Expression of the HPV16 E7 Gene Generates Proliferation in Stratified Squamous Cell Cultures Which Is Independent of Endogenous p53 Levels

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Abstract
Monolayer cultures of human foreskin and ectocervical epithelial cells were infected with retroviral vectors expressing HPV16 oncogenes, selected for G418 resistance, and cultured organotypically so that they reformed the fully differentiated, stratified squamous tissues from which they were originally derived. Expression of HPV16 E7 prevented cell cycle withdrawal in the suprabasal layers of these stratified cultures but had no effect on terminal differentiation. Cultures expressing E7 alone and those coexpressing E6 and E7 were identical in terms of suprabasal proliferation and terminal differentiation, but they differed in expression of the endogenous tumor suppressor protein p53. Immunohistochemically detectable p53 protein localized to the proliferative compartment in normal and E7-containing cultures but was undetectable in those cultures which coexpressed E6 and E7. This result suggests that E7-induced suprabasal proliferation is independent of the steady-state level of p53.

Introduction
Among the many HPV types which commonly infect the genital or mucosal epithelium, only the so-called high risk types, such as HPV16, are strongly associated with progression to malignant disease. The viral genes designated E6 and E7 are specifically implicated in this process since they are frequently retained in an integrated, functional form in malignant cells originating from HPV-infected tissues. This association with malignancy is also reflected in the transforming properties of these viral genes, since together the E6 and E7 genes of high risk, but generally not low risk HPVs, can cooperate to transform or immortalize a variety of cell types in culture (for reviews, see Refs. 1 and 2). Both the E6 and E7 genes from high risk HPVs can separately induce some aspects of the transformed phenotype in established rodent fibroblast cultures (3, 4), and in primary foreskin epithelial cells, E7 alone can extend the life span of these cells beyond senescence (5), such that fully immortalized cell lines occasionally emerge after an extended crisis phase (6, 7). Although high risk E6 alone can immortalize primary human breast epithelial cells (8), cooperation between the E6 and E7 genes is apparently required to efficiently immortalize epithelial cells from stratified squamous tissues such as foreskin (5, 9, 10).

There is compelling evidence that two significant cellular targets of the HPV E6 and E7 proteins are the p53 and pRB tumor suppressor proteins, respectively. Both p53 and pRB appear to negatively regulate cell proliferation, and loss or mutation of either or both of the genes that encode these proteins is associated with a variety of malignancies (11, 12). E7 proteins encoded by high risk HPV types appear to inactivate the tumor suppressor activity of pRB by binding pRB with high affinity (13, 14), and many of the in vitro transforming properties of E7 have been linked to its pRB-binding domain (15–17). Similarly, E6 proteins from high risk HPV5 bind p53 with high affinity, but this interaction appears to inactivate p53 by targeting it for rapid degradation by the ubiquitin pathway (18–20). The transforming proteins of adenovirus and SV40 also bind to one or both of these tumor suppressor proteins (13, 21, 22), suggesting that inhibition of negative proliferation controls is a common mechanism of viral transformation among these viruses.

pRB is a nuclear phosphoprotein whose phosphorylation state and nuclear affinity changes in a cell cycle-dependent manner (23, 24). Quiescent G0 cells or cells in G1 contain a hypophosphorylated form of pRB which appears to be the active, proliferation-suppressing form, and recent evidence suggests that the unphosphorylated form of pRB may limit cell cycle progression, in part by sequestering or inactivating the E2F transcription factor and other cellular proteins involved in cell cycle progression (25, and references therein). The mechanism by which p53 acts to regulate proliferation is less clear. p53 is phosphorylated by cdc2 (26), and like pRB, its phosphorylation state, subcellular location, and tertiary conformation change in a cell cycle-dependent manner (26–29), suggesting that the function of p53 is regulated within the cell cycle. Furthermore, the characterization of p53 as a tumor suppressor protein suggests that p53 normally functions to negatively regulate cellular proliferation, and this notion is supported by various transfection studies in which transduction of a WT p53 gene inhibited proliferation or reduced the tumorigenicity of cultured cells which were lacking normal p53 function (30–32). In spite of the fact that p53...
function is strongly associated with negative regulation of proliferation, endogenous expression of p53 is commonly detected in proliferating, but not quiescent cells (28, 29, 33–36). It seems reasonable, therefore, that p53 might specifically function in actively cycling cells to maintain specific constraints on the proliferation process.

In stratified squamous tissues, such as foreskin and ectocervix, proliferation is normally limited to the undifferentiated basal layer. As proliferating cells are displaced from the basal layer, they withdraw from the cell cycle, assume a flattened or squamous morphology, and sequentially express a variety of tissue-specific differentiation functions as they progress through increasingly mature tissue layers, undergo terminal differentiation, and are ultimately sloughed from the apical surface of the tissue (37, 38). Organotypic cultures provide a means to reconstitute these differentiated stratified tissues in vitro with cultured cells (39, 40). In these cultures, epithelial cells are supported by a collagen gel embedded with stromal fibroblasts and are maintained at the air-liquid interface such that the epithelial cells are exposed to air and fed via the underlying collagen gel. Under these conditions, cells from normal stratified squamous epithelium will grow, stratify, and differentiate into a multilayered epithelial sheet which closely resembles the tissue of origin. HPV-immortalized epithelial cell lines cultured organotypically display a wide range of abnormalities in both growth and differentiation. However, many of these cell lines rapidly acquire additional abnormalities with passage in culture, and it has not been possible with established cell lines to determine which abnormalities are a direct consequence of HPV oncogene expression and which are a consequence of subsequent passage in culture (41–45).

To sort out abnormalities directly attributable to HPV oncogene expression, we have utilized recombinant retroviruses to integrate the HPV16 E7 gene alone, or the contiguous HPV16 E6 and E7 genes together, into both HFE and HCE cells. This approach allows for the selection of G418-resistant cell populations, and their subsequent organotypic culture, prior to senescence of vector-containing controls. In this report, we demonstrate that HPV16 E7 specifically prevents cells from becoming quiescent in the suprabasal layers of organotypic cultures of stratified squamous epithelia, and that the continued cycling of these cells does not interfere with their ability to mature and terminally differentiate. Furthermore, cultures expressing E7 alone and those coexpressing E6 and E7 were identical in terms of suprabasal proliferation, terminal differentiation, and overall tissue morphology, in spite of the fact that both E6 and E7 are generally required for efficient immortalization of cultured stratified squamous cells. The one property of presenescent organotypic cultures which did correlate with long term immortalization potential was loss of immunohistochemically detectable p53 protein in proliferating cells, since p53 protein localized to the proliferative compartment in organotypic cultures of normal and E7-containing cells but was undetectable in those that coexpressed E6 and E7.

**Results**

**Expression of HPV16 E6 and E7 in Epithelial Cells.** The LXSN, LXSN16E6E7 and LXSN16E7 retroviral constructs utilized in these studies are diagrammed in Fig. 1. HFE and HCE cells were infected with these constructs, selected for G418 resistance, and analyzed by immunoprecipitation for the ability to synthesize HPV16 E6 and E7 proteins. All pooled and clonal G418-resistant HCE cell populations expressed the appropriate HPV proteins (Fig. 2), and identical results were obtained with HFE cells (data not shown). Previous studies have shown that the level of expression of E6 and E7 generated in genital epithelial cells with these retroviral constructs is sufficient for high frequency immortalization (6).

**Morphological Consequences of HPV16 Oncogene Expression in Organotypic Cultures of HCE Cells.** Ectocervical epithelium is a nonkeratinizing stratified epithelium with a cuboidal proliferative basal layer, several homogeneous intermediate layers of immature squamous cells, and many layers of terminally mature squamous cells. These terminally differentiated cells are characterized by the presence of pyknotic nuclei and the accumulation of large glycogen stores which give their cytoplasm a characteristic pale and distended appearance (37). To determine the effects of HPV oncogenes in a tissue-like setting, pooled and clonal populations of HCE cells which had been infected with the LXSN, LXSN16E6E7, or LXSN16E7 retrovirus constructs and selected for G418 resistance were cultured organotypically (Fig. 3). Parental uninfected HCE cells and pooled G418-resistant HCE colonies infected with the LXSN vector alone formed a differentiated epithelium in organotypic culture which was identical in appearance to the ectocervix of a sexually mature female. In contrast to the normal controls, pooled G418-resistant colonies of HCE cells containing either the HPV16 E7 gene alone (LXSN16E7), or the contiguous HPV16 E6 and E7 genes together (LXSN16E6E7), generated organotypic cultures which closely resembled premalignant low grade intraepithelial neoplasias associated with squamous cell carcinoma (46). In these oncogene-containing cultures, the normally quiescent squamous cells of the immature su-
prabasal layers were replaced by cuboidal mitotic cells (Fig. 3), and the presence of these apparently proliferating cells in the suprabasal layers suggested that expression of HPV16 E7, either by itself or in the context of the E6/E7 retroviral construct, specifically prevents cells from becoming quiescent once they leave the basal layer. At the same time, these cultures retained the ability to terminally differentiate in the upper layers of the reconstituted tissue (Fig. 3), and periodic acid-Schiff staining has confirmed that these morphologically differentiated cells are fully glycogenated (data not shown).

Suprabasal cell cycle withdrawal is widely presumed to be a prerequisite for terminal differentiation in stratified squamous tissues, yet the results presented above suggest that E7 expression specifically blocks cell cycle withdrawal with no concomitant loss of terminal differentiation. However, since the pooled cell populations were derived from approximately 5000 individual G418-resistant colonies, they did not exclude the possibility of two distinct cell populations in primary HCE cultures, one of which responds to E7 by failing to withdraw from the cell cycle, and a separate population which does not respond to E7 in this manner and therefore retains the ability to terminally differentiate. Similarly, extreme variations in differentiation capacity have been observed between HPV-immortalized foreskin epithelial cell lines in organotypic culture (41-43), and it is unclear whether this variation is due to selective loss of differentiation capacity with passage in culture or to intrinsic variations in the differentiation potential of the individual epithelial cells which originally founded the various cell lines. To address these issues, individual G418-resistant colonies containing either the LXSN16E6 or LXSN16E6E7 constructs were also analyzed in organotypic culture, and representative cultures are shown in Fig. 3. All clonal cultures contained cuboidal, mitotic cells in the cell layers normally occupied by immature quiescent squamous cells, but the apical portion of these cultures consisted of four to five fully differentiated and glycogenated cell layers, suggesting that the same cell population which continued to proliferate above the basal layer in oncogene-containing cultures also retained the ability to terminally differentiate.

The number of terminally differentiated cell layers in these clonal cultures was somewhat reduced relative to the pooled cultures, and the reason for this is unclear. However, previous studies have demonstrated that epithelial cell populations containing HPV oncogenes frequently undergo rapid secondary changes resulting in loss of differentiation potential with passage in culture (41, 43), and since the clonal cultures utilized in organotypic cultures have been amplified 8 to 14 population doublings more than the pooled cultures at the time of organotypic culture (see "Materials and Methods"), it is possible that these secondary changes are responsible for the reduction in the number of terminally differentiated cell layers detected in clonal cultures.

Morphological Consequences of HPV16 Oncogene Expression in Organotypic Cultures of HCE Cells. The terminal differentiation program in foreskin epidermis differs from that in ectocervix. After leaving the proliferative basal layer, epidermal cells pass through two morphologically distinct quiescent squamous layers, known as the spinous and granular layers, where cells first begin to express early and late markers of differentiation, respectively. At the boundary of the granular and cornified layers, these cells undergo the terminal differentiation step, or keratinization, characterized by the accumulation of covalently cross-linked keratin bundles and by the degradation and loss of nuclei and other organelles.

Organotypic experiments similar to the ones described above for HCE cells were also done with HCE cells, except that with foreskin cells, clonal analysis was limited to the LXSN16E6E7 construct. Uninfected parental cells and pooled G418-resistant, vector-containing cultures regenerated a normal epidermis in organotypic cultures (Fig. 4). Pooled G418-resistant cultures containing E7 and pooled and clonal cultures containing both E6 and E7 all retained the ability to keratinize or terminally differentiate to the same extent as normal controls, but the quiescent squamous cells of the spinous and granular layers of these cultures were replaced by cuboidal mi-
Mitotic cells (Fig. 4) in a manner similar to that seen in cervical cells (Fig. 3), demonstrating that in foreskin as well as ectocervix, E7 prevents suprabasal cell cycle withdrawal without affecting terminal differentiation.

**Immunohistochemical Staining for Differentiation Markers in Organotypic Cultures of HFE Cells.** To further define the extent of differentiation present in organotypic cultures of HFE cells, expression of keratin K1 and profilagrin was assayed by immunohistochemical staining of serial sections of the cultures shown in Fig. 4 (Fig. 5). K1 is an early marker of keratinizing differentiation whose expression is normally limited to cells of the spinous layer and above (47). Profilagrin expression appears later and is limited to the keratinohyalin granules of the granular layer (48). All pooled and clonal cultures containing HPV16 oncogenes expressed both of these differentiation markers to the same extent as the uninfected and vector-containing controls. In cultures containing HPV onco-
genes, however, mitotic figures were visible within the cell layers expressing K1 (Fig. 5), and with high magnification, it is clear that these mitotic cells are themselves coexpressing K1 at the periphery of the mitotic spindle (data not shown), suggesting that the same cells which are not quiescent in the suprabasal layers are capable of initiating the terminal differentiation program.

**HPV16 E7 Prevents Suprabasal Quiescence in Stratified Squamous Organotypic Cultures.** The extent of proliferation was also monitored in organotypic cultures. PCNA is a cofactor of DNA polymerase delta, and it is present to varying degrees throughout the cell cycle in proliferating cells. In quiescent cells, synthesis of PCNA is inhibited, but it can be rapidly induced when cells are stimulated to proliferate (49, 50). PCNA is also induced by several viral transforming proteins which bind pRB (51–53), and previously, we have demonstrated that suprabasal PCNA expression is a common feature of organ-

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**Fig. 4.** Hematoxylin and eosin-stained cross-sections of methacarn-fixed organotypic cultures of HFE cells. Upper left panel (parent) represents normal cells cultured organotypically at passage 2. All other panels represent cells derived from the same primary foreskin culture as the parent, but which have been infected with various retrovirus constructs, selected for G418 resistance, and cultured either as individual G418-resistant clones or pooled cultures prior to organotypic culture. Vector, HFE cells containing LXSN. E7 pooled, HFEs containing LXSN16E7. E6/7 pooled and E6/7 clone 1 and 2, pooled and clonal HFEs containing LXSN16E6E7. Arrows, mitotic figures.
Typically cultured, established HPV-immortalized HFE cell lines (44). Immunohistochemical staining for PCNA expression in organotypic cultures of HCE and HFE is shown in Fig. 6. PCNA staining in parental cells and vector-containing controls for both HCE and HFE was limited primarily to the nuclei of cells in the basal layer, whereas pooled and clonal cultures containing HPV oncogenes had PCNA-positive nuclei throughout the suprabasal layers as well.

Although the presence of mitotic figures and PCNA staining in the suprabasal layers of organotypic cultures containing HPV16 oncogenes suggested that these cells were actively cycling, it was also formally possible that these cells had arrested randomly within the cell cycle. To substantiate the presence of actively cycling suprabasal cells in these cultures, BrdUrd incorporation studies were also performed. In these experiments, culture medium was supplemented with 100 μg/ml of BrdUrd for 24 h prior to fixation of organotypic cultures, and after sectioning, newly synthesized DNA was visualized immunohistochemically with antibodies to BrdUrd (Fig. 7). Positive staining for BrdUrd was limited to nuclei in the basal layer in parental and vector-containing HFE controls, but in cultures containing LXSN16E7 or LXSN16E6E7, BrdUrd-positive nuclei were distributed throughout the suprabasal layers. These BrdUrd-positive suprabasal nuclei were less intensely stained than BrdUrd-positive basal cell nuclei, but in light of the fact that these cultures float basal cell side down on the surface of the culture medium, it is likely that this relatively pale staining in the suprabasal layers is due to poor penetration of BrdUrd into the interior of the epithelial sheet.

It is noteworthy that in HPV oncogene-containing cultures, BrdUrd incorporation was detected throughout the living layers and even within the lower regions of the terminally differentiating keratinized layer, both in nuclei undergoing degradation and in the residual nuclear material left at the end of this degradation process (Fig. 7). This result implies that these oncogene-containing cells remain capable of DNA synthesis until around the time of nuclear breakdown late in the terminal differentiation process. The precise point in terminal differentiation at which cells can no longer enter S phase is unclear from these studies, since the 24-h labeling interval used here exceeds the length of the average S phase. However, in normal control cultures, virtually no BrdUrd-positive cells have left the basal layer during this interval, and therefore, the differences in maturation of cells from the beginning to the end of the 24-h labeling period are likely to be reasonably small.

**Immunohistochemical Staining for p53 in Organotypic Cultures.** Although a large body of evidence indicates that WT p53 plays a negative role in the control of proliferation, endogenous p53 expression in cultured cells is associated with proliferating but not quiescent cells (28, 29, 33–36). To determine the distribution of p53 in organotypic cultures, serial sections were stained with the 1801 antibody to human p53 (Fig. 8). Positive staining with this antibody was limited primarily to the nuclei of basal cells in parental and vector-containing cultures, with a lesser number of positive nuclei in the immediately adjacent suprabasal layer. This parallels results in intact epidermis, where p53 expression has been localized to the proliferating basal layer (54). E7-containing cultures displayed strong nuclear p53 staining in both the basal and suprabasal layers. This pattern of staining

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*Fig. 5.* Differentiation markers expressed in organotypic cultures of HFE cells. Serial sections of the cultures shown in Fig. 1 were stained for keratin K1 (A) or for profilaggrin (B) using an avidin-biotin immunoperoxidase procedure. Panels are labeled as in Fig. 4.
closely parallels that seen for PCNA (Fig. 6) and BrdUrd (Fig. 7) in E7-containing organotypic cultures, and together these results suggest that stable endogenous p53 expression is associated with the proliferating compartment in both normal and E7-containing stratified squamous organotypic cultures.

In contrast to normal and E7-containing cultures, organotypic cultures containing both E6 and E7 were essentially negative for immunohistochemically detectable p53 in both the basal and suprabasal layers. In vitro studies have demonstrated that binding of p53 by E6 results in rapid degradation of the p53 protein by the ubiquitin pathway (19, 20), and therefore, the simplest and most likely explanation for the absence of detectable p53 in stratified organotypic cultures is E6-mediated degradation of p53 protein. We have not, however, formally ruled out other possibilities, including the possibility that E6 binding to p53 can mask the epitope recognized by the 1801 antibody, and therefore, at least some residual p53 may be present but undetectable in cultures containing HPV16 E6 and E7. In any case, it is striking that in a background of E7 expression, gross changes in the level of p53 protein as a consequence of E6 expression does not result in obvious alterations to either cellular proliferation or to the terminal differentiation of organotypic cultures.

**Discussion**

Organotypic culture provides a means to identify and study parameters of proliferation control which are fundamental to stratified epithelia, but which are either poorly defined or obscured in undifferentiated, homogeneously proliferating monolayer cell cultures. The results presented here demonstrate that expression of HPV16 E7 generates continued suprabasal proliferation in stratified squamous cultures, that this effect is specific and accompanied by other changes such as loss or reduction of terminal differentiation, and that this E7-regulated proliferation is independent of steady-state p53 levels, at least when these levels are altered by E6.

Two interrelated sets of observations support the notion that the interaction of E7 with pRB contributes to the failure of suprabasal cells to withdraw from the cell cycle. First, the hypophosphorylated form of pRB normally acts to regulate the G1-G0, transition in the cell cycle (23, 55, 56), at least in part by sequestering the E2F transcription factor and other cellular factors in inactive complexes (for a review, see Ref. 25). Binding of pRB by the adenovirus E1A protein releases transcriptionally active E2F (57), which then initiates a cascade of transcriptional events involved in cell cycle progression (58, 59). Although as yet there is no direct proof that HPV E7 can similarly displace pRB, the functional and structural similarities between viral pRB-binding proteins make this a likely possibility (60). Secondly, TGF-β, reversibly inhibits proliferation of cultured epidermal cells, and in intact epidermis, TGF-β expression is limited to suprabasal cells, suggesting that TGF-β may play a similar role in establishment of suprabasal quiescence in situ (for a review, see Ref. 38). TGF-β induces the dephosphorylation of pRB in mink lung cells (61), and it is likely that TGF-β has a similar activity in other epithelial cells, since TGF-β-induced quiescence of HFE monolayer cultures is blocked by any of several viral transforming proteins, including E7, which bind pRB (62). Taken together, these results suggest that TGF-β induces cell cycle withdrawal by inducing dephosphorylation of pRB, which then se-

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**Fig. 6.** Immunohistochemical staining for PCNA in organotypic cultures. A, serial sections of HCE cells from the cultures shown in Fig. 3. B, serial sections of HFE cells from the cultures shown in Fig. 4.
questers cellular factors required for cell cycle progression, and that viral transforming proteins which bind pRB prevent this sequestration. If this model is correct, and if TGF-β is the physiological signal involved in induction of suprabasal quiescence, then viral proteins which bind to and displace pRB from cellular complexes should specifically block cell cycle withdrawal in the suprabasal layers.

The results reported here confirm that expression of one pRB-binding protein (i.e., HPV16 E7) has precisely this effect in organotypic cultures.

The results described here challenge the widely held notion that withdrawal from the cell cycle is a prerequisite for expression of the terminal differentiation program in stratified squamous tissues. Demonstrations of suprabasal...
basal PCNA staining, BrdUrd incorporation (in HFE cells), and mitotic figures in cultures containing HPV oncogenes together constitute a strong argument for the continued cycling of suprabasal cells in these cultures. At the same time, terminal differentiation in these cultures is neither absent nor delayed, and there are several indications that proliferation and terminal differentiation are occurring simultaneously. For example, in oncogene-containing HFE cultures, nuclei in the later stages of differentiation incorporated BrdUrd with a frequency similar to that seen in lower cell layers (Fig. 7), and the differentiation marker K1 was expressed in mitotic cells (Fig. 5). Taken together, these observations argue that cells retain the ability to cycle well beyond initiation of the differentiation program, and it is possible, at least in the case of foreskin cells, that the ability to cycle may not be abrogated until nuclear breakdown and keratinization render the issue moot.

The distribution of p53 in organotypic cultures raises several interesting points. First, these studies reinforce the observation that p53 is expressed in proliferating cells. Previously, p53 expression has been associated with proliferating cells in cultures of lymphocytes and fibroblasts (28, 33–36), and recently p53 expression has been localized to the basal and immediate suprabasal layers of intact epidermis (54). Furthermore, an increase in p53 expression in the suprabasal epithelial layers similar to that seen in E7-containing organotypic cultures has been demonstrated in the benign hyperproliferative skin disease, psoriasis (63).

In spite of the fact that p53 is normally found in proliferating cells, E7-mediated suprabasal proliferation is unaffected when p53 levels are drastically reduced as a consequence of E6 expression (Fig. 8). This result contrasts with a variety of transduction experiments in which expression of a transduced exogenous WT p53 gene frequently, but not always (64, 65), reduced the proliferation and/or tumorigenicity of cultured cells (30–32, 66). For example, Mercer et al. (67) have introduced an exogenous inducible WT p53 gene into malignant glioblastoma cells and demonstrated that WT p53 expression results in both growth arrest and disappearance of PCNA in the transduced cell population. One explanation for this discrepancy might be that E7 specifically inactivates p53 function; however, this seems unlikely, since transduced WT p53 can block the transformation of BRK cells mediated by cooperating E7 and ras genes (68). Since both ras and E7 are required for transformation of BRK cells, this result does not distinguish whether WT p53 is overriding the effects of E7, or of ras3, or of some interaction between these two oncogenes. However, some immortalized cell lines, such as NIH 3T3, can be transformed by E7 alone, and in this case, cotransduced WT p53 has no effect on transformation (68). This latter case is similar to the situation in organotypic cultures in the sense that it involves increased proliferative capacity mediated by E7 alone, which is apparently independent of p53.

Immortalization studies have shown that both E6 and E7 are required, and that together they are sufficient, to immortalize HFE cells (5, 6, 10). Immortalized HFE cell lines occasionally emerge after an extended crisis from cultures containing E7 alone (6, 7, 42), but the frequency with which they emerge is consistent with the notion that a secondary genetic change is required for immortalization of E7-containing HFE cells. It is striking, therefore, that the feature which distinguishes these two cell populations in presenescent organotypic cultures is not the presence of abnormal suprabasal proliferation, but the amount of detectable p53 in the abnormally cycling cells. The point in the cell cycle at which p53 exerts its regulatory effects is unclear, and it is possible that p53 functions at more than one point in the cell cycle (for a review, see Ref. 11). Most observations, however, are consistent with the idea that p53 exerts at least some of its regulatory effects early in the cell cycle sometime prior to S phase, and it is tempting to speculate that cells which do not enter or pass through Go, such as E7-containing cells, proliferate independently of endogenous p53 levels because they never pass through the point at which p53-mediated regulation occurs. This may be true for some regulatory effects of p53 and may possibly be the underlying reason that proliferation in E7-containing cells is independent of p53 levels. However, our data are also consistent with the possibility that, in some situations, the decision to proliferate or not is not directly governed by p53; in other words, p53 may not act as a simple inducer or suppressor of proliferation, but instead may play a more subtle role in the modulation of proliferation. Indeed, the differences in p53-linked immortalization potential between E7 and E6/E7-containing cultures imply that although suprabasal proliferation is unaffected by fluctuations in p53 levels in these cells, the cells remain sensitive to the negative regulatory effects of p53 in terms of long term proliferation.

Materials and Methods
Monolayer Culture of Human Foreskin and Cervical Epithelial Cells and Fibroblasts. Primary and secondary HFE and dermal fibroblast cultures were established in monolayer culture from fresh foreskins as previously described (41). HCEs and stromal fibroblasts were prepared from surgical specimens by identical procedures. Epithelial monolayer cultures were maintained in keratinocyte-SFM (GIBCO) and split at a ratio of 1:3 at confluence. Stromal fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 10 μg/ml of hydrocortisone (Sigma). All cultures were frozen at passage 2 in their respective growth media supplemented with 20% FBS and 10% dimethyl sulfoxide.

Radioimmunoprecipitations. Subconfluent cells were incubated at 37°C in 5% CO2, for 30 min in methionine-free growth medium and then supplemented with 100 μCi/ml of 35S-methionine for 1.5 h. Cells were lysed in radioimmunoprecipitation assay buffer, and immunoprecipitations were carried out as described by Harlow and Lane (69). Rabbit polyclonal antibodies to HPV16 E6 and E7 bacterial fusion proteins were generated in our laboratory (70). Resultant immunoprecipitates were electrophoresed in 17% acrylamide gels. Gels were fixed in glacial acetic acid, saturated with 2,5-diphenyloxazole, dried under vacuum, and exposed to X-ray film.

Infection of Epithelial Cells with Recombinant Retroviruses. Aliquots of virus stocks were added directly to the culture fluid of subconfluent monolayers in the pres-

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* R. A. Blanton and M. D. Coltrera, unpublished results.
ence of 25 μg/ml Polybrene and incubated at 37°C and 5% CO₂ overnight. Cells were trypsinized, resuspended, and replated at 90% of their original density to obtain pooled populations of G418-resistant cells, and at 10% of their original density to select for clonal G418-resistant populations. Cultures were maintained in the presence of 50 μg/ml G418 (Calbiochem) for 5 days, during which time, G418-sensitive cells died and detached from the dishes. After initial selection, pooled cultures were split 1:3, allowed to approach confluence, and frozen for later use. Individual clones were trypsinized, plated into 24-well dishes (Falcon), and further amplified into 100-mm dishes before freezing. All cultures were maintained under continuous selection for G418 resistance until used in organotypic culture. Based on the numbers of G418-resistant colonies obtained in these infections, initial multiplicity of infection were approximately 0.1 to 0.025, and individual pooled cultures are derived from 4–7 × 10⁴ colonies.

Organotypic Cultures. Organotypic cultures were generated using procedures modified from those described previously by us and others (39–41). Confluent fibroblast cultures derived from foreskin dermis or cervical stroma were trypsinized and resuspended in FBS to a final concentration of 1 to 2 × 10⁶ cells/ml. Seven parts collagen type I (Collaborative Research), 1 part 10X E medium (consisting of 3 parts Dulbecco’s modified Eagle’s medium to 1 part Ham’s F-12 medium), 1 part reconstitution buffer (consisting of 2.2% NaCO₃, 0.05% NaOH, and 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 1 part fibroblasts in FBS were mixed on ice, pipetted into culture dishes, and allowed to gel. Epithelial cells were trypsinized, resuspended in Dulbecco’s modified Eagle’s medium supplemented as described by Kopan et al (40), and overlaid onto the collagen gel. Cervical cultures were further supplemented with 10 ng/ml of β-estradiol during organotypic culture. Composite gels were cultured submerged for 4 days and then floated on 40-mesh stainless steel screens (Alaska Copper and Brass, Seattle, WA) in 60-mm organ culture dishes (Falcon, Lincoln Park, NJ) and cultured for 8 days at the air-liquid interface prior to fixing in methacarn (30% chloroform-60% methanol-10% acetic acid) and embedding in paraaffin.

Immunohistochemistry. Five-μm sections of organotypic cultures were deparaffinized and rehydrated. Serial sections were incubated with monoclonal antibodies 34bb4 for the keratin K1 (71), AKH1 for filaggrin (48), 19A2 for PCNA (72), 1801 for p53 (73), or BU-1 for BrdUrd (74). Localization of the antibodies was via avidin-biotin (ABC) immunoperoxidase for K1 and filaggrin, or via the streptavidin biotin immunoperoxidase technique in the case of PCNA and p53.

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