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Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7

(breast cancer/p53/Rb/keratins/tumor-suppressor genes)

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ABSTRACT **Multiple mammary epithelial cell (MEC) types are observed both in mammary ducts *in vivo* and in primary cultures *in vitro*; however, the oncogenic potential of different cell types remains unknown. Here, we used human papilloma virus 16 E6 and E7 oncogenes, which target p53 and Rb tumor suppressor proteins, respectively, to immortalize MECs present in early or late passages of human mammary tissue-derived cultures or in milk. One MEC subtype was exclusively immortalized by E6; such cells predominated in late-passage cultures but were rare at early passages and apparently absent in milk. Surprisingly, a second cell type, present only in early-passage tissue-derived cultures, was fully immortalized by E7 alone. A third cell type, observed in tissue-derived cultures and in milk, showed a substantial extension of life span with E7 but eventually senesced. Finally, both E6 and E7 were required to fully immortalize milk-derived MECs and a large proportion of MECs in early-passage tissue-derived cultures, suggesting the presence of another discrete subpopulation. Identification of MECs with distinct susceptibilities to p53- and Rb-targeting human papillomavirus oncogenes raises the possibility that these cells may serve as precursors for different forms of breast cancer.**

The majority of breast cancers arise from epithelial cells of the mammary gland. Both *in vivo* and in cell culture, two major types of epithelial cells are seen: a continuous layer of glandular cells (luminal cells) lines the duct, whereas a discontinuous layer of cells near the basement membrane (basal cells) shares certain features of myoepithelial cells (1, 2). Intermediate phenotypes have also been observed (1). Basal cells predominantly express keratin (K) 5, K6, K7, K14, and K17, whereas luminal cells predominantly express K8, K18, and K19 (1–4). The relative oncogenic potential of various mammary epithelial cells (MECs) remains unknown. Myoepithelial cells rarely if ever give rise to breast cancers, but it is possible that luminal and basal cell subtypes give rise to distinct carcinomas.

To circumvent the inherent limitations on *in vivo* analyses of human tumor progression, this laboratory has established a cell culture system to oncogenically transform mammoplasty-derived MECs (5–8). These cells represent a selected population that emerges from a mixed initial culture after 4 or 5 passages and grows further for 10–20 passages (5); these selected cells are immortalized by human papilloma virus (HPV) 16 E6 but not by HPV-16 E7 (6). In contrast, both E6 and E7 are required to efficiently immortalize keratinocytes (9). Here, we used retroviral infection of early (before selection) or late (after selection) passages of mammoplasty-derived and milk-derived cells to demonstrate multiple epithelial cell subtypes with distinct susceptibilities to immortalization by E6, E7, or both E6 and E7.

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MATERIALS AND METHODS

Cell Culture. Normal MECs were derived in DFCI-1 medium, as described (5). In DFCI-1, the outgrowing cells are heterogeneous and proliferate rapidly for 4 or 5 passages (referred to as "early passage"); this is followed by a selection when most cells senesce and the resulting homogeneous population can be propagated for 10–20 passages (referred to as "late passage"). Milk-cell strains M1, M2, M3, and M4 were derived from milk samples of healthy lactating women, as described (4), and cultured in DFCI-1 medium with 10% (vol/vol) fetal calf serum. M1 and M2 are from a single donor.

Retroviral Infection of MECs. PA317 amphotrophic packaging cell lines stably transfected with pLXSN (vector), pLXSN16E6 (HPV-16 E6), or pLXSN16E7 (HPV-16 E7) (provided by D. Galloway, Fred Hutchinson Cancer Research Center, Seattle) (10) were grown to 70–80% confluence, and supernatants were collected for 16 hr and stored in aliquots at −80°C. Approximately 10^5 cells were plated per 25-cm^2 flask for 18 hr and infected with 100–200 μl of virus stock in 2 ml of medium containing Polybrene (Sigma) at 4 μg/ml for 8 hr.

Immunoprecipitation of HPV-16 E6 and E7 Proteins. Detergent lysates of [^{35}S]cysteine-labeled cells were immunoprecipitated with rabbit anti-E6 [against glutathione *S*-transferase (GST)–HPV-16 E6 fusion protein] and anti-E7 (provided by D. Galloway) antibodies and electrophoresed on a 12.5% polyacrylamide gel, as described (6, 11).

Radiation-Induced G_1-Phase Arrest. Exponentially growing cells were γ-irradiated with 8 Gy from a ^{137}Cs source, harvested at 36 hr, stained with propidium iodide, and analyzed for cell cycle distribution on a fluorescence-activated cell sorter (FACScan, Becton Dickinson), as described (12).

Western Blot Analysis. Cell lysate (25 μg) was resolved on 7.5% gels, transferred to poly(vinylidene difluoride) membrane (Immobilon-P, Millipore), incubated with anti-p53 (PAb 1801, Oncogene Science) or anti-Rb (PharMingen) antibodies followed by horseradish peroxidase-conjugated goat anti-mouse (Pierce), and developed by enhanced chemiluminescence (Amersham), as described (13).

Northern Blot Analysis. Hybridization of total RNA with a 2.1-kb WAF1 (14), a 0.8-kb mdm-2 (15), a 1.2-kb Rb (16), and a 0.7-kb GST π (17) cDNA was as described (8).

Two-Dimensional Gel Analysis of Keratin Expression. The Triton X-100-insoluble fraction of [^{35}S]methionine- and [^{35}S]cysteine-labeled cells was solubilized in isoelectric focusing buffer and analyzed by nonequilibrium pH-gradient gel electrophoresis followed by SDS/PAGE, as described (3).

RESULTS

HPV-16 E6 Alone Efficiently Immortalizes MECs in Late-Passage but not Early-Passage Cultures. Since HPV-16 E6

Abbreviations: MEC, mammary epithelial cell; HPV, human papilloma virus; GST, glutathione *S*-transferase; PD, population doubling; K, keratin.

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alone immortalizes the late-passage MECs (6, 7), it was of interest to determine whether the additional cell types seen at early passages showed a similar or a distinct requirement for immortalization. We introduced HPV-16 E6, E7, or both E6 and E7 into early-passage MECs by retroviral infection, which could be expected to transduce these genes even into relatively rare cell types present in the cultures. After 48 hr, cultures were transferred to D2 medium, which selects for outgrowth of immortal cells (6–8).

In contrast to efficient immortalization of late-passage MECs by E6 (6, 7), seven out of nine early-passage cells infected with E6 senesced within three to five passages (Table 1). In two samples (76N and 70N), a crisis period with cell death was observed, followed by outgrowth of cells that have been in culture for >600 PDs. The viral supernatants were highly efficient at immortalizing the late-passage MECs, excluding the possibility of low titers. Thus, E6 alone is inefficient at immortalizing cells that predominate in early-passage cultures, indicating the rarity of E6-susceptible population.

HPV-16 E7 Immortalizes a Fraction of MECs in Early-Passage Cultures. While E7 alone was incapable of immortalizing late-passage E6-susceptible MECs (6) (Table 1), selection of E7-infected early-passage MECs in D2 medium resulted in a rapidly growing cell population (doubling time, ≈24 hr), followed by a crisis period when many cells senesced. While all cells in one sample senesced by passage 20, growing cells emerged in eight other samples; these have been in culture for >40 passages (>400 PDs). Thus, early-passage cultures contain MECs that undergo immortalization or a dramatic extension of life span (normally <5 passages) when infected with E7 alone.

Table 1. Immortalization of MEC strains by HPV-16 E6, E7, or both E6 and E7

Sample	Passage	Outcome of infection			
		Vector	16E6	16E7	16E6+E7
Mammoplasty-derived					
7VN	Early	S	S	I	I
	Late	S	I	S	I
8VN	Early	S	S	I	I
	Late	S	I	S	I
9VN	Early	S	S	I	I
	Late	S	I	S	I
32VN	Early	S	S	I	I
70N	Early	S	I	Ex(S)	I
	Late	S	I	S	I
76N	Early	S	I	I	I
	Late	S	I	S	I
81N	Early	S	S	I	I
	Late	S	I	S	I
Mastectomy-derived					
21VN	Early	S	S	I	I
	Late	S	I	S	I
28VN	Early	S	S	I	I
Milk-derived					
M1*		S	S	EX(S)	I
M2*		S	S	EX(S)	I
M3		S	S	EX(S)	I
M4		S	S	EX(S)	I

Cells were infected with virus stocks of vector (LXSN), 16E6, 16E7, or 16E6+E7 retroviral constructs. Mammoplasty-derived cells were shifted to D2 medium 2 days after infection. Milk-derived cells were grown in DFCI-1 plus 10% fetal calf serum. I, immortal cells; S, senesced (tissue-derived cells of <5 passages and milk-derived cells of <3 passages); EX(S), extension of life span [200 population doublings (PDs), mammoplasty-derived; 60–100 PDs, milk-derived], followed by senescence.
*Two samples from the same person.

Both E6 and E7 Are Required to Immortalize a Large Fraction of MECs in Early-Passage Tissue-Derived Cultures and in Milk. Selection of E6- and E7-infected early-passage cultures in D2 medium led to rapidly growing cell lines in all samples, with minimal or no crisis period. These cells have been in culture for >60 passages with no signs of senescence. Thus, the combination of E6 and E7 is markedly more efficient at immortalizing early-passage MECs than either gene alone.

In addition to tissue-derived MECs, we also infected four milk-derived MEC strains with E6, E7, or both E6 and E7 retroviruses. Similar to vector-infected milk cells, E6-infected cells failed to grow beyond 2 or 3 passages and senesced. E7-infected cells grew rapidly for ≈10 passages (60–100 PDs) and then senesced in all cases. In contrast, E6- and E7-infected cells continued to grow without crisis and are currently at >80 passages (800 PDs) (Table 1). Thus, the analyses of tissue- and milk-derived cells strongly suggest that the combination of E6 and E7 target additional MECs that are not immortalized by E6 or E7 alone.

Similar to E6-immortalized cells studied earlier (6, 7), tissue-derived cells immortalized with E6, E7, or both E6 and E7 showed anchorage-dependent growth and did not grow as transplants in nude mice (data not shown). However, E6- and E7-immortalized milk cells could grow in soft agar but were nontumorigenic in nude mice (data not shown).

Expression of E6 and E7 Proteins in Immortal Cells. To assess E6 and E7 protein expression, detergent lysates of metabolically labeled cells were immunoprecipitated with anti-E6 and anti-E7 antibodies. The antisera immunoprecipitated HPV-16 E6 (18 kDa) and E7 (20 kDa) proteins, respectively, from the E6- and E7-expressing cervical carcinoma cell line Caski (used as a positive control) but not from normal MECs, 76N (Fig. 1). Importantly, E6 or E7 proteins were readily detectable in all cells immortalized by E6 or E7 genes, respectively.

Abrogation of Radiation-Induced G_1-Phase Arrest in Both E6- and E7-Immortalized Cells. To assess the functional inactivation of p53 and Rb by E6 and E7 (18, 19), respectively, we compared immortalized cells with their parent cells for G_1-phase cell cycle arrest in response to γ-irradiation. Irradiated normal parental cells (76N, 70N, and 81N) failed to exit the G_1 phase (little change in G_1 with a decrease in S and a small increase in G_2/M). In contrast, irradiated E6-immortalized cells continued to exit G_1 (marked decrease in G_1, smaller decrease in S, and a marked increase in G_2/M). Interestingly, E7-immortalized cells also showed a lack of G_1 arrest (Fig. 2). Similar results were obtained with E6- and E7-immortalized 7VN, 8VN, and 21VN cells (data not shown). Thus with recently published reports in other cell types (20–22), our data show that both E6 and E7 abrogate radiation-induced G_1 arrest, indicating a functional inactivation of p53 and Rb, respectively.

Decreased p53 and Rb Protein Levels in E6- or E7-Immortalized Cells, Respectively. To directly correlate the loss of p53- and Rb-mediated G_1 arrest with the expression of these proteins, we performed Western blot analyses of lysates prepared before and after exposure of cells to γ-irradiation. As expected (6, 7), levels of p53 protein in E6-immortalized cells were drastically reduced compared to normal cells but were

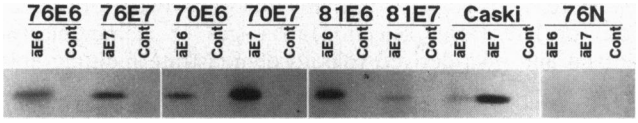

FIG. 1. Immunoprecipitation analysis of E6 and E7 proteins. Metabolically labeled cells were immunoprecipitated with preimmune (Cont), anti-E6 (āE6), or anti-E7 (āE7) antibodies, resolved on 12.5% gels, and autoradiographed.

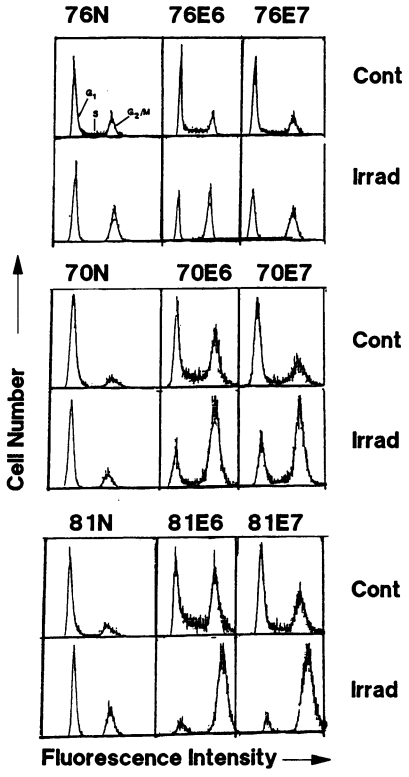

FIG. 2. Cell cycle distribution of γ-irradiated normal cells and E6- and E7-immortalized cells from a flow cytometry analysis. Cells were plated in DFCI-1 medium for 48 hr, treated with 8 Gy of γ-irradiation, and then processed for cell cycle analysis 36 hr later. Different phases of cell cycle (G_1, S, and G_2/M) are shown.

normal in E7-immortalized cells (Fig. 3). γ-Irradiation significantly increased the p53 levels (seen clearly at 3 hr) in normal and E7-immortalized cells. In contrast, p53 levels in E6-immortalized cells failed to increase upon γ-irradiation (Fig. 3).

Differences in phosphorylation result in a 110-kDa (hypophosphorylated) form and a 112- to 114-kDa (hyperphosphorylated) form of Rb on SDS/PAGE gels (23); E7 is known to interact with the hypophosphorylated form that mediates cell cycle arrest (19). Both Rb forms were observed in growing normal MECs. Twenty-four hours after irradiation, Rb protein was primarily in a hypophosphorylated form. The basal levels of Rb protein were markedly reduced in all E7-immortalized cells compared to normal or E6-immortalized cells (Fig. 3 and data not shown). Longer exposures of immunoblots showed that irradiation did not change relative proportions of the two Rbs forms in these cells (data not shown). Interestingly, in most cases (such as 70E6 and 81E6 and data not shown), both Rb forms were observed 24 hr after irradiation of E6-immortalized cells. Thus, E6 and E7 led to specific biochemical alterations of p53 and Rb proteins, respectively, which are likely to account for the loss of their function.

The lower levels of Rb in E7-immortalized cells are likely to be due to E7-induced changes in Rb protein rather than selection of cells with intrinsically lower Rb expression, since a number of early- and late-passage normal MEC strains (76N, 70N, 81N, and 21VN) showed no differences in the levels of Rb protein (data not shown). Consistent with this suggestion, Rb mRNA levels in normal and E6- or E7-immortalized cells were comparable (Fig. 4).

Induction of WAF1 and mdm-2 in E7- but Not E6-Immortalized Cells. The p53 protein regulates transcription of genes, such as WAF1 and mdm-2 (14, 15). Indeed, irradiation of normal and E7-immortalized MECs rapidly increased the WAF1 and

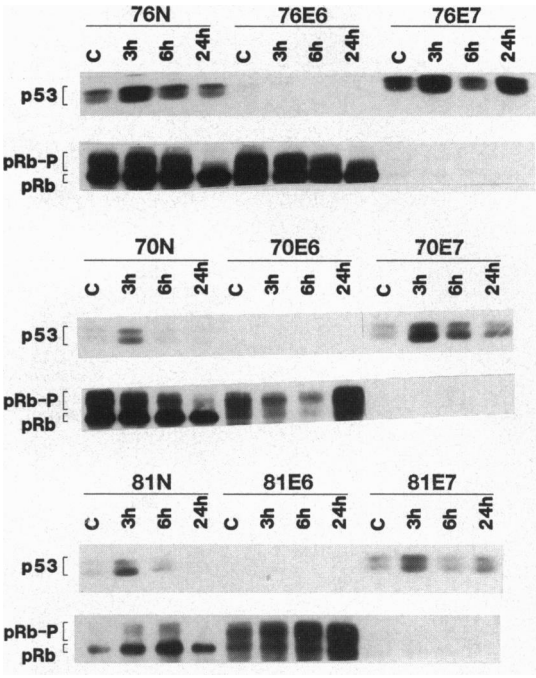

FIG. 3. Western blot analysis of p53 and Rb proteins in normal cells and E6- and E7-immortalized cells. Cells were irradiated as in Fig. 2, and lysates prepared at the times indicated were processed for Western blot analysis. pRb-P, hyperphosphorylated Rb; pRb, hypophosphorylated Rb.

mdm-2 mRNA (Fig. 4). In contrast, E6-immortalized cells failed to show an increase in WAF1 or mdm-2 mRNA levels. These results further demonstrate that p53 function is selectively lost in E6-immortalized cells.

Keratin Profiles of E6- or E7-Infected MECs. To begin to assess whether E6- and E7-susceptible MECs are distinct, we examined the patterns of keratin expression on selected sam-

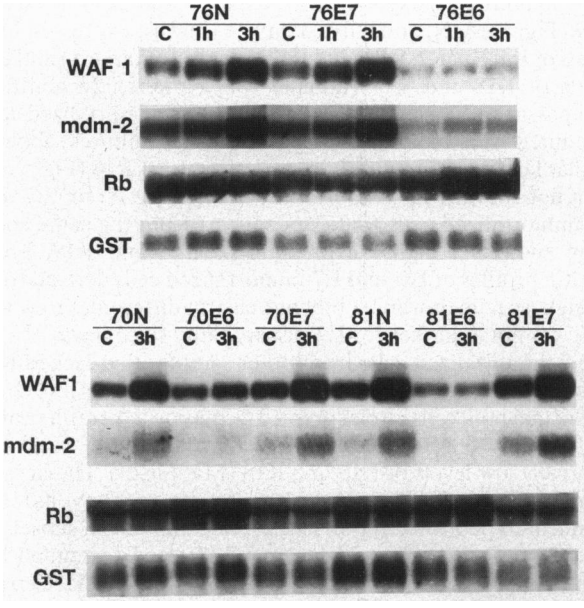

FIG. 4. Expression of WAF1, mdm-2, and Rb mRNA in 8-Gy γ-irradiated normal cells and E6- and E7-immortalized cells. Ten micrograms of total cellular RNA was loaded per lane. Membranes were hybridized with cDNA probes specific for WAF1, mdm-2, and Rb. GSTπ (GST) was used as a loading control.

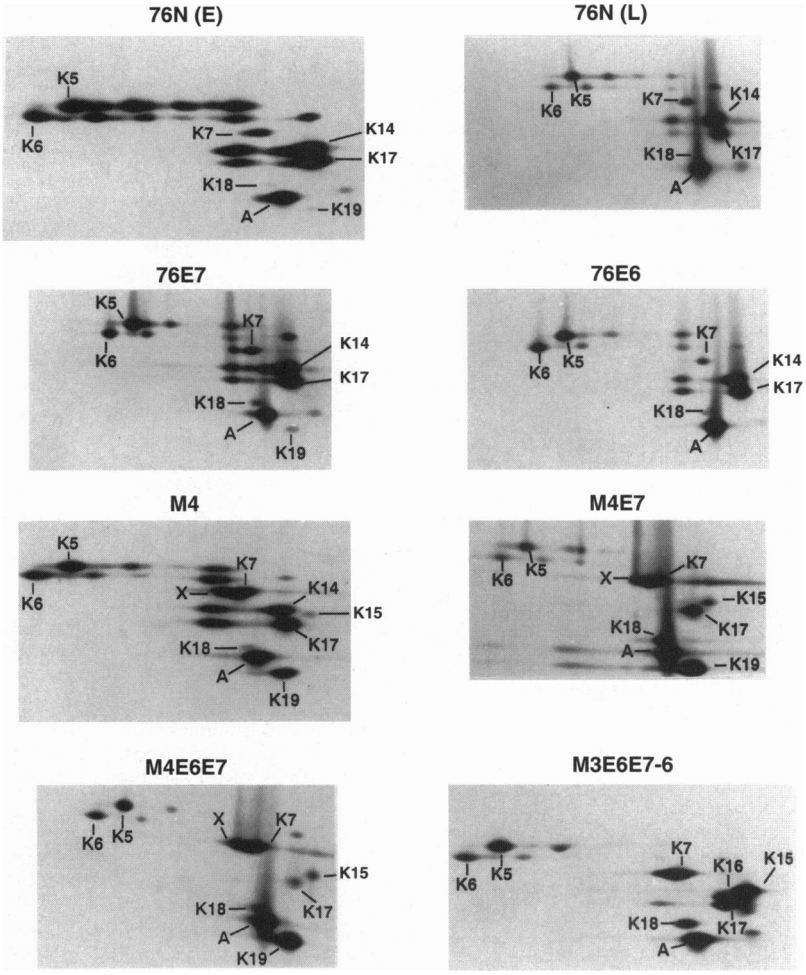

FIG. 5. Keratin analysis of normal cells and E6-, E7-, and E6+E7-immortalized cells. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and keratins were extracted and analyzed by nonequilibrium pH-gradient gel electrophoresis in first dimension, followed by SDS/PAGE in second dimension. Various keratins are indicated. A, actin; X, an unassigned keratin probably K8.

ples of these immortalized MECs. Consistent with the presence of multiple cell types, early-passage tissue-derived MECs, 76N (Fig. 5), 81N, and 70N (data not shown), expressed high levels of K5, K6, K7, K14, and K17 (basal cell markers) and low levels of K18 and K19 (luminal cell markers). In contrast, late-passage MECs lacked K19 (Fig. 5). E6-immortalized cells (obtained from either early- or late-passage cultures) showed similar keratin profiles with increased levels of K18 (Fig. 5 and data not shown). E7-immortalized (76E7, Fig. 5) or E6- and E7-immortalized cells (data not shown) from the same specimen showed a higher expression of K18 and K19. Thus, keratin profiles of E6- and E7-immortalized cells derived from a single specimen were subtly but clearly different. However, K19 was not a marker for E7 susceptibility since it was absent in E7-immortalized cells from other samples that lacked K19 to begin with (e.g., 7VN and 8VN; data not shown).

Normal milk cells also expressed a composite keratin profile with high levels of K5, K6, K7, K14, K17, and K19 and relatively low levels of K15 and K18 (M4, Fig. 5). The keratin marked X is probably K8, although its mobility did not conform to published reports (1, 2). In comparison, subsets of these keratins were expressed on E7-infected (extended life span) or E6- and E7-immortalized pooled milk cells. Certain clones of pooled E6- and E7-immortalized milk cells did not express K19 but expressed high levels of K5, K6, K7, K15, K16, K17, and K18 (M3E6E7-6, Fig. 5). Thus, keratin profiles are consistent with the presence of multiple MEC subtypes but do not predict E6 or E7 susceptibility.

DISCUSSION

The relationship between the heterogeneity of normal precursor cells and the evolution and behavior of human cancers in general and those of the breast in particular is not well understood. Such analyses are of substantial significance, as they may be related to such poorly understood characteristics of tumors as growth rate, drug/radiation sensitivity, invasive potential, and propensity to recur.

Here, we demonstrate that distinct MECs show a remarkably different susceptibility to oncogenes that target two important tumor suppressor gene products, p53 and Rb (18, 19). In contrast to efficient immortalization of late-passage MECs by E6 alone and their resistance to immortalization by E7 (6), early-passage MECs were very inefficiently immortalized by E6, consistent with the idea that E6-susceptible population is rare. Whether selection of this cell type is due to preferential growth in currently used media, intrinsically higher proliferative potential, *in vitro* differentiation from an E6-resistant precursor, or the possibility that these cells represent stem cells with longer life span is unknown at present.

Interestingly, use of early-passage tissue-derived cultures revealed a distinct cell population whose life span could be dramatically extended by E7 alone. In many cases these cells have been in culture for up to 400 PDs, strongly indicative of their immortalization. E7 immortalization was typically associated with a crisis period during which a proportion of cells was lost. Use of both E6 and E7, however, resulted in highly efficient immortalization with little or no crisis period. This

result suggests that the combination of E6 and E7 may immortalize additional cell types that are not susceptible to immortalization by either gene alone. Consistent with this suggestion milk-derived epithelial cells could not be fully immortalized by either E6 or E7 but were immortalized by the combination of E6 and E7. Although subtle differences in keratin expression were observed in certain cases, it is clear that additional studies are needed to identify phenotypic and/or genotypic markers that define the various MEC subtypes targeted by HPV-16 oncogenes.

Biochemical analyses showed an expected loss of p53 protein in E6-immortalized cells, with abrogation of p53-dependent G_1 cell cycle response to DNA damage and lack of induction of p53-regulated genes WAF1 and mdm-2. Unexpectedly, all E7-immortalized cells showed a dramatic reduction in the levels of the Rb protein, whereas Rb mRNA levels were unaltered. It is possible that E7 protein inhibits Rb protein synthesis or targets it for degradation, analogous to E6-induced degradation of p53 protein (7, 18).

Differential susceptibility of different epithelial cell subtypes from a single organ to oncogenes is unprecedented. In addition to providing initial evidence for different oncogenic potentials of these cell types, our results suggest that different tumor suppressor pathways may each contribute in the homeostasis of a single tissue type by predominating either in a particular cell type or at various stages of differentiation. Moreover, in certain cell types multiple tumor suppressor proteins may be functional necessitating their concurrent inactivation for efficient immortalization. Importantly, the MECs immortalized with E6, E7, or both E6 and E7 provide critical reagents to examine the possible relationship between different cellular subtypes and different histologic forms of human breast cancer.

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