Mutant p53-induced Immortalization of Primary Human Mammary Epithelial Cells

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ABSTRACT

Mutations of the p53 gene are the most frequent genetic lesions in breast cancer, suggesting a critical role for p53 protein in normal mammary cell growth control. Indeed, the p53-targeting human papillomavirus oncogene E6 induces efficient immortalization of normal human mammary epithelial cells (MECs). To assess whether selective loss of p53 is sufficient for MEC immortalization, we introduced seven missense mutants and one single-amino acid deletion mutant (del239) of p53 into the 76N normal MEC strain. Although the missense mutants failed to immortalize MECs, the del239 mutant reproducibly immortalized these cells. The immortal cells were anchorage dependent and nonmitogenic, indicating a p53-dependent transformation. γ-Irradiation of these cells failed to induce G1 cell cycle arrest and did not lead to an increase in WAF1 and mdm-2 mRNA levels, demonstrating a loss of the endogenous p53 function. These results demonstrate that selective ablation of p53 function by a dominant-negative mutant is sufficient for immortalization of MECs. Availability of an immortalizing as well as several nonimmortalizing p53 mutants should help identify functions critical for cell growth control by p53 in mammary epithelial cells.

INTRODUCTION

Development of fully malignant tumors is a multistep process in which immortalization is thought to be an early and essential event (1). Although certain rodent cells exhibit a significant level of spontaneous immortalization in vitro, normal human cells in general exhibit a finite life span in culture and eventually senesce (2). We and others have shown that normal MECs in culture proliferate for about 10–20 passages (when cultured at a 1:10 split ratio) and then invariably senesce without any spontaneous immortalization (3, 4). Remarkably, however, introduction of the HPV 16 or 18 genome into these cells induces efficient immortalization in every case (5). The E6 gene of HPV-16 was sufficient for immortalization, providing a simple system to study the biochemical mechanisms of immortalization in MECs (6, 7). Consistent with the in vitro p53-degrading activity of the E6 protein (8), we demonstrated a marked degradation of the p53 protein in E6-immortalized MEC (6, 7). Moreover, the use of E6 genes of low-risk papillomaviruses or HPV-16 E6 mutants demonstrated an essential role of the in vivo p53 loss for MEC immortalization (7, 9). In another study, we have demonstrated that immortalization of the MECs induced by γ-radiation was also associated with an early loss of p53 protein (10). Together, these results suggest an essential role for p53 inactivation in MEC immortalization. This suggestion is consistent with the occurrence of mutations or loss of the p53 gene in a large proportion of breast cancers (11, 12). More importantly, the breast cancer-prone Li-Fraumeni syndrome patients possess a germ line mutation in the p53 gene (13, 14), and normal epithelial cells from such patients exhibit spontaneous immortalization (15). However, it was not known whether abrogation of p53 function is sufficient for immortalization of the MECs. Here, we demonstrate that a unique cancer-derived p53 mutant with a point deletion of Asp293 can by itself induce immortalization of MECs, indicating that the loss of p53 function is sufficient for immortalization of MECs.

Wild-type p53 is a DNA-binding protein that regulates the transcription of several key cell growth and cell cycle-related genes, such as WAF1 and mdm-2 (16, 17). This function is thought to be critical for p53-mediated G1 arrest in response to DNA damage, which is hypothesized to allow DNA repair prior to DNA replication, thus ensuring a mutation-free genome (18, 19). We show that MECs immortalized by the del239 p53 mutant indeed show a drastic abrogation of radiation-induced cell cycle arrest and induction of the expression of p53-regulated genes mdm-2 and WAF1. This unique model should help delineate the p53 function(s) that are critical for mammary cell growth control and for which abrogation is essential for immortalization.

MATERIALS AND METHODS

Cells and Cell Culture. Reduction mamplasty-derived MEC strain 76N and cell lines (76R-30 and 21PT) were grown in DFCI-1 medium as described previously (4, 10, 20).

Transfection and Immortalization. Various mutant p53 cDNAs (del239, R273L, N247I, V173L, C277F, G154V, and H179Q, obtained from Dr. Peter Howley, Harvard Medical School, Boston, MA; and V143A, obtained from Dr. Bert Vogelstein, Johns Hopkins University of Medicine, Baltimore, MD) were cloned into the pCMVneo vector (from B. Vogelstein) and transfected into immortalized MECs. Cells were also obtained by calcium phosphate-mediated transfection and G418 selection (50 µg/ml for 10 days), cells were transferred into D2 medium (DFCI-1 medium lacking FCS and bovine pituitary extract including 0.05% BSA) for selection of the immortal phenotype (5–7). Cells were passaged by subculturing at 1:3–1:10 ratios. Stable mutant p53 transfectants of 76R-30 and 21PT were also obtained by calcium phosphate-mediated transfection and G418 selection, as described earlier (10, 20).

Western Blot Analysis. Cell lysis was prepared in SDS-PAGE sample buffer, and 50 µg of each lysate (quantitated by Pierce protein assay) were resolved on 7.5% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes were incubated with a mAb against p53 (PAb 1801; Neomarkers, Fremont, CA) followed by goat antimouse antibody conjugated to horseradish peroxidase (Pierce). Enhanced chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham).

Immunoperoxidase Staining of p53 Protein. Cells (1 x 10^6/well) were plated in eight-well chamber slides (Nunc) for 48 h. The immunoperoxidase staining procedure was as recommended by the supplier (Vectastain avidin-biotin complex kit; Vector Laboratories, Burlingame, CA). Briefly, cells were fixed with acetone and methanol (1:1) for 5 min, blocked with normal horse serum, and incubated with anti-p53 mAb (1801; Neomarkers) or control mAb (P3) for 60 min in a humidified chamber. Cells were washed with PBS containing 0.05% Tween 20, incubated with biotinylated goat antimouse immunoglobulin for 30 min, washed, and then incubated with avidin coupled to horseradish peroxidase (Pierce). Enhanced chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham).

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4 The abbreviations used are: MEC, mammary epithelial cell; HPV, human papillomavirus; mAb, monoclonal antibody; del, deletion.
to horseradish peroxidase for 30 min. The color reaction was developed by adding diaminobenzidine tetrahydrochloride and hydrogen peroxide.

**Determination of p53 Half-Life.** Exponentially growing cells were pre-starved for 30 min in cysteine- and methionine-free medium and pulse labeled with 250 \(\mu\)Ci 35S-methionine and 35S-cysteine (Expre35S35S; New England Nuclear) for 15 min followed by chase for various times. At each time interval, cells were washed with cold PBS and lysed in NETN lysis buffer (20 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride). Lysates were precleared, and equal cpm of lysates were immunoprecipitated with PAb 122, an anti-p53 antibody (hybridoma was obtained from the American Type Culture Collection), and resolved on SDS-polyacrylamide gels as described earlier (21).

**Radiation-Induced G1 Arrest.** Exponentially growing cells were treated with 8 Gy \(\gamma\)-radiation using a Mark 17Cs irradiator and harvested with trypsin/EDTA at various time points. Harvested cells were fixed in 70% ethanol for 30 min at 4°C, washed twice with PBS, and treated with 10 \(\mu\)g/ml RNase for 1 h at 37°C. The treated cells were washed once with PBS and stained with propidium iodide (69 \(\mu\)M in 38 mM sodium citrate) for 30 min at room temperature, and the proportion of cells in different phases of the cell cycle was analyzed by FACSscan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as described previously (10).

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated from 50–70% confluent cell monolayers using the guanidinium isothiocyanate method (22). Northern blot hybridization was carried out using a nylon membrane (Hybond-N; Amersham) as described previously (5, 7). Probes used were a 2.1-kb WAF1 cDNA (16), a 1.8-kb \(\alpha\)53 cDNA (23), and a 0.8-kb mdm-2 cDNA (nucleotides 578-1462; Ref. 24).

## RESULTS

**Immortalization of Normal MECs by a p53 Mutant.** To examine the possible immortalization of MECs by selective inactivation of p53 function, we transfected 76N normal MECs (at passage 12) with seven missense mutants and one point deletion (Asn239) mutant of the p53 gene, each derived from human tumors. Our extensive analyses with 76N cells have demonstrated that these cells do not undergo spontaneous immortalization. Furthermore, both p53 alleles in 76N cells are wild type, as demonstrated by sequence analyses of the entire coding region of the p53 gene (21). The p53 mutants used in this study had been shown to dominantly inhibit p53 function through analyses in colon carcinoma and osteosarcoma (Saos-2) cells (25, 26).

G418-resistant cells were plated in D2 medium, which does not support the growth of normal MECs but does allow the growth of immortal cells (5–7). In three separate experiments, 76N cells transfected with each of the seven missense p53 mutants senesced and failed to give rise to immortal cells (Table 1). Strikingly, the cells transfected with the deletion mutant of p53 (del239) gave rise to a cell population that did not senesce in D2 medium; instead, these cells continued to divide and have been in continuous culture for >100 passages (split ratio, 1:10) without any signs of senescence (Table 1). Immortal cells were obtained in each of the three separate experiments. Morphologically, the immortal cells grow more densely and are polygonal compared with the spindle-shaped appearance of the normal 76N cells (Fig. 1). Hereafter, these immortal cells are referred to as MP1 cells.

MP1 cells did not exhibit anchorage-independent growth in soft agar when plated in DFCI-1 medium that contained various critical growth factors, including epidermal growth factor, insulin, and estradiol (4), and did not form tumors when transplanted in nude mice (data not shown). Together, the characteristics of MP1 cells indicate that these cells have undergone a neoplastic transformation.

**Expression of Mutant p53 Proteins in Transfected Cells.** To rule out the possibility that nonimmortalizing p53 mutants may have failed to express a stable protein, we used three different methods. The lysates of p53 transfecants after G418 selection were subjected to Western blot analysis. All transfecants revealed a similar increase in the levels of p53 protein compared with parental 76N cells, reflecting a substantial expression of the transfected mutant p53 genes (Fig. 2A). In addition, intense p53 signals were observed in mutant p53 transfecants compared with relatively low levels of staining in parental cells by immunoperoxidase staining using anti-p53 antibody PAb 1801 (Fig. 2B). As a third measure of expression of the transfected p53 mutants, we carried out pulse-chase labeling of transfecants, followed by p53 immunoprecipitation to determine the half-life of the p53 protein. As expected from our prior analyses that the p53 protein in 76N cells has a half-life of about 3 h (21), the p53 signal in vector-transfected 76N cells showed a substantial decrease by 4 h of chase period (Fig. 2C). In contrast, both the immortalizing (Fig. 2C, del239) and nonimmortalizing (Fig. 2C, N247L and V173L) p53 mutants were expressed at substantially higher levels with longer half-lives unique to each mutant (Table 1 and Fig. 2C). As summarized in Table 1, all other mutant p53 genes also encoded longer-living p53 protein. The half-lives of individual p53 mutants were similar when these were expressed in 76R-30 cells, a p53-null derivative of 76N cells (10; data not shown). Thus, the inability of missense

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**Table 1** Immortalization of 76N cells with del239 p53 mutant

<table>
<thead>
<tr>
<th>p53 construct</th>
<th>Experiments I–III, passages in D2</th>
<th>Immortalization</th>
<th>p53 half-life (h)</th>
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<tbody>
<tr>
<td>Vector</td>
<td>3, 2, 4</td>
<td>NI</td>
<td>3</td>
</tr>
<tr>
<td>N239 del</td>
<td>100, 100, 80, 60</td>
<td>I</td>
<td>6</td>
</tr>
<tr>
<td>R273L</td>
<td>3, 2, 2</td>
<td>NI</td>
<td>8</td>
</tr>
<tr>
<td>R247L</td>
<td>3, 4, 3</td>
<td>NI</td>
<td>6</td>
</tr>
<tr>
<td>V173L</td>
<td>3, 3, 2</td>
<td>NI</td>
<td>8</td>
</tr>
<tr>
<td>C277F</td>
<td>5, 2, 2</td>
<td>NI</td>
<td>8</td>
</tr>
<tr>
<td>G154V</td>
<td>2, 2, 2</td>
<td>NI</td>
<td>6</td>
</tr>
<tr>
<td>V143A</td>
<td>4, 4, 3</td>
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<td>6</td>
</tr>
<tr>
<td>H179Q</td>
<td>6, 4, 5</td>
<td>NI</td>
<td>ND</td>
</tr>
</tbody>
</table>

a NI, nonimmortal; I, immortal.

b These cells are referred to as MP1 cells.

c ND, not done.
Fig. 2. A, Western blot analyses of p53 protein in mutant p53-transfected 76N cells. 76N cells were transfected with vector or various p53 mutants and selected in DFCI-1 medium containing G418, and lysates were prepared in sample buffer. An equal amount of protein (50 µg) was processed for Western blot analysis. Note that all mutants are expressed at very high levels compared with vector-alone transfectants, which show low levels of endogenous p53. B, immunoperoxidase staining of p53 protein in vector- and various mutant p53-transfected cells. Vector- or mutant p53-transfected cells after selection in G418 were stained with anti-p53 antibody PAb 1801 using a Vectastain avidin-biotin complex kit. Note that all mutant p53 proteins are expressed at much higher levels compared with vector-transfected cells [Vector (p53 ab)]. C, pulse-chase analysis of p53 protein in p53 mutant-transfected 76N cells. G418-resistant 76N cell transfectants were pulse labeled with 35S-methionine/cysteine for 15 min and chased for various time periods. Equal cpm were immunoprecipitated with anti-p53 PAb 122 and resolved on SDS-7.5% polyacrylamide gels. Note that 76N cells transfected with vector control show the expected levels of p53 protein, with a half-life of <4 h. In contrast, the cells transfected with the indicated mutant p53 genes express markedly higher levels of p53 protein, with longer half-lives. Three representative mutant transfectants and vector transfectant are shown. Half-life data are included in Table I.

**DISCUSSION**

Our previous studies with HPV E6- and radiation-immortalized MECs indicated a critical role for the loss of p53 function in MEC immortalization (6, 7, 10). Here, we addressed the possibility that loss of p53 function alone may be sufficient to immortalize MECs. Prior studies have demonstrated that the p53 protein is an oligomer, and oligomerization of wild-type with a mutant p53 protein can drive it into a mutant conformation (30). Thus, expression of mutant p53 proteins in MECs could be expected to selectively ablate the function of endogenous p53 (31). Therefore, we transfected a number of mutant p53 cDNAs derived from human tumors (25, 26, 32, 33) into 76N normal MECs and assessed whether immortalization was induced. Our results showed that seven of eight p53 mutants that we tested did not induce immortalization. Analysis of p53 expression demonstrated that the inability of seven mutants to immortalize MECs was not due to lack of expression of a stable protein.

**Abrogation of Radiation-induced G1 Arrest in MPI Cells.** A well-defined function of the wild-type p53 protein is to mediate G1 arrest in response to DNA damage (18, 19). We used this response to assess the functional status of the endogenous wild-type p53 protein in MPI cells. As shown in Fig. 3, A and B, γ-irradiation of 76N normal parent cells induced the expected G1 arrest, resulting in a decrease in the proportion of cells in S-phase with little change in G1. In contrast, MPI cells failed to exhibit G1 arrest after γ-irradiation, as shown by a decrease in G1 phase (Fig. 3, A and B). As expected, both the 76N and MPI cells exhibited the p53-independent, radiation-induced G2-M arrest, as seen by accumulation of cells in G2. These results demonstrate a loss of wild-type p53 function in MPI cells.

**Abrogation of Radiation-Induced WAF1 and mdm-2 mRNA Expression in MPI Cells.** The ability of p53 protein to transactivate cellular genes has been closely linked to its ability to serve as a cell cycle checkpoint control (27). Recent studies have shown that the expression of p53-regulated WAF1 and mdm-2 genes is increased in response to DNA damage in a p53-dependent manner (28, 29). Therefore, we examined the expression of WAF1 and mdm-2 mRNA in 76N (normal) and MPI cells after γ-irradiation. The γ-irradiated 76N cells showed an increase in the levels of the WAF1 and mdm-2 RNA (Fig. 4), which was accompanied by an increase in the level of the p53 protein (data not shown). In contrast, γ-irradiation of MPI cells failed to induce an increase in the levels of either the WAF1 or mdm-2 mRNA (Fig. 4). Probing of the same blot with β-actin shows equal mRNA loading. These results support a lack of wild-type p53-mediated transactivation function in MPI cells.
have been shown to abrogate transactivation mediated by p53 in other cell types (26), and one particular mutant (V143A) was shown to extend the life span of senescent human diploid fibroblasts (34). In fact, we confirmed this by examining the effects of p53 mutants on cotransfected wild-type p53 function following transient transfection into 76R-30 cells, a p53-null cell line derived by radiation transformation of 76N normal MECs (10). Recent studies have demonstrated that abrogation of wild-type p53 functions by p53 mutants is cell type dependent (35). It is possible that the nonimmortalizing mutants are unable to abrogate p53 functions in the milieu of the primary MECs. In this context, it is notable that primary MECs in culture express substantially higher levels of wild-type p53 compared with isogenic mammary fibroblasts (21). Furthermore, the half-life of the p53 protein in mammary fibroblasts was about 15 min, whereas that in MECs was about 3 h (21). At present, direct analyses of the effects of nonimmortalizing p53 mutants on p53 functions in primary breast epithelial cells have been technically difficult, due to markedly lower transfection efficiency and high levels of endogenous wild-type p53 proteins.

It has been reported that some p53 mutants exhibit a gain of function similar to certain oncogenes, which can be assayed by enhanced anchorage-independent growth of cells expressing these mutants (36). However, we did not detect any change in growth of two p53-null mammary epithelial cell lines 76R-30 and 21FT (10, 20) when stably transfected with the del239 p53 mutant. Thus, we believe that a gain of function is unlikely to be the explanation for del239-induced immortalization.

At present, it is not known whether the immortalizing ability of the del239 mutant is related to its being deletional or because residue 239 is more critical for p53 function in MECs.Codon 239 is within the hot spot region for p53 mutations (37). The del239 mutant was originally derived from a lung cancer specimen (32) but has also been observed occasionally in leukemia and breast cancer samples, whereas its mutation (Asn → Ser) has been reported in colon cancer (38, 11). Interestingly, this mutant has been reported to be capable of binding to SV40 large T antigen as well as to HPV16 E6, although at significantly lower levels than the wild-type p53 (33). More extensive analyses of both the missense and deletional mutants should clarify the nature of p53 mutations that confer MEC-immortalizing ability.

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