interpret these data, along with unpublished sister chromatid exchange results, to indicate that the hormonal induction of aneuploidy was not a direct consequence of hormone-induced DNA damage.

The results of the comparative genomic hybridization studies are shown in Fig. 4. The hormone-stimulated (via pituitary isograft) p53-null cells showed an average of 7.3 alterations per sample compared with 2.0 for the hormone-stimulated p53 WT cells. The alter-

ations were distributed randomly over the genome with the exception of a consistent gain in the distal region (H2–3) of mouse chromosome 2 (3/3). The majority of the alterations (18/22) were gains.

The results of the Western blot analysis for mitotic proteins are shown in Fig. 5. Consistent with previous experiments, hormone stimulation increased the level of DNA synthesis to \sim 8% compared with 2% observed in the transplants in the nonpituitary isograft bearing mice. Several mitotic proteins in their native form either increased from undetectable or very low levels to high levels (separate and securin) or increased in posttranslational modified forms (p55CDC, Cdh1, and Mad2) as a consequence of hormone stimulation. Bub1 was decreased significantly (Fig. 5A). The effect of hormones on mitotic-related proteins appeared to be selective as expression of a number of other proteins involved in various phases of the cell cycle remained unchanged (Fig. 5B). Rad21(M), cyclin E1(G₁-S), Rad51(S), Cdc2(G₂-M), and cyclin B1(G₂-M) protein levels remained at similar levels. The lack of an effect on cohesion Rad21, a sister chromatid cohesion protein, indicates the specificity and selective effect of hormones on this process. In addition, additional increases of separate and Mad2 were detected in p53-null cells compared with p53 WT cells in both untreated transplants (Mad2) and hormone-stimulated transplants (Mad2 and separate; Fig. 5C). Of interest, securin was not increased additionally in hormone-stimulated p53-null cells compared with hormone-stimulated WT cells (Fig. 5D). The quantitative analysis of these changes is shown in Fig. 5D.

To determine whether the increase in protein reflected a direct effect of hormones on gene expression or an indirect effect because of alveolar cell differentiation over the 5-week period, we evaluated the mammary transplants after short-term hormone stimulation. Seven weeks after transplantation, mice were exposed to chronic hormone treatment (50 μ g E2 and 20 mg P) via silastic implants, and 8 mice were exposed to blank implants. At 18 and 48 h of hormone stimulation, the transplants were collected, epithelial cell pellets prepared, and RNA isolated. Northern blot analysis revealed marked increases (\geq 4x) in *ESPL1* mRNA at 18 and 48 h posttreatment compared with controls (Fig. 6).

To obtain a more precise picture of the time course of hormone-induced gene transcription of *mESPL1*, we used an established *in vitro* model of human breast cells, the MCF7 cancer cell line. This was

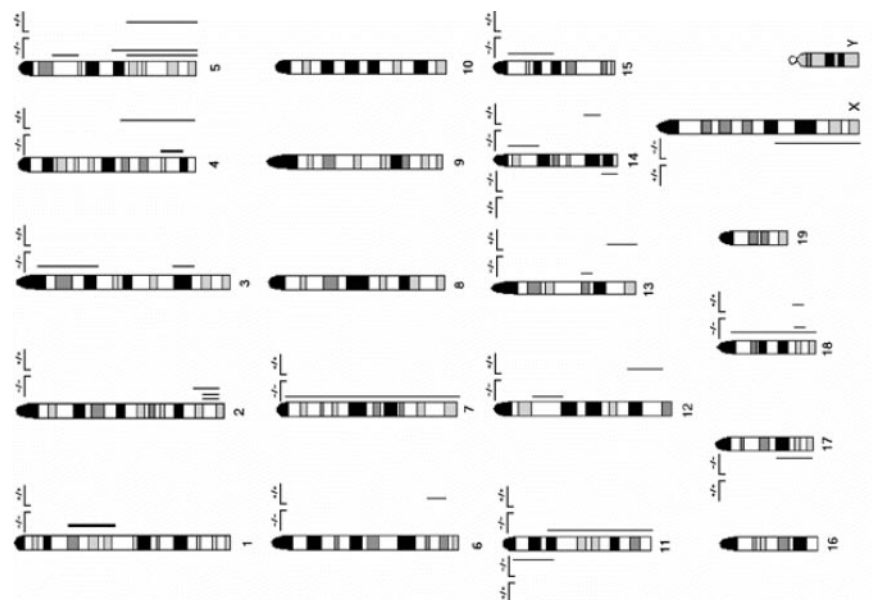


Fig. 4. Karyogram of the DNA copy number changes observed in the p53-null mammary cells obtained from 3 different samples compared with p53 wild-type mammary cells obtained from 3 different samples. Bars on the left side of the chromosome ideogram indicate losses, and bars on the right side indicate gains. Bold bars on the right side indicate amplifications. For each chromosome, the bars have been grouped to summarize the results of the p53^{-/-} cells and the p53^{+/+} cells.

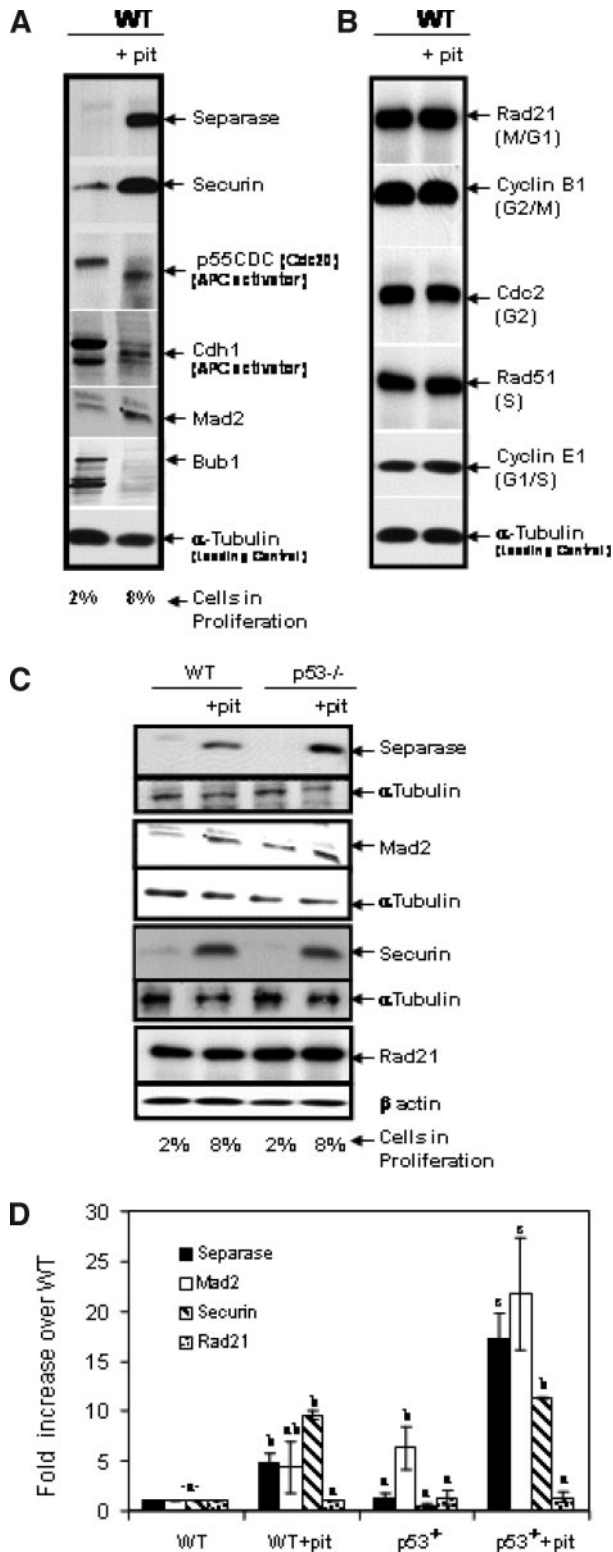


Fig. 5. Western blot analysis of the effect of steroid hormone on the expression of mitotic proteins involved in sister chromatid cohesion and separation and spindle damage checkpoint. *A*, represents the expression of separase, mitotic inhibitor securin, anaphase promoting complex/C regulators p55Cdc and Cdh1, and anaphase promoting complex inhibitor Mad2 and Bub1 in mammary glands with WT p53 in the absence and presence of pituitary (+*pit*) isograft. *B*, represents effect of hormones on expression of a number of other proteins involved in various phases of cell cycle control. *C*, shows the differential expression of separase, Mad2, securin, and Rad21 in p53 WT versus null p53^{-/-} glands. *D*, represents expression of separase, Mad2, securin, and Rad21 as fold increases over p53 WT glands after normalization to the expression of a housekeeping gene α -tubulin/ β -actin to compensate for loading control. Data are the average of the means from two (Mad2 and securin) to four (separase and Rad21) experiments. Individual values were compared using Student's *t* test. Values with dissimilar symbols are different ($P < 0.05$). Bars, \pm SE.

necessary because mouse mammary cells are notoriously unresponsive to estrogen and progesterone in monolayer cell culture. Fig. 7 shows a time-dependent increase in *hESPL1* mRNA and protein levels with hormone treatment. The mRNA reached a maximum 4 \times increase by 8 h of hormone treatment that persisted for 48 h. Increases in protein started at 8 h with continuing increases at 20 and 48 h.

In view of the hormonal stimulation of separase and Mad2 protein expression (Fig. 5, *A* and *C*, and separase mRNA, Figs. 6 and 7), we examined the sequence of the 5' upstream region of both mouse and human *ESPL1* (encodes separase protein) and *MAD2* genes for potential transcription start sites and potential transcriptional factor binding site (Fig. 8) using bioinformatics tools (35).¹⁰ In mouse *ESPL1*, we identified a strong promoter sequence with a score of 0.99 (a score of 0.85 has a 0.1–0.4% false positive prediction rate; Fig. 8A). A TATA box (TATAT) is found 30 bp 5' of the putative transcriptional initiation site. Along with a number of putative transcriptional binding sites, two sequences that have 100% homology to the consensus sequence of estrogen responsive element and progesterone responsive element at 203 bp and 93 bp 5' of transcription start sites, respectively, and a sequence that is 90% homology to the consensus sequence p53 transcriptional activation element were identified (Fig. 8A). Sequence analysis also indicated a putative p53 transcriptional repressor element, at position 1636 bp 5' of transcription start sites, that is identical to the consensus sequence. The p53 transcriptional repressor element is a recently identified *cis*-acting sequence element identified on the basis of its involvement in the suppression of p53-mediated promoter activation (37). Analysis of human *ESPL1* sequence also indicated presence of p53, p53 transcriptional repressor element, progesterone responsive element, and estrogen receptor binding elements (data not shown). The presence of the p53 and steroid responsive elements signify a potential transcriptional control of separase expression by p53 and steroids hormones. It is interesting to note that compared with WT glands, p53-null mammary cells have a significantly higher level of Mad2 and a slightly higher separase (Fig. 5D). Analysis of both mouse and human *MAD2* promoter sequences (Fig. 8B) indicated the presence of a putative progesterone responsive element, estrogen responsive element, and p53 transcriptional repressor element, but not p53 transcriptional activation element sites.

The increase in Mad2 levels was initially surprising to us; therefore,

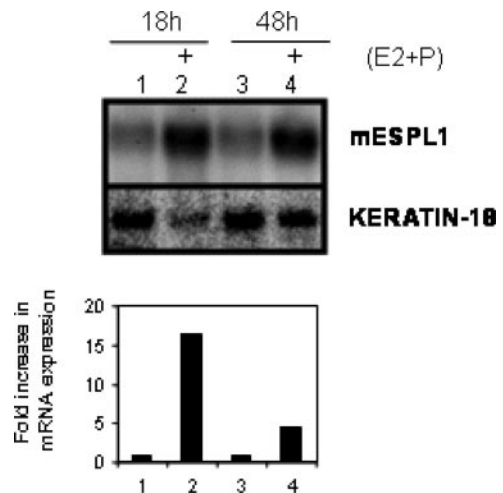


Fig. 6. Transcriptional activation of *mESPL1* mRNA by progesterone (*P*) and estrogen (*E2*) treatment in p53-null mammary gland cells *in vivo*. Mammary gland cells were treated *in vitro* with *E2+P* for 18 h and 48 h, and Northern blot analyses were performed. Keratin-18 is shown to compare loading. Optical densities of the bands were quantified using ImageQuant software. The quantified *mESPL1* mRNA levels were divided by the intensity of the corresponding keratin-18 bands.

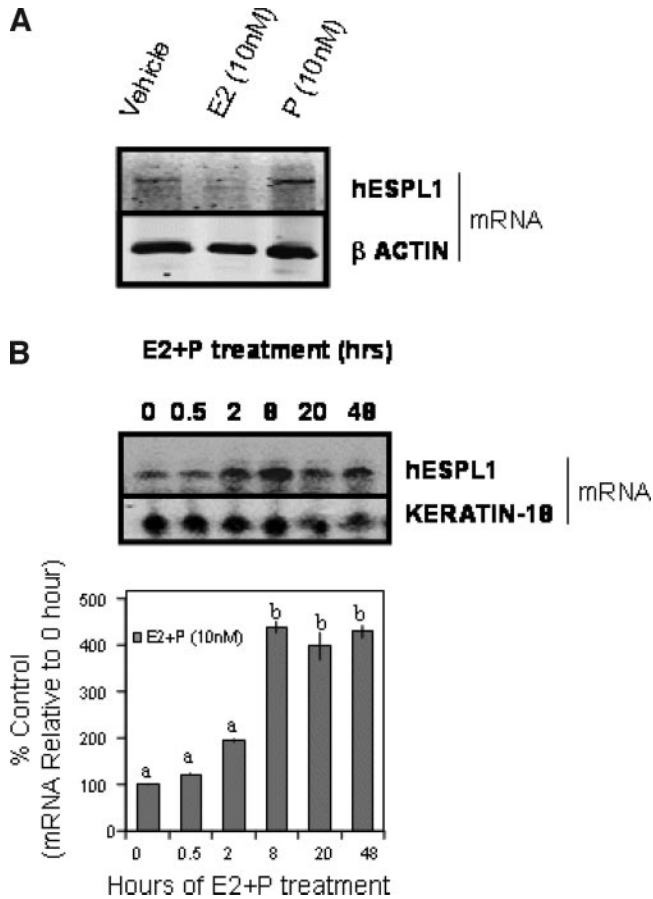


Fig. 7. Transcriptional activation of *hESPL1* mRNA by progesterone (P) and estrogen (E2). Northern blot analysis of *hESPL1* mRNA levels in MCF-7 cells. Cells were seeded in 5% charcoal-stripped serum IMEM for 24 h, seeded in serum-free IMEM for another 48 h, and treated with ethanol (vehicle) or P (10 nM), E2 (10 nM; A), or E2+P (10 nM each; B) in fresh serum-free media as indicated. The mRNA levels were determined as described in the text below. Time course of the combined treatment of E2 and P at a dose of 10 nM each on the expression of *hESPL1* mRNA in MCF7 cells. Keratin-10 is shown as loading control. Bottom panel B represents relative *hESPL1* mRNA levels from two independent experiments with respect to the housekeeping gene keratin-10 that was normalized and presented as % control; bars, \pm SE. Values associated with dissimilar symbols are significantly different ($P < 0.05$ from one another).

we pursued this observation using immunohistochemistry of normal and tumor tissues. Fig. 9 illustrates the patterns of MAD2 expression and localization. Mad2 is detectable at low frequency and intensity of staining in normal cells of p53 WT and p53-null (Fig. 9A). However, in small hyperplasias (Fig. 9, A and B), Mad2 frequency and intensity is markedly increased, and localization in the cytoplasm as well as the nucleus becomes apparent. In tumors, Mad2 continues to be highly expressed, and cytoplasmic localization can be significant (Fig. 9, C and D).

DISCUSSION

The presence of aneuploidy in tumor cells is undisputed. However, the significance of aneuploidy as an obligatory mechanism of tumorigenesis and tumor progression is controversial (15, 16). In an earlier study, we reported that hormones greatly increased the frequency of aneuploidy in normal mammary cells that had lost the p53 tumor suppressor gene (8). Additionally, these same hormones increased the frequency of tumorigenesis, and the resultant tumors were also highly aneuploid (36). Beyond this initial correlation, the possible mechanisms by which hormones might induce aneuploidy were not addressed. The results described herein address this major question.

First, the results show that the p53-null and p53 WT cells exhibit a similar proliferative response to estrogen and/or progesterone. These results, together with previous results demonstrating a similar degree of dependence on these ovarian hormones for growth in ovariectomized mice as well as for tumor development (36), argue strongly that the cellular responses to these hormones with respect to control of proliferation are normal and should not be considered as dysregulated. Additionally, analysis of the RNA transcriptome demonstrates that the p53-null mammary cell responds normally to these hormones with respect to milk protein synthesis (27). Thus, an altered proliferative response is not likely the cause of the marked increase in aneuploidy.

Second, the hormone-induced aneuploidy in the p53-null cells was not correlated with increases in chromosomal breaks, dicentrics, or translocations, which are markers for DNA damage. The lack of any increase in DNA damage as determined by two different assays (sister chromatid exchange and oxidative damage) also does not support the idea that the hormones were increasing these biochemical events.¹¹ Previously, we ruled out the mechanism of centrosome duplication (8) as a source of aneuploidy; thus, the data thus far suggest that these two mechanisms (*i.e.*, DNA damage and centrosome duplication) are not important in the hormone-mediated induction of aneuploidy in normal p53-null mammary cells.

Given the above results, we examined the mechanism proposed by Pihan and Doxsey (19) that increased aneuploidy in these cells is a consequence of mitogenesis. Lengauer *et al.* (38) argue that the rate of chromosomal missegregation with normal mitosis is on the order of 1% of mitotic events and that this missegregation would elicit a cell cycle checkpoint, thereby preventing accumulation of aneuploid cells in the organism. However, in the absence of a normal G₂ checkpoint, as in p53-null cells, there would be an accumulation of cells with abnormal mitoses. Although some of these would be nonviable cells, it is likely that viable, aneuploid cells would accumulate in the cell population. An increased frequency of proliferation would shorten the time for aneuploid cells to accumulate in the cell population. This scenario is exactly what is indicated by our results. In the virgin animal, proliferation is low, and the appearance of detectable aneuploid cells increases with host age so that there is no detectable aneuploidy in p53-null cells at 8–14 weeks of age but low levels of aneuploidy at 22–26 weeks of age (8). With hormone-induced increases in proliferation in p53-null cells, there is a marked induction of aneuploidy. Importantly, there is no aneuploidy in p53 WT cells.

These results raise the question whether the absence of p53 confers merely a passive mechanism for generation of aneuploidy or whether p53 is more directly involved by altering the transcriptional regulation or functional activities of genes that are important for proper chromosomal segregation. There have been considerable advances in understanding the genes involved in sister chromatid separation and chromosome segregation (17). The relationship between p53 and the genes involved in regulating chromosome segregation is not well established. The results reported herein indicate that the levels of several proteins important for the regulation of orderly sister chromatid separation are increased in mitosis. More importantly, the levels of two of these proteins (Mad2 and separase) are increased in hormone-treated p53-null cells compared with hormone-treated p53 WT mammary cells. Hormone treatment also increased separase RNA expression. The increase in separase RNA was observed in normal p53-null mouse cells *in vivo* by 18 h and in p53 WT MCF7 human cancer cells *in vitro* by 8 h. These results support the idea that hormones were directly causing an increase in separase gene expression. The significance of the increase in separase is inferred from the knowledge of its

¹¹ Unpublished data.

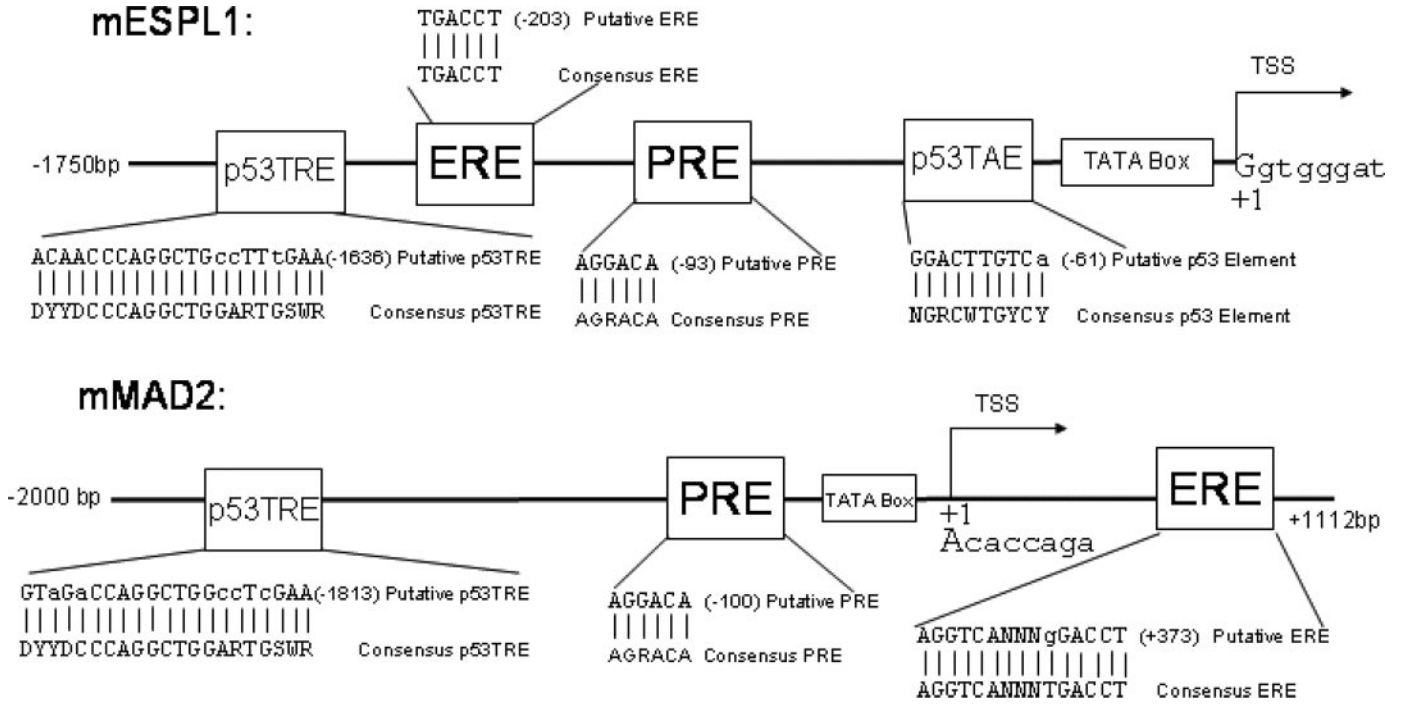


Fig. 8. Schematic drawing showing the putative sequence motifs of the separate and Mad2 promoters. Arrow, the predicted transcription start site (TSS); and C, the +1 position. T ATAT TATA-box at -30, putative progesterone responsive element (PRE), estrogen responsive element (ERE), p53 transcriptional activation element (p53TAE) and p53 transcriptional repressor elements (p53TRE) are also shown. The following IUPAC-IUB codes for nucleotides were used: R, purine (A or G); Y, pyrimidine (C or T); W(A or T); S(G or C); D (G, A, or T); N, any nucleotides.

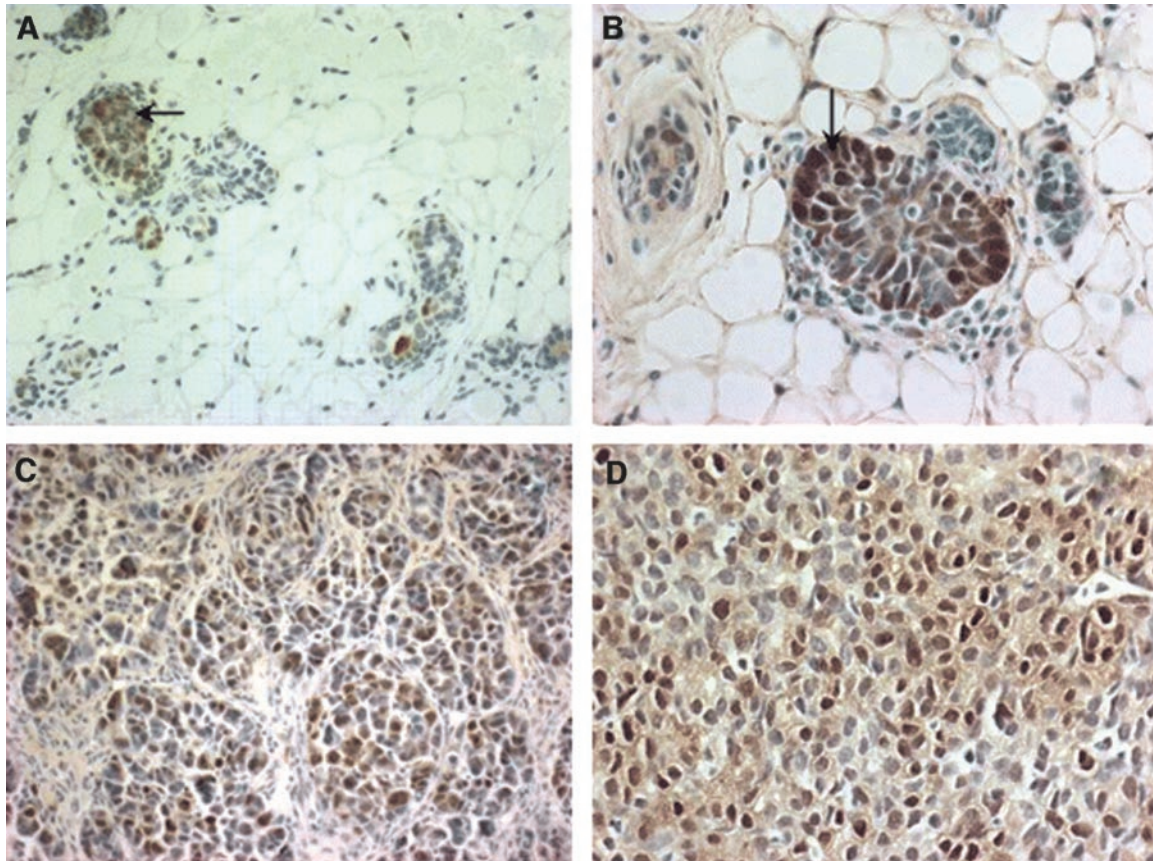


Fig. 9. Mad2 immunohistochemical staining of p53-null normal mammary cells (A and B), ductal hyperplasia (arrows in A and B), and mammary cancers (C and D). The staining is low in normal appearing ducts but strongly enhanced in hyperplasias and cancer. The cytoplasmic staining is readily apparent in D. Magnification is $\times 20$ (A and C) and $\times 40$ (B and D).

function, because separase activity is necessary for sister chromatid separation (17). A direct effect of estrogen on Mad2 gene expression has been reported also by an *in vivo* study in mouse uterus using microarray analysis, where a 6.5-fold increase in Mad2 RNA was reported after estrogen treatment (39).

Interestingly, securin and Rad21 protein levels were not increased in the hormone-stimulated p53-null cells compared with the hormone-stimulated WT cells. Securin is already elevated as a consequence of hormone stimulation; however, Rad21 protein remains at basal levels. If securin and separase are high, one might predict lower levels of Rad21 because of the increased protease activity represented by separase. One explanation for this paradox might be found in the dynamics of Rad21 localization in mammalian cells. The majority of Rad21 dissociates from the centromere in prophase and localizes as unbound protein in the nucleoplasm (40). The cleaved protein from the centromere will represent a small fraction of the total Rad21 pool and is unlikely to alter the signal detected by the antibody on a Western blot. Alternatively, the increased separase protein may represent a catalytically inactive protein. This question remains to be answered.

Why the two proteins (Mad2 and separase) are differentially regulated by p53 is explained by the analysis of the respective promoters of the two genes. Both promoters contained p53 and steroid hormone response elements. The *ESPL1* promoter differed from the *MAD2* promoter in the additional presence of a p53 transcriptional activation element. Analysis of p53-regulated gene expression from a published study using oligonucleotide arrays identified *MAD2* as one of the genes that is repressed by p53 (41). Previous observations suggest that the p53 transcriptional repressor element sequence can modulate p53-dependent transcriptional activation in a position and promoter-independent manner (37). These results support the hypothesis that in WT mammary cells, p53 can act to dampen steroidal induction of separase and Mad2 gene transcription. In the absence of p53 function, steroid hormones manifest their uninhibited effects by significant induction of *ESPL1* and *MAD2* gene expression that facilitates aneuploidy. The physiological significance and mechanism of p53 repression and its coregulation, if any, with the p53 activation binding elements is yet to be elucidated.

The significance of elevated Mad2 is more difficult to understand because this protein functions to inhibit the anaphase promoting complex, and the functional activity of the latter complex is necessary for releasing separase activity (17). Haploinsufficiency of Mad2 results in chromosome instability (42). Also, the appropriate functioning of the spindle checkpoint requires a critical ratio of Mad1 and Mad2 to ensure a pool of Mad1-free Mad2 (43). An excess of Mad2 as well as insufficient Mad2 might result in an altered spindle checkpoint. Another possibility is that the increased form of Mad2 is an inactive form, but this remains to be tested experimentally. Finally, it is possible that the increased levels of Mad2 are only related indirectly to the increased aneuploidy but rather have effects on other cellular functions (44–46). It has been reported recently that increased levels of Mad2 are present in gastric tumors (47) and ovarian tumors (48). The results reported herein demonstrated increased levels of Mad2 in mammary hyperplasias and tumors. Additionally, the localization of Mad2 in these lesions was both nuclear and cytoplasmic compared with just nuclear in normal cells. These results along with reported interactions of Mad2 with proteins thought to be unrelated to control of mitosis (44–46) suggest that Mad2 affects multiple cellular functions. In either event, the observation that proteins involved in regulation of sister chromatid separation are altered in p53-null cells provide a mechanism that is consistent with aneuploidy occurring in the absence of other suggested mechanisms (*i.e.*, DNA damage and centrosome duplication).

Are the effects of hormones more encompassing than just increased levels of separase and Mad2 driving chromosome missegregation? We have tried to assess this question using two different approaches. First, analyzing the pattern of chromosomal gains and losses by comparative genomic hybridization revealed one consistent chromosomal alteration in hormone-stimulated p53-null cells compared with hormone p53 stimulated WT cells. The most consistent change occurred in the distal region of chromosome 2, a region in the mouse harboring genes such as MMP9, kinesin, β -2 microglobulin, and intracisternal A particles. This region is syntenic to human chromosome 20q11.21 and 20q13.32. Genes of interest in this region include aurora kinase A, breast carcinoma amplified sequence 1, ubiquitin-related protein sumo-1, protein tyrosine phosphatase 1B, and CCAAT/enhancer binding protein β . We are examining the expression of some of these genes at the protein level; namely, aurora kinase A and CCAAT/enhancer binding protein β .

The second approach used was serial analysis of gene expression, which showed alterations in ~1% of the transcriptome of the hormone-stimulated p53-null mammary cells (27). These genes included known p53-regulated genes such as gelsolin, Gadd45b, and Igfbp5, and other genes not thought to be directly regulated by p53, such as intracisternal A particles, MMP9, β -2 microglobulin, and wdm1 (27). The first three of these genes are located on the distal arm of chromosome 2. The role of any of these genes in regulation in sister chromatid separation and/or mitosis is not suspected.

The amplification of intracisternal A particles, a gene that is a transposon, suggests another possibility for generating aneuploidy and/or increased RNA expression of the genes. The gain of this region was also observed as increases in intracisternal A particles by electron microscopy.¹² The increase in transposon activity has been shown to activate specific gene functions in other mouse models of mammary tumorigenesis (49, 50).

Based on the current accumulated data, we are proposing that hormone facilitation of aneuploidy in p53-null mammary epithelial cells occurs by several mechanisms. First, we suggest that hormones have an indirect role in the induction of aneuploidy by markedly increasing the mitotic frequency of these normal cells and a direct role by increasing the expression levels of proteins regulating chromosome segregation. We hypothesize that the absence of p53 function results in both an aberrant separase activity and an aberrant G₂ checkpoint that together allows the accumulation of cells with aneuploidy as a function of time and mitotic frequency. The presence of aneuploidy has an indirect effect on tumorigenesis by increasing stochastically the frequency of altered expression of genes involved in growth regulation and invasiveness. Second, we speculate that hormones specifically increase expression of genes localized in the distal region of chromosome 2, which contains genes related to control of chromosome segregation (*e.g.*, aurora kinase A), as well as genes involved in premalignant progression (*e.g.*, CCAAT/enhancer binding protein β and SUMO-1). Future experiments will test these hypotheses.

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¹² D. Medina, unpublished result.

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