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neu/ERBB2 Cooperates with *p53-172H* during Mammary Tumorigenesis in Transgenic Mice

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Thirty percent of human breast cancers have amplification of *ERBB2*, often in conjunction with mutations in p53. The most common p53 mutation in human breast cancers is an Arg-to-His mutation at codon 175, an allele that functions in a dominant oncogenic manner in tumorigenesis assays and is thus distinct from loss of p53. Transgenic mice expressing mouse mammary tumor virus-driven *neu* transgene (MMTV-*neu*) develop clonal mammary tumors with a latency of 234 days, suggesting that other events are necessary for tumor development. We have examined the role of mutations in p53 in tumor development in these mice. We have found that 37% of tumors arising in these mice have a missense mutations in p53. We have directly tested for cooperativity between *neu* and mutant p53 in mammary tumorigenesis by creating bitransgenic mice carrying MMTV-*neu* and 172Arg-to-His *p53* mutant (*p53-172H*). In these bitransgenic mice, tumor latency is shortened to 154 days, indicating strong cooperativity. None of the nontransgenic mice or the *p53-172H* transgenic mice developed tumors within this time period. Tumors arising in the *p53-172H/neu* bitransgenic mice were anaplastic and aneuploid and exhibited increased apoptosis, in distinction to tumors arising in p53-null mice, in which apoptosis is diminished. Further experiments address potential mechanisms of cooperativity between the two transgenes. In these bitransgenic mice, we have recapitulated two common genetic lesions that occur in human breast cancer and have shown that p53 mutation is an important cooperating event in *neu*-mediated oncogenesis.

A central goal of current cancer research is the identification of the genes involved in tumorigenesis and the definition of the precise role that these genes play in tumor development. Analysis of human breast carcinomas has implicated a number of genes in the genesis of these tumors, including *ERBB2/neu* (64), *HST* and *INT2* (1), *p53* (26), *src* (60), and *Rb* (37). It is suggested by a number of studies that the development of breast cancer in humans requires changes in more than one of these genes, which may in part explain the long latency associated with this disease (26).

neu encodes a receptor tyrosine kinase (RTK) related to the receptor for epidermal growth factor (EGFR or ErbB) and is amplified in nearly 30% of human cancers, particularly intraductal carcinomas (29, 65). Numerous studies suggest that this amplification leads to increased mitogenic signaling in the cell. The importance of this amplification is supported by the finding that 70% of transgenic mice that overexpress rat *neu* in the mammary gland develop mammary carcinomas (20). However, the latency of tumorigenesis is relatively long (over 200 days), suggesting that other oncogenic events are necessary. Analysis of these tumors revealed small in-frame deletions in the *neu* transgene in 65% of tumors analyzed (63). These deletions resided in the extracellular domain adjacent to the transmembrane domain and resulted in activation of Neu tyrosine kinase activity. These findings indicate that activation of Neu tyrosine kinase activity plays an important role in the development of these tumors. This observation is consistent with previous experiments showing that mice carrying a mouse mammary tumor virus (MMTV)-driven rat *neu* transgene (MMTV-*neu*) with an activating mutation in the transmembrane domain develop multifocal mammary carcinomas with a significantly shorter latency (52).

Another mechanism that can accelerate tumorigenesis in MMTV-*neu* transgenic mice is coexpression of the gene encoding transforming growth factor α (TGFα) (51). TGFα is a ligand for EGFR and can activate Neu tyrosine kinase activity through transmodulation. Thus, the activation of Neu tyrosine kinase activity by any of several different mechanisms can lead to mammary tumor progression.

In 30% of human breast carcinomas, expression of *ERBB2/neu* is associated with the presence of mutant *p53*, suggesting that activated tyrosine kinase receptors cooperate with mutant p53 in the development of these tumors (26). p53 is a multifunctional protein that is involved in the regulation of growth of nearly all cell types within mammalian organisms (reviewed in reference 34). The wild-type p53 protein can suppress tumor cell growth (14) and likely functions as a regulatory protein in two capacities: as a key component of apoptosis pathways within the cell (74) and as a checkpoint protein to control the G_1-to-S transition in the presence of genotoxic stress (35). Structural domains of p53 include an amino-terminal transcriptional activation domain, a central DNA binding domain, and a carboxyl-terminal domain important for oligomerization (reviewed in reference 34). Genetic alterations at the *p53* locus are common in human cancers and are primarily either missense mutations or allele loss (5, 25, 53). While the majority of human tumors with altered *p53* have one allele bearing a missense mutation and one null allele, occasionally tumors are found to have one mutated allele and one normal allele (53).

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These findings suggest a progression model in which the initial event is a missense mutation in one *p53* allele, leading to a proliferative advantage, and then loss of the other allele, which confers a further selective advantage.

p53 point mutations are highly clustered into four regions that correspond to evolutionarily conserved domains of the protein that function in DNA binding. Some of the most commonly mutated amino acids are those that make direct contact with the DNA (8). p53 proteins bearing these mutations have been found to have altered DNA binding and transactivation properties (31, 32). Some mutant proteins fail to activate normal target genes, such as *p21*, but can activate atypical targets, such as *MDR1* (7). Thus, certain mutations in *p53* may lead to the acquisition of novel and dominant activities within the cell. It is evident from a number of studies that certain missense mutations in *p53* function as dominant negative alleles that encode proteins that lack transcriptional activation potential but retain the ability to oligomerize and thus can pull wild-type p53 into nonfunctional complexes (49). An example of this is the 135V mutation, which can accelerate tumor development in heterozygous but not nullizygous *p53*-deficient mice (22). Other alleles, such as 143A, 175H, 248W, 248Q, 273H, and 281G, act as dominant oncogenic alleles, since they can confer new malignant phenotypes upon gene transfer into cells that lack *p53* (11, 27). These phenotypes include the ability to grow in soft agar and to form invasive tumors in nude mice. The molecular mechanisms that underlie the ability of mutant *p53* alleles to induce these changes are unknown.

p53 alterations are common in human breast carcinomas (10, 57). Missense mutations have been identified at many of the hot spot regions, including codons 175(R to H) and 248(R to Q). 175H represents approximately 8% of all p53 mutations in human breast cancers. These alleles are dominantly oncogenic in cell culture and nude mouse tumorigenicity assays (11, 27). To obtain a more accurate picture of the effect that the *p53-175H* allele has on mammary cell growth, we used transgenic mice in which expression of the corresponding murine allele (*p53-172H*) was targeted to the mammary epithelium by using the whey acidic protein (*WAP*) promoter. It was somewhat surprising to find that despite high level expression in the mammary gland, mice carrying the *WAP* promoter-driven *p53-172H* were not abnormally susceptible to mammary carcinomas; only one mouse developed a mammary carcinoma, and this was with a latency of 11 months (41). These data suggested that this allele is not dominantly oncogenic on its own in this setting and requires other cooperating events. Indeed, these mice were much more susceptible than nontransgenic control mice to mammary tumors induced by carcinogens that are known to activate Ha-Ras (36, 42, 47). This finding suggests that activated Ras is one molecule that can cooperate with p53-172H in this system.

It is known that Neu can initiate a mitogenic signal within the cell and that this signal utilizes the same pathway as activated Ras. This finding suggested that if p53-172H can cooperate with activated Ras, it may also cooperate with Neu. In this study, we demonstrate cooperativity between *neu* and *p53-172H* in the development of mammary carcinomas and offer this as a model system that closely mimics the genetic changes in human breast cancers and that allows for further studies to uncover the mechanism of cooperativity between these two genes.

MATERIALS AND METHODS

Transgenic mice. The *p53-172H* transgenic mice, in which a mutant *p53* transgene was preferentially overexpressed in the mammary epithelium by use of the *WAP* promoter, were created and characterized as will be described elsewhere (41). Unactivated *neu* transgenic mice (line N#202) which contain the wild-type rat *neu* gene driven by MMTV have been described previously (20). Both lines are on an FVB background. *p53-172H/neu* bitransgenic mice were generated by crossing female and male offspring of line 8512 *WAP-p53-172H* transgenic mice to offspring of line N#202 of MMTV-*neu* transgenic mice. Mouse tail DNA from the offspring of this cross was isolated as described previously (38). Mice carrying both *WAP-p53-172H* and MMTV-*neu* transgenes were identified by multiplex PCR. The screening primers for *p53-172H* transgene utilized a 5′ primer on the WAP promoter (5′-CCGTCGACGGCCACAGTGAAGACCTCCGGCCAG-3′) and a 3′ primer on exon 2 of murine *p53* (5′-GCCTGAAAATGTCTCCTG GCTCAGAGGG-3′) and yielded a 1.2-kb PCR product. Primers for the rat *neu* cDNA (5′-GGAAGTACCCGGATGAGGAGGGCATATG-3′ and 5′-CCGGG CAGCCAGGTCCCTGTGTACAAGCCG-3′) were used to identify *neu* transgenes, yielding a 0.7-kb PCR product, which corresponds to nucleotides 1872 to 2578 of rat *neu* cDNA. PCR primers for mouse β-*casein* exon 7 (5′-GATGTG CTCCAGGCTAAAGTT-3′ and 5′-AGAAACGGAATGTTGTGGAGT-3′) provided an internal control for the PCR. The PCR (100-μl volume, containing 2.5 mM $MgCl_2$, 1× PCR buffer [Promega], 0.2 mM each deoxynucleoside triphosphate, 0.1 μM each primer, 2.5 U of *Taq* polymerase [Promega], and 2.0 μg of template DNA) consisted of 31 cycles of 1 min 15 s at 94°C, 2 min 15 s at 60°C, and 3 min 15 s at 72°C (RoboCycler Gradient 40; Stratagene). PCR-positive *p53-172H/neu* bitransgenic mice were confirmed by Southern blot analysis as described previously (40).

Screening of *p53* mutation in *neu*-induced mammary tumors. DNA extracted from MMTV-*neu*-induced mammary tumors were subjected to PCR to amplify exons 5 and 6 and exons 7 and 8 of murine *p53* for sequencing. The primers for amplifying exons 5 and 6 were 5′-CGTTACTCGGCTTGTCCCCGACCT-3′ and 5′-CAACTGTCTCTAAGACGCACAAC-3′ (which reside on introns 4 and 6, respectively, of murine *p53*). The primers for amplifying exons 7 and 8 were 5′-GAGGTAGGGAGCGACTTCACCTGG-3′ and 5′-TGAAGCTCAACAGG CTCCTCCGCCTCC-3′ (on introns 6 and 8, respectively, of murine *p53*).

RNA extraction and analysis. Mammary gland and mammary tumor biopsies were performed under anesthesia (Avertin, intraperitoneally) as described previously (38). Tissues were frozen immediately in liquid nitrogen and kept at −80°C until isolation of RNA. RNA was isolated by homogenization of frozen tissues with a homogenizer (Janke & Kunkel KIKA-Labortechnik), using the TRIzol protocol as described by the manufacturer (GIBCO BRL). RNA was fractionated by electrophoresis in a 1.2% agarose gel containing 0.66 M formaldehyde with 1× 4-morpholinepropanesulfonic acid buffer and then transferred to Zetaprobe membranes (Bio-Rad) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and hybridized as described previously (40), using an *Xho*I-*Kpn*I fragment excised from mouse *p53* cDNA as a probe to detect the expression of the *p53* transgene and a *Bam*HI-*Bam*HI fragment excised from rat *neu* cDNA as a probe to detect the expression of the *neu* transgene.

PCR analysis of deletion in *neu* transgenes in mammary tumors. DNA was isolated from mammary tumors of *neu* transgenic mice and *p53-172H/neu* bitransgenic mice as described previously (39). Hot-start PCR was used to analyze deletions in *neu* transgenes in mammary tumors. The PCR was performed with two primers, 5′-CGGAACCCACATCAGGCCCTGCTCCACAGT-3′ and 5′-C TCAGTTTCCTGCAGCAGCCTACGCATCG-3′, which amplify the region corresponding to nucleotides 1487 to 2116 of rat *neu* cDNA and yield a 629-bp PCR product. The forward primer was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The PCR conditions used were the same as those used for screening bitransgenic mice, but with 1.5 μg of tumor DNA as the template. Six-microliter aliquots of labeled PCR products were mixed with 4 μl of Sequenase stop buffer and then heated to 75°C for 5 min; 5 μl of this mixture was separated by electrophoresis through 5% polyacrylamide sequence gels and exposed to X-ray film.

Immunoprecipitation and immunoblotting. Tissue lysates were prepared as described previously (63). Immunoprecipitation were performed by incubating 500 μg of the cleared protein lysate with 500 ng of anti-Neu antibody (Ab-4; Oncogene Science) for 2 h at 4°C, with 4,600 ng of rabbit anti-mouse immunoglobulin G for 1 h at 4°C, and then with protein A-Sepharose for 1 h at 4°C on a rotating platform. ErbB-2 immunoprecipitates were washed three times with lysis buffer and resuspended in 75 μl of sodium dodecyl sulfate (SDS)-gel loading buffer; 50 μl of each sample was electrophoresed on an SDS–7.5% gel. After being electrophoresed, the protein was transferred onto a Biotrace NT membrane (Gelman Sciences) with an immunoblot transfer apparatus (Hoefer). The membrane was blotted with antiphosphotyrosine antibody (4G10; Upstate Biotechnology) first, stripped, and reblotted with anti-Neu antibody (sc-284; Santa Cruz Biotechnology). Proteins were visualized with an ECL kit (Amersham).

Histologic analysis, BrdU labeling, and TUNEL staining. Mammary glands and mammary tumors were surgically removed, fixed in 10% neutral buffered formalin (ANATECH Ltd., Battle Creek, Mich.) for 6 h, and placed in 70% ethanol until processed. These tissues were embedded in paraffin, and 5-μm sections were placed on regular slides and stained with hematoxylin and eosin (H+E). Three ErbB-2-alone tumors and eight bitransgenic tumors were analyzed by H+E staining. Relative nuclear size was quantitated by calculating the product of two orthogonal measurements of nuclear diameter, taken on six nuclei per specimen. For bromodeoxyuridine (BrdU) labeling, mice were injected intraperitoneally with BrdU labeling reagent (Amersham RPN 201, 3 mg/ml; 2 ml injected per 100 g of body weight). After 2.5 h, the mice were

sacrificed and tumor samples were harvested, fixed in 10% neutral buffered formalin, and then sectioned and stained as described previously (67). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining for apoptotic cells was performed as described previously (39).

Flow cytometry. DNA content of mammary tissues was analyzed by flow cytometry on paraffin sections as described previously (23).

RESULTS

Analysis of the *p53* gene in MMTV-*neu*-induced tumors reveals mutations in *p53*. Mice carrying MMTV-*neu* (line N#202) express *neu* at a high level in the mammary gland and develop mammary tumors with a latency of 7 to 8 months (20). We wished to determine if mutations in *p53* could be a cooperating event in the genesis of these mammary tumors and may help to explain the long latency of tumor development. To that end, we examined eight mammary tumors arising in these mice for the presence of mutations in exons 5 to 8 of *p53*. We performed direct sequence analysis of two different amplification products obtained by PCR using primers bracketing exons 5 and 6 or exons 7 and 8. Three of eight tumors showed a G-to-A transition at codon 256 in exon 7, which changed the coding potential from Asp to Asn. Codon 256 in mouse *p53* is equivalent to codon 259 in human *p53*. This codon resides immediately adjacent to domain IV of the DNA binding region (8) and is a site of mutation in human T-cell acute lymphoblastic leukemias (6). The remaining five tumors showed no changes in DNA sequence within the interval examined. Further analysis of these tumors by reverse transcription (RT)-PCR, using primers for *neu*, showed that none of the eight tumors had activating deletions in the *neu* transgene of the type described by Siegel et al. (63) (data not shown).

Expression of 172H mutant *p53* and unactivated *neu* in the mammary glands of transgenic mice. The finding of *p53* mutations in mammary tumors arising in MMTV-*neu* transgenic mice argues that *p53* mutation can be a cooperating event in *neu*-induced tumors in this model and is thus consistent with data from the analysis of human tumors (26). To further test this cooperativity, we sought to coexpress both genes in the mammary epithelium of transgenic mice and to determine the effect of this coexpression on susceptibility to mammary carcinomas. We previously developed a line of transgenic mice (line 8512) in which murine *p53-172H* was targeted to express in the mammary gland under control of the rat *WAP* promoter (41). WAP is a prominent constituent of rodent milk; its expression is restricted to the mammary gland, where it is normally turned on at day 10 of pregnancy, remaining elevated through lactation (2, 55). Codon 172 of murine *p53* is equivalent to codon 175 of human *p53* (3, 9), and the majority of *p53* mutations on codon 175 in human primary mammary tumors were found to be Arg to His (25, 57, 69). This allele (rather than the 256N allele) was chosen for study because 175H is the most prevalent *p53* mutation in human breast cancers (54). Overexpression of murine *p53-172H* in the mammary glands of transgenic mice induced a mammary tumor in only one of five female founders, with a latency of 11 months, and no other tumors have been observed in F_1 to F_3 mice despite continuous breeding over more than 2 years. However, when the mice were treated with dimethylbenzanthracene (DMBA), mammary tumors developed with shorter latency than in nontransgenic mice (41). The fact that overexpression of *p53-172H* alone rarely causes mammary tumors but can markedly accelerate mammary tumor formation with DMBA treatment suggests that an initiating event, or elevated signaling from a mitogenic pathway, was needed to cooperate with *p53-172H* for mammary tumorigenesis. We postulated that the MMTV-*neu* transgene could provide such a stimulus.

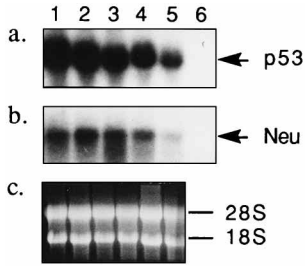

FIG. 1. Expression of mutant *p53-172H* and unactivated *neu* transgene in the mammary glands of *p53-172H/neu* bitransgenic mice. Shown is RNA blot analysis (20 μg) of total RNA isolated from the mammary gland at day 2 of lactation. Panels a and panel b were probed with *p53-172H* and *neu*, respectively. Both panels were exposed for 16 h. Lanes 1 to 5, five RNA samples isolated from five different *p53-172H/neu* bitransgenic mice; lane 6, RNA from nontransgenic mouse as a control. (c) Ethidium bromide-stained RNA gel.

To directly test for cooperativity between *neu* and *p53-172H* in mammary tumorigenesis, we generated *p53-172H/neu* bitransgenic mice in which both transgenes were expressed in the mammary gland. *p53-172H* transgenic mice were mated to line N#202 MMTV-*neu* transgenic mice, and *p53-172H/neu* bitransgenic offspring were identified by DNA analysis. In all, 26 female *p53-172H/neu* bitransgenic, 25 *p53-172H*-alone female transgenic, and 20 *neu*-alone female transgenic mice were identified from the same group of offspring. All transgenic mice were kept either pregnant or lactating by continued housing with male FVB mice, in order to maintain expression of *WAP* promoter-driven transgene. To confirm coexpression of *p53-172H* and *neu* transgenes, we performed Northern blot analysis of RNA from mammary gland biopsies performed at 2 days postpartum during lactation from five *p53-172H/neu* bitransgenic mice. This analysis showed that both *p53* and *neu* mRNAs were readily detected in 20 μg of total RNA after a 16-h exposure (Fig. 1), with some variability in different individuals.

Development of mammary tumors is accelerated in *p53-172H/neu* bitransgenic mice. At 112 days of age, after two rounds of pregnancy and lactation, mammary tumors began to appear in the *p53-172H/neu* bitransgenic mice. In the *neu*-alone transgenic mice, mammary tumors began to emerge at 163 days of age. At age of more than 300 days after three and four rounds of pregnancy, no tumors had appeared in *p53-172H*-alone transgenic mice. The median age of tumor development was 154 days for *p53-172H/neu* bitransgenic mice, whereas it was 234 days for MMTV-*neu* singly transgenic mice (Fig. 2). These data indicate a strong cooperation between *neu* and the dominant oncogenic 172H allele of *p53*.

To check the expression status of *p53-172H* and *neu* transgenes, RNA isolated from both mammary tumor and adjacent mammary glands of *p53-172H/neu* bitransgenic mice were subjected to Northern analysis (Fig. 3). The expression levels of p53 appeared reasonably constant in both mammary tumors and adjacent mammary glands from three different *p53-172H/neu* bitransgenic mice (Fig. 3a, paired samples in lanes 1 and 2, 5 and 6, and 7 and 8) and were similar to the level of nonneoplastic mammary gland from singly transgenic *p53-172H* mice (lane 10). Expression of *neu* transgene in mammary tumors (Fig. 3b, lanes 2 and 8) was much higher than in adjacent mammary gland, but the level of expression did not appear to correlate with the presence or absence of the *p53-172H* transgene (compare lanes 3 and 4 [*p53-172H* absent] with lanes 5 and 6 [*p53-172H* present]).

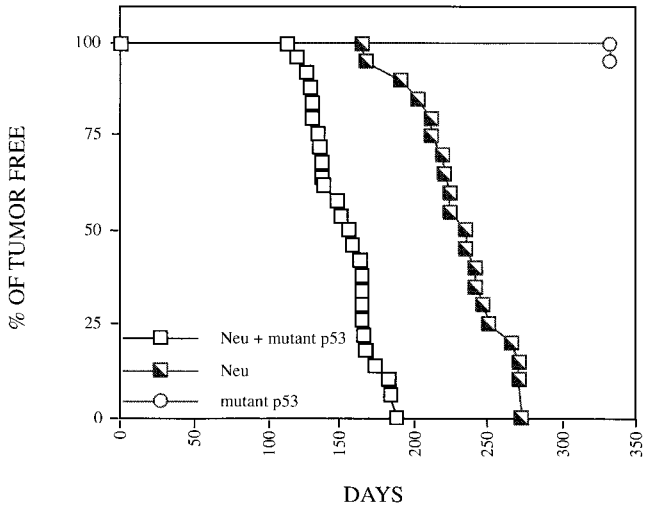

FIG. 2. Comparison of the kinetics of tumor incidence among female 172His mutant *p53-172H*-alone transgenic, *neu*-alone transgenic, and *p53-172H/neu* bitransgenic mice. Mice were scored positive for tumors when mammary nodules were first identified by palpation.

Tumors with p53-172H exhibit a higher grade and have higher rates of mitosis and apoptosis. Histological examination of the tumors revealed that the presence of the *p53-172H* transgene had a marked effect on tumor morphology (Fig. 4). Tumors arising in the MMTV-*neu* singly transgenic mice are consistently typical mammary adenocarcinomas, exhibiting focal gland formation, solid clusters of tumor cells, and abundant tumor angiogenesis. While the nuclear-to-cytoplasmic ratio was high, nuclear size was rather uniform, and the majority of tumor cells had nearly round to ovoid nuclei with smooth nuclear borders. In contrast, the *p53-172H*-expressing tumors arising in the bitransgenic mice exhibited greater cytologic variability, with most having a much larger cellular and nuclear size: relative nuclear size on cross section was 3.9 times greater than for *neu*-alone tumors ($P < 0.05$). Nuclear morphology in the bitransgenic tumors was more variable than in the *neu*-alone tumors, showing frequent indentations. In addition, bitransgenic tumors had pronounced anaplasia and, as assessed by morphology alone, markedly higher rates of both apoptosis and mitosis. These features are consistent with a much higher grade of neoplasm and a higher growth fraction, and they suggest aneuploidy or polyploidy. Thus, the expression of *p53-172H* in this setting appeared to have a marked effect on tumor cell morphology and tumor growth.

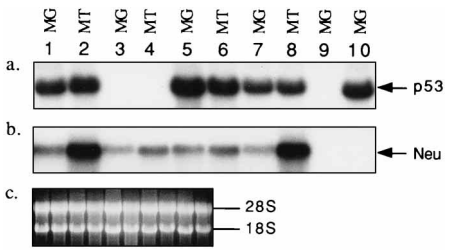

FIG. 3. Expression of the *p53-172H* and *neu* transgenes in mammary tumors (MT) and adjacent mammary glands (MG) of *p53-172H/neu* bitransgenic mice at day 2 of lactation. Twenty micrograms of total RNA was subjected to Northern analysis. Panels a and b were probed with p53 and *neu*, respectively. Panel a was exposed for 2 h, and panel b was exposed for 21 h. (c) Ethidium bromide-stained RNA gel. Lanes 1 and 2, 5 and 6, and 7 and 8 are paired RNA samples from three independent *p53-172H/neu* bitransgenic mice. Lanes 3 and 4 are RNA samples from *neu*-alone transgenic mice. Lanes 9 and 10 are RNA samples from a nontransgenic mouse and a *p53-172H*-alone transgenic mouse, respectively.

From the histologic appearance, it is evident that tumors expressing *p53-172H* have higher rates of apoptosis and mitosis. To confirm this, we assayed the relative rate of mitosis with BrdU labeling and the apoptosis index with the TUNEL assay. We injected *neu* singly transgenic and *p53-172H/neu* bitransgenic mice harboring equal-size tumors in parallel with BrdU, sacrificed the mice, and immunostained the mammary tumors for BrdU incorporation into DNA. The results (Fig. 5A) show a clearly higher mitotic rate in tumors expressing *p53-172H* than in those without. Similar analysis was performed on phenotypically normal (nonneoplastic) mammary glands of singly and bitransgenic mice, and no significant differences were found between the different genotypes (data not shown). We also assessed the rate of apoptosis on similar tumor samples, as well as nonneoplastic mammary glands from mice of the genotypes under study, and these results show a markedly higher rate of apoptosis in mammary tumors expressing both *p53-172H* and *neu* (Fig. 5B; compare bar 6 [*neu* tumor] with bar 9 [*p53-172H/neu* tumor]). These data confirm the impression obtained from examination of the H+E-stained slides. The bitransgenic tumor also showed a higher apoptosis rate than the adjacent nonmalignant tissue (Fig. 5B; compare bar 8 with bar 9). Interestingly, premalignant mammary glands from bitransgenic mice (bar 7) had a significantly higher rate of apoptosis than similar tissue from *neu* transgenic mice (bar 5).

Bitransgenic tumors exhibit aneuploidy and tetraploidy. On the basis of the large nuclear size seen on the H+E-stained sections of the most mammary tumors arising in bitransgenic mice, we suspected that the tumors expressing *p53-172H* had greater than 2n DNA content. We thus investigated the ploidy of the nonmalignant and malignant mammary tissue by flow cytometry of nuclei derived from paraffin-embedded tissue. We first determined the ploidy of cells in nonmalignant mammary cells, on the second day of lactation, and found all genotypes to have 2n DNA content, with a similar fraction of cells in G_2/M and S (Fig. 6A and B and data not shown). These specimens exhibited a high fraction of cells in G_2/M (4n peak) which is attributed to the proliferative nature of the mammary gland at this stage. In tumor specimens, while *neu*-alone tumors were euploid (four of four tumors [Fig. 6C]), all four bitransgenic tumors analyzed were markedly aneuploid, with a minority of cells having 2n DNA content: the majority had 4n DNA or were intermediate in DNA content (Fig. 6D, representative tumor). The bitransgenic tumors had an additional peak at 8n (not shown) that represented tetraploid cells in G_2/M.

Bitransgenic tumors exhibit increased Neu tyrosine phosphorylation. We were interested in exploring the mechanism of *p53-172H*-induced tumor acceleration in the bitransgenic mice. One possible role of *p53-172H* is to alter the intrinsic tyrosine kinase activity of Neu, through either a direct or an indirect effect. Muller and coworkers have found that the induction of mammary tumors in transgenic mice expressing the unactivated *neu* alone is associated with activation of the receptor's intrinsic tyrosine kinase activity (20). To determine if this was the case with mammary tumors arising in the bitransgenic animals, we performed immunoprecipitations using anti-Neu antisera followed by Western blot analysis using antisera against either phosphotyrosine or Neu. This analysis revealed an elevation in levels of Neu protein in all of the bitransgenic tumors relative to that in the adjacent nonmalignant mammary gland, and in five tumors (Fig. 7A, lanes 9, 10, 12, 14, and 15), the level was comparable to that seen in *neu* singly transgenic mice (lanes 1 to 5). In addition, the level of tyrosine-phospho-

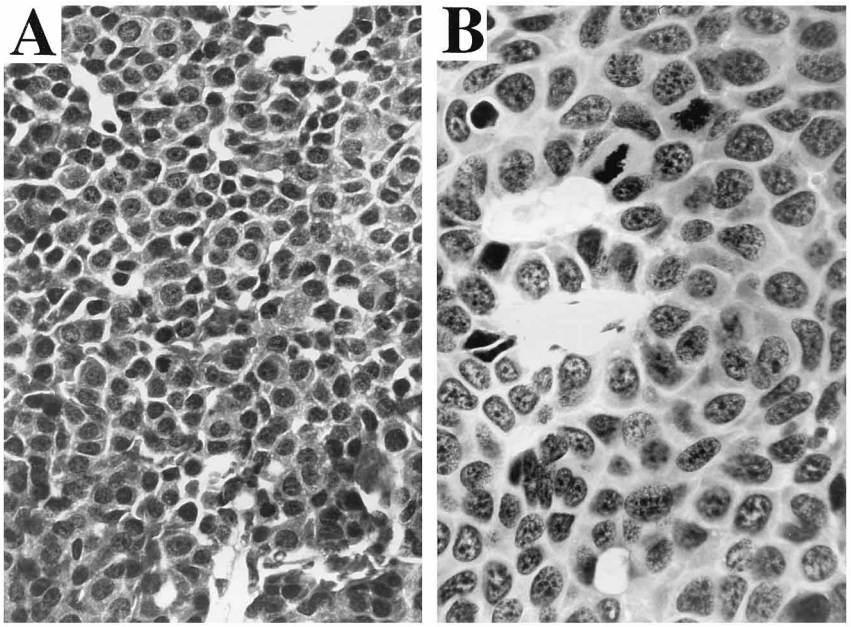

FIG. 4. Histopathology of mammary tumors from *neu* transgenic and *p53-172H/neu* bitransgenic mice. (A) Representative mammary tumor from an unactivated *neu* transgenic mouse. (B) Representative mammary tumor from a *p53-172H/neu* bitransgenic mouse. This particular tumor exhibited a nuclear size that was about twice the average for the eight bitransgenic tumors analyzed. Magnification, ×276.

rylated Neu in these tumors was comparable to that in *neu*-alone transgenic tumors (Fig. 7B). However, the level of Neu expression in the bitransgenic tumors was not as consistent as that seen in this sampling of *neu* singly transgenic tumors. There also appeared to be little correlation between the level of Neu protein in the tumor and the level of tyrosine phosphorylation, for either set of tumors. Nonetheless, these data indicate that like the singly transgenic tumors, the ones arising in *p53-172H* bitransgenic mice exhibit elevated levels of tyrosine-phosphorylated Neu.

Deletions in *neu* transgenes are not detectable in mammary tumors of *p53-172H/neu* bitransgenic mice. One mechanism of Neu activation in mammary tumors arising in MMTV-*neu* mice is through small (7- to 12-amino-acid) somatic deletions in unactivated *neu* transgenes (63). The finding of these mutations in 65% of the tumors argues that activation of Neu tyrosine kinase activity is a rate-limiting step in tumor development. We wondered if the presence of the *p53-172H* transgene abrogated the need for these activating mutations in the *neu* transgene, and thus we analyzed tumor RNA and DNA for the presence of activating deletions of the *neu* transgene. The RNA was subjected to RT-PCR analysis using radioactive

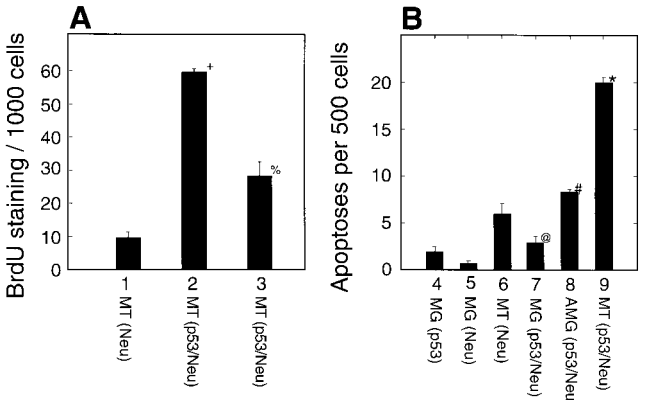

FIG. 5. Coexpression of *p53-172H* and *neu* caused an increase in both apoptosis and mitosis. (A) Average numbers of BrdU-labeling cells from different tumor samples. Bar 1, mammary tumor (MT) from *neu* mice; bars 2 and 3, mammary tumors from *p53-172H/neu* mice; +, significantly greater than 1 ($P < 0.01$); %, significantly greater than 1 ($P < 0.05$). Three *neu*-alone tumors and six *p53-172H/neu* bitransgenic tumors were analyzed. Representative analyses are presented. (B) Average numbers of apoptotic cells in different tissues. Bar 4, 5, and 7, mammary glands (MG) from 2-day-lactating *p53-172H*, *neu*, and *p53-172H/neu* mice, respectively; bars 6 and 9, mammary tumors from *neu* and *p53-172H/neu* mice, respectively; bar 8, the adjacent histologically normal mammary gland from *p53-172H/neu* mice; @, significantly greater than 5 ($P < 0.05$); #, significantly greater than 7 ($P < 0.01$); *, significantly greater than all others ($P < 0.01$). Three different mice were analyzed for each genotype; representative analyses are presented.

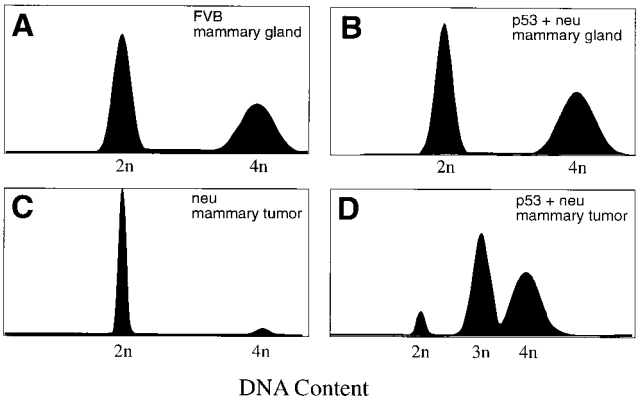

FIG. 6. Coexpression of unactivated *neu* and *p53-172H* caused aneuploidy and tetraploidy in mammary tumor cells. The results shown are representative of flow cytometric analysis of DNA content of mammary gland cells and mammary tumor cells from nontransgenic mice (FVB), *neu* transgenic mice, and *p53-172H/neu* bitransgenic mice. Nuclei were isolated from paraffin blocks, stained with propidium iodide, and subjected to flow cytometric analysis.

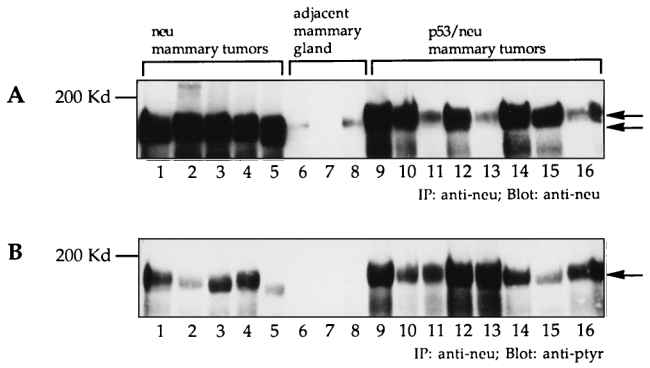

FIG. 7. Neu is highly phosphorylated in mammary tumors of *p53-172H/neu* bitransgenic mice. (A) Protein lysates were immunoprecipitated (IP) for Neu with antibody (Ab-4), fractionated by SDS-polyacrylamide gel electrophoresis, and then subjected to immunoblotting with anti-Neu antibody (sc-284). The upper arrow shows the Neu protein in adjacent mammary gland (lanes 6 to 8) and mammary tumors of *p53-172H/neu* mice; the lower arrows shows the Neu protein with deletion in the tumors of *neu* mice. (B) Same membrane subjected to immunoblotting with antiphosphotyrosine (anti-ptyr) antibody (4G10). Phosphorylated Neu proteins are indicated by the arrow.

primers that generated a fragment spanning from nucleotides 1487 to 2116 of rat *neu* cDNA, which is the region where deletions of *neu* transgene were found in MMTV-*neu*-induced mammary tumors (63). DNA samples were subjected to PCR with the same primers. Both RT-PCR and PCR results revealed that the deletions in the *neu* transgene did not occur in the mammary tumors of *p53-172H/neu* bitransgenic mice (Fig. 8), while a deletion was detected in the DNA and RNA from a mammary tumor that arose in a MMTV-*neu* singly transgenic mouse. This result suggests that unlike *neu*-alone-induced mammary tumors, *neu* deletions are not associated with the mammary tumor formation in *p53-172H/neu* bitransgenic mice and that the presence of the *p53-172H* allele abrogates the need for these mutations.

Higher levels of TGFα were detected in *p53-172H/neu*-induced tumors. Unlike mammary tumors from *neu*-alone transgenic mice, no somatic deletions were detected in *neu* transgenes from the mammary tumors of *p53-172H/neu* bitransgenic mice. One possible mechanism is activation via ligand stimulation. We have addressed this possibility by assessing the level of expression of two ligands known to activate Neu through transmodulation: amphiregulin and TGFα. Northern blot analysis of *TGFα* expression in the mammary glands and the mammary tumors from *p53-172H*, *neu*, and *p53-172H/neu* mice

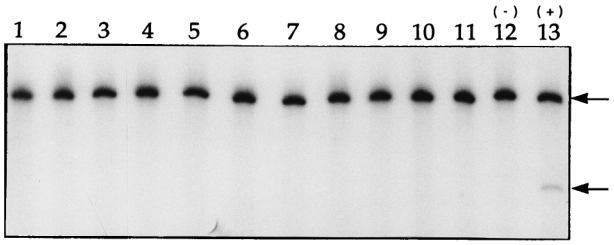

FIG. 8. Analysis of deletions in *neu* transgenes in *p53-172H/neu*-induced mammary tumors. DNA samples were subjected to hot-start PCR as described in Materials and Methods. The upper arrow points the wild-type *neu* transgene, and the lower arrow points the *neu* transgene with a deletion. Lanes 1 to 11, late-stage mammary tumors from 11 different *p53-172H/neu* bitransgenic mice; lane 12, normal mammary gland of an unactivated *neu* transgenic mouse as a negative control; lane 13, late-stage mammary tumor in an unactivated *neu* transgenic mouse as a positive control.

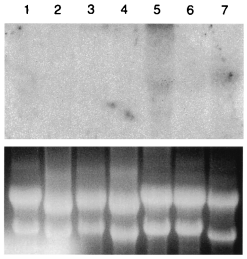

FIG. 9. Expression of *TGFα* in mammary glands and mammary tumors from transgenic mice. Shown is RNA blot analysis of total RNA (20 μg) from mammary glands and tumors, hybridized with a probe for *TGFα*. Samples are normal mammary glands from *p53-172H* transgenic (lane 1), *p53-172Hneu* bitransgenic (lane 2 and 6), FVB (lane 3), and *neu*-alone (lane 4) mice and tumor samples from *neu*-alone (lane 5) and *p53-172H* bitransgenic (lane 7) mice. The hybridized filter was exposed for 12 days.

revealed a higher levels of *TGFα* expression in the mammary tumors of both *neu*-alone and *p53-172H/neu* bitransgenic mice relative to nonmalignant mammary tissue of the same genotype or to tumor tissues of the other genotypes (Fig. 9). When corrected for differences in RNA loading (Fig. 9, bottom panel), expression of TGFα in the bitransgenic tumor was considerably higher than that in the *neu*-alone tumor and may partially explain the higher level of Neu receptor tyrosine phosphorylation. No expression of amphiregulin was detected in either mammary tumors or nonmalignant mammary tissue of all genotypes (data not shown).

DISCUSSION

This report describes the creation of a mouse mammary tumor model in which two of the most frequent changes in human breast cancers, amplification of *neu* and a dominant oncogenic mutation of *p53*, have been recapitulated. This model serves to address two important issues in tumor development: the mechanism of cooperation of genes in mammary tumorigenesis, and the effect of dominant oncogenic alleles of *p53* on tumor growth in an in vivo experimental model.

To address the possibility that p53 mutations play a cooperating role in *neu*-mediated mammary tumors, we document the presence of *p53* point mutations in three of eight mammary tumors that arose in MMTV-*neu* transgenic mice. To directly address a genetic interaction between *p53* and *neu*, we then crossed MMTV-*neu* transgenic mice with mice transgenic for the dominant oncogenic *p53-172H* allele (equivalent to the human *p53-175H* allele). Strikingly, while we observed only a single mammary tumor among 25 *p53-172H* transgenic mice, we found strong cooperation between the *p53-172H* allele and *neu*. We further show that unlike tumors induced by *neu* alone, the *p53-172H/neu* tumors exhibit no activating deletions in the *neu* transgene. Nonetheless, the tumors have increased tyrosine phosphorylation of the Neu protein, indicating receptor activation. This result indicates that the presence of the dominant oncogenic *p53* allele abrogates the need for activating mutations of *neu* in mammary tumorigenesis. It is unlikely that the etiology of the increased Neu receptor activity is a direct effect of p53-172H, since the nonmalignant bitransgenic mammary tissue does not exhibit it. Thus, this feature emerges during tumorigenesis.

It is known that dominant oncogenic mutants of *p53* such as 175H can cause immortalization of primary cells (61), can cooperate with Ras in transforming primary cells (12, 24), and can enhance the tumorigenic potential of cells lacking *p53* (11). *p53-175H* is particularly potent, being able to induce growth of

SAOS-2 cells in agar, where other mutant alleles are not (11). The rapid kinetics and high efficiency of cooperation in these assays by dominant oncogenic alleles of *p53* indicate a direct effect on tumor cell growth. That these effects can be seen in the absence of endogenous p53 argues that these alleles are not acting simply as dominant negative alleles, by inactivating wild-type p53 function. These features of cellular transformation mediated by mutant *p53* alleles suggest that these alleles act not only by interfering with p53-dependent functions such as apoptosis, senescence, or genomic instability (all of which have been suggested as important tumor-promoting sequelae of p53 loss [30, 45, 48, 66]) but also by exerting a dominant effect on cell growth. The nature of this effect is unknown. Recent data from skin tumorigenesis studies of mice support the distinction between *p53* null alleles and dominant oncogenic mutations. Tetradecanoyl phorbol acetate-treated transgenic mice specifically expressing TGFα in the skin develop skin tumor with about 8 weeks' latency. The onset of skin tumor was delayed in $p53^{-/-}$ mice bearing the same *TGF*α transgene (19). However, in transgenic mice specifically expressing 172H mutant p53 in the skin, the latency of DMBA-tetradecanoyl phorbol acetate-induced skin tumors was shortened to 3 to 4 weeks (70a).

In our bitransgenic model, we do not observe the emergence of tumors with kinetics that indicate direct and immediate malignant transformation by coexpression of *p53-172H* and *neu*: tumors arise following the second pregnancy rather than the first and are unifocal, indicating the necessity for other events. This observation is thus distinct from the cell culture results described above and is likely due to several things, including the lower transforming potential of native Neu relative to Ras, the presence of endogenous *p53* alleles in our transgenic mice, as well as other tumor control mechanisms that exist in the intact animal, such as tumor immunity, the inhibitory influence of surrounding tissue, and the requirement for tumor angiogenesis. Nonetheless, the *p53-172H* allele accelerates *neu*-induced tumorigenesis, albeit by an unknown mechanism. We address several possible mechanisms that our bitransgenic model will allow us to test. These models are based on the known or suggested functions of p53, which include an effect on apoptosis, on genome stability, and on transcriptional regulation of cell growth-regulatory genes.

A role for p53 in programmed cell death is well established and is likely mediated, at least in part, through its ability to transcriptionally activate the cell death agonist *bax* (50). It has been proposed that the loss of p53-mediated cell death is an important tumor-promoting mechanism in $p53^{-/-}$ tumors (66). Tumors that arise in one simian virus 40 T-antigen model exhibit lower levels of apoptosis relative to control tumors, suggesting that p53 plays an essential role in apoptosis (66). The 135V allele, which acts as a dominant negative allele, can block E1A-induced apoptosis (62). However, this allele cannot cooperate with *neu* in mammary carcinogenesis (50a), which suggests that one cannot accelerate *neu*-induced murine mammary tumorigenesis by decreasing apoptosis. Similarly, p53-dependent apoptosis in the mammary cells appears not to be required for normal mammary gland development (28, 43). Our data indicate an increased rate of apoptosis *p53-172H*-induced tumors, making the loss of apoptotic cell death an unlikely mechanism for *p53-172H* cooperativity in mammary tumorigenesis.

In *neu*-alone tumors, activation of *neu* through mutations in the *neu* transgene is an important, rate-limiting step in tumorigenesis (63). It is likely that Neu activation is also rate limiting in the *p53-172H/neu* bitransgenic tumors. Thus, understanding the mechanism underlying this increase in RTK activity is a possible route to understanding the role of p53-172H in accelerating tumor formation in this model. By Northern blot analysis, we have documented that both the *neu* transgene and the activating ligand *TGF*α are expressed at higher levels in the bitransgenic tumors than in nonmalignant mammary tissue of the same genotype. These data provide two possible mechanisms for increased Neu RTK activity but are not likely to be direct effects of p53-172H, given the low expression of the genes in nonmalignant bitransgenic tissue. Thus, these changes in the level of *neu* and *TGF*α gene expression may accompany malignant progression rather than cause it. We are currently determining if other ligands for the ErbB family of receptors may be transcriptionally altered in a direct manner by p53-172H. An alternative mechanism is that p53-172H could cause an increase in the expression of a receptor critical for Neu function, such as EGFR, ErbB-3, or ErbB-4.

The data from cell culture experiments described above suggest a direct effect of p53-172H on tumor cell growth, and such an effect may indeed play an important role in our system. However, other effects of this allele are also possible. One is that p53-172H increases the likelihood of additional mutational events in genes other than the *neu* transgene in the nonmalignant cells expressing *neu* and thus accelerates tumor formation. One type of genetic alteration known to contribute to mammary tumorigenesis is gene amplification. While an increased frequency of gene amplification is seen in p53-null cells, it is not observed in Li-Fraumeni cells (mutated at position 184 or 248) that retain one wild-type *p53* gene (44). Since our *p53-172H/neu* bitransgenic tumors appear by Southern blot analysis to retain a wild-type copy or copies of *p53* (data not shown), this mechanism may not apply to our model. We are currently assessing the frequency of other types of alterations, e.g., deletions and point mutations, in these bitransgenic tumors.

One notable feature of the tumors expressing *p53-172H* is their large nuclear size and >2n DNA content, which occurred despite the retention of the endogenous wild-type *p53* allele(s). Aneuploidy was found by some investigators in tumors driven by *p53*-null alleles (58) and in primary $p53^{-/-}$ fibroblasts following extended culture (21, 44, 68) but not in primary $p53^{-/-}$ hematopoietic cells, in $p53^{-/-}$ erythroid tumors, or in the majority of cell lines derived from these tumors, even following 150 passages (48). It is known that polyploid nuclei can result from the uncoupling of S phase and mitosis. One way in which this can occur is through loss of the p53 target gene, *p21*, which encodes a negative regulator of cyclin-dependent kinases. In the absence of p21, or in the presence of mutant p53 (in which case p21 is not induced by DNA-damaging agents), cells fail to arrest at G_1/S and will replicate their DNA. Cells then proceed into additional rounds of DNA replication, culminating in apoptosis (70). At a low frequency, this can occur in $p53^{-/-}$ cells in the absence of DNA-damaging agents (48). This p53- and p21-dependent G_1/S checkpoint may play an important role in vivo to arrest cell growth in the setting of tumor hypoxia (18), and the loss of this pathway may then result in chromosomal reduplication, a hallmark of malignant tumors (70) and a feature of tumors expressing p53-172H. p53 is also thought to play an important role in centrosome duplication. In $p53^{-/-}$ mouse embryo fibroblasts, multiple copies of functionally competent centrosome are generated during a single cell cycle, which is thought to result in unequal segregation of chromosomes (16). These data suggest that loss of wild-type p53 function may cause chromosomal instability. It is important to note that in our system, aneuploidy does not arise prior to tumor formation, indicating that either (i) other genes need to be mutated

in order to allow polyploidization or (ii) epigenetic events, such as tumor hypoxia, must occur (46).

An alternative mechanism of p53-172H action in this model is that it may promote other aspects of tumor growth, such as tumor angiogenesis. The finding that mutant, but not wild-type, p53 can synergize with protein kinase C to stimulate vascular endothelial growth factor (33) suggests that the p53-172H allele could stimulate vascular ingrowth, which is known to be a rate-limiting step in tumorigenesis. Another potential mechanism to explain the cooperativity between *p53-172H* and *neu* is that the mutant p53 may have a negative effect on the antiproliferative signaling of TGFβ, a factor that can cause slowing of growth, G_1 arrest, or apoptosis, depending on the cell line. TGFβ inhibition of cell growth can be observed in p53 null cells (73) and in cells expressing the *E6* gene of human papillomavirus, which causes the degradation of p53 protein (15), indicating that wild-type p53 does not play a role in TGFβ signaling. However, lack of responsiveness to TGFβ has been correlated with certain mutations at p53 (72), and transfer of a mutant p53 allele, either murine 132F (59) or 135V (4, 13) or human 143A (17), causes reduced responsiveness to TGFβ in some cells but not others (56, 71). These data suggest that dominant oncogenic alleles of p53 may act to interfere with TGFβ signaling, either through a decrease in TGFβ type I or type II receptor or through interference with intracellular TGFβ signaling. Specifically, TGFβ has been shown to decrease cdk4 levels, and mutant p53 can block this effect (13).

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