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Epithelial Cells Immortalized by Human Papillomaviruses Have Premalignant Characteristics in Organotypic Culture

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Three HPV-16– and four HPV-18–immortalized human foreskin keratinocyte cell lines were analyzed on organotypic epidermal raft cultures at various passage levels. This culture system allowed normal cultured keratinocytes to stratify and differentiate in a manner similar to normal epidermis. All seven HPV-immortalized cell lines displayed epidermal morphologies on organotypic cultures, which were clearly abnormal and resembled premalignant lesions* in vivo. *Features of premalignant lesions that were shared by all of the HPV-immortalized cell lines included disorganized tissue architecture, mitotic cells present throughout the living layers of the epidermal sheet, abnormal mitoses, enlarged nuclei, and variable cell size and shape. Most HPV-immortalized cell lines were stable in terms of epidermal morphology with long-term passage in culture. Two of the HPV-18–immortalized cell lines, however, lost all morphologically apparent terminal squamous differentiation potential after long-term passage in monolayer culture. These results strongly support the idea that immortalization of squamous epithelial cells in culture by HPV-transforming genes generates a morphologically premalignant cell. (Am J Pathol 1991, 138:673–685)

Human papillomaviruses (HPV) are associated with a variety of benign, premalignant, and malignant lesions of the cutaneous and mucosal epithelia.[1,2] More than 60 HPV types have been identified to date,[3] and those HPVs that have been well characterized display type specificities for both the anatomic site and the histopathology of the associated lesions.[4] The evidence that HPV has a role in the development of human genital carcinoma is strong. In carcinoma of the uterine cervix, more than 90% of the tumors contain integrated HPV sequences, with HPV-16 and HPV-18 the most frequently detected types.[1,3] Transcriptionally active sequences from the contiguous E6 and E7 viral open reading frames (ORF) are specifically retained by tumors[5–11] and E7 protein is expressed abundantly,[9,12] while the other viral genes often are deleted, interrupted, or inactivated. These observations in tumors and tumor-derived cells correlate well with *in vitro* studies that implicate the E6/E7 region of oncogenic HPV types 16 and 18 in the processes of cellular transformation and immortalization. The E7 ORF alone or in concert with the E6 ORF of HPV-16 and 18 appear to code for transforming functions when introduced into a variety of cells.[13–18]

In vitro transformation studies showed that HPV-16 and 18 sequences can immortalize normal human keratinocytes from both neonatal foreskin[19–24] and adult cervix.[25–27] The immortalizing function for foreskin keratinocytes has been localized to the E6/E7 region,[21,26,28,29] and E7 expression can be demonstrated in all immortalized keratinocyte lines that have been analyzed.[24,30,31] Although HPV-16 and 18 can immortalize human genital epithelial cells, the resultant cell lines usually are not tumorigenic and retain many of the properties associated with normal cultured keratinocytes.[19,22,24,27] Introduction of activated *ras* oncogene into HPV-immortalized keratinocyte lines can render them tumorigenic in nude mice.[32] These results correlate well with the natural history of HPV-associated carcinoma. The high incidence of HPV infections without apparent malignant disease[33,34] and the long latency period between HPV infection and the appearance of malignant lesions[35] imply that although HPV infection may play a significant role in oncogenesis, other diverse factors are required for full malignancy. These considerations have led to the idea that HPV-immortalized keratinocytes might represent an *in vitro* counterpart to premalignant cells that arise *in vivo* as a result of stable

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expression of the HPV E6/E7 region. Support for this notion has come from the direct isolation from a premalignant cervical lesion of a nontumorigenic, immortalized keratinocyte cell line that contains HPV-16 sequences.[36] Keratinocyte lines immortalized *in vitro* therefore appear to be both a convenient and a *bona fide* model for analysis of the role of HPV in multistep carcinogenesis.

Mechanisms underlying immortalization *in vitro* and neoplastic progression *in vivo* are poorly understood. At the molecular level, both the E6 and E7 proteins have characteristics of DNA-binding proteins[37] and have been shown to bind cellular tumor suppressor proteins.[38–40] At the cellular and tissue levels, however, specific alterations resulting from the expression of HPV-transforming genes have not been determined.

In normal stratified squamous epithelia such as foreskin and cervix, cells leaving the basal layer cease to divide and undergo an ordered pattern of stratification and differentiation. Intraepithelial neoplastic progression in these tissues involves alterations to these processes and is diagnosed at early stages as dysplastic changes in the lower layers of the epithelium. As these lesions progress, dysplastic changes involve sequentially higher layers of the tissue until ultimately the entire thickness of the epithelium appears dysplastic. These high-grade dysplasias or carcinomas *in situ* are considered to be premalignant lesions. Dysplastic cells are characterized by disruption of tissue organization, variable size and shape of individual cells, enlarged and irregularly shaped nuclei, the ability to undergo mitosis in the suprabasal layers of the tissue, and the presence of abnormal mitoses.

Human papillomavirus-immortalized keratinocyte cell lines should share many characteristics of dysplastic cells if they, in fact, represent a tissue culture counterpart to naturally occurring premalignant cells. Most characteristics of dysplastic cells, however, require the context of epithelial tissue architecture to be identified as such. Recently a variety of organotypic culture systems have been developed for epithelial cells[41,42] that involve culturing epidermal cells at the air–liquid interface on a dermal equivalent support. Normal keratinocytes derived from stratified squamous tissues stratify and fully differentiate in a manner similar to the normal epithelial tissues,[43] while a variety of squamous carcinoma cells and established squamous carcinoma cell lines exhibit dysplastic morphologies on epidermal rafts similar to those seen *in situ*.[44–46]

We used an epidermal raft culture system to assess the alterations in epidermal morphology of a variety of human neonatal foreskin epidermal cell lines that previously were immortalized with HPV-16 or HPV-18 in our laboratory. We find that all of these HPV-immortalized cell lines generate epidermal sheets in organotypic culture that closely resemble premalignant squamous lesions.

Materials and Methods

Culture of Normal Foreskin Keratinocytes and Foreskin Dermal Fibroblasts

Primary and secondary foreskin keratinocyte cultures were established and maintained as previously described[47] in keratinocyte growth medium (Clonetics, San Diego, CA). Primary foreskin dermal fibroblasts were prepared from neonatal foreskins by incubation at 4°C overnight in 25 mg/ml dispase (Boehringer-Mannheim, Indianapolis, IN) prepared in phosphate-buffered saline (PBS). The epidermis was removed with forceps and the dermis was washed in PBS, minced, and incubated at 37°C with gentle agitation in 10 ml of a 25 mg/ml solution of collagenase (Worthington, Freehold, NJ). Cells were pelleted at 1000*g* for 5 minutes, washed twice with PBS, and cultured in Dulbecco's modified Eagle's essential medium (DMEM), plus 10% fetal calf serum (FCS). Cells were fed routinely every 4 days and split at a ratio of 1:3.

Immortalized Foreskin Keratinocyte Lines

All HPV-16– and HPV-18–immortalized cell lines were generated after introduction of HPV-16 or HPV-18 sequences by the Ca_2PO_4 technique. Characterization of the FEA, FEH, FEPEIL8, FEPEIL9, and FEPEIL13 cell lines was published previously.[20,21,48] The 185 and 1811 cell lines were isolated after introduction of HPV-18 sequences into the same population of keratinocytes used to generate the FEPEIL8 and FEPEIL9 cell lines.[31] Cell lines were maintained in keratinocyte growth medium and split at a split ratio of 1:3 every 3 to 7 days.

Epidermal Raft Cultures

Raft cultures were generated using procedures slightly modified from those described previously.[41,42] Confluent dermal fibroblast cultures were trypsinized, counted, washed in PBS, and resuspended in cold FCS to a final concentration of 1 to 2 × 10^6 cells per milliliter. Seven parts bovine dermal collagen (Cellmatrix A, Seikugaku Inc., St. Petersburg, FL), two parts 5 × DMEM, one part reconstitution buffer (consisting of 2.2% $NaHCO_3$, 0.05N NaOH, and 200 mmol/l (millimolar) HEPES, and one part fibroblasts in FCS were mixed on ice in the order given according to instructions from the manufacturer. Two milliliter of the collagen mixture were pipetted into 35-mm dishes and allowed to gel at 37°C in a humid 5% CO_2 atmosphere, then the cultures were overlayed with 1 ml DMEM containing 10% FCS and 0.4 μg/ml hydrocortisone (DMEM + HC). The liquid medium was removed 12 to 24 hours later and 5 × 10^5 cells of various keratinocyte populations were seeded on top of the gels in 1 ml

DMEM + HC. The cultures were submerged for 5 or 6 days and fed every other day with DMEM + HC supplemented as described by Kopan et al.[42] Composite collagen gels were removed from their culture dishes, washed gently in PBS, and floated onto grids cut from 40 mesh stainless steel screens (Alaska Brass and Copper, Seattle, WA). Grids were placed onto the center well of a 60-mm organ culture dish (Falcon, Lincoln Park, NJ) that contained supplemented DMEM + HC. Raised organotypic cultures were fed daily. The composite gels were fixed in 10% neutral buffered formaldehyde, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E), 10 to 12 days after raising cultures to the air–liquid interface.

Results

Normal Foreskin Keratinocytes Cultured on Epidermal Rafts

Figure 1 shows a representative photomicrograph of a cross-section through an epidermal raft culture generated with normal foreskin epithelial cells. Cells were established and maintained in monolayer culture before culture on epidermal rafts. Normal keratinocytes stratified, granulated, and cornified on epidermal raft cultures in a manner similar to normal epidermis at all passage levels before senescence, and well-defined basal (B), spinous (S), granular (G), and cornified (C) layers were seen readily.

HPV-16–Immortalized Cell Lines Cultured on Epidermal Rafts

The FEPEIL8, FEPEIL9, and FEPEIL13 cell lines were isolated after transfection of tumor-derived HPV-16 sequences into normal foreskin keratinocytes.[21] The sequences used to generate these three cell lines contained HPV-16 sequences starting within the L1 ORF and continued through the noncoding region (NCR), the E6 and E7 ORFs, and terminated within the E1 ORF. The only intact ORFs present in these cells were the E6 and E7 ORFs. Expression of the E6 and E7 gene products has been demonstrated previously in these cell lines.[21] Epidermal raft cultures generated with these cell lines are shown in Figures 2, 3, and 4. Each cell line was analyzed at intermediate passage levels and at the earliest and latest passage levels available to identify changes that occur with passage in culture. At all passage levels analyzed, these cell lines retained the ability to keratinize in a manner similar to normal cultured foreskin keratinocytes. However the epidermal sheets formed with these cell lines differed from normal cultured keratinocytes in several ways. First, although the superficial

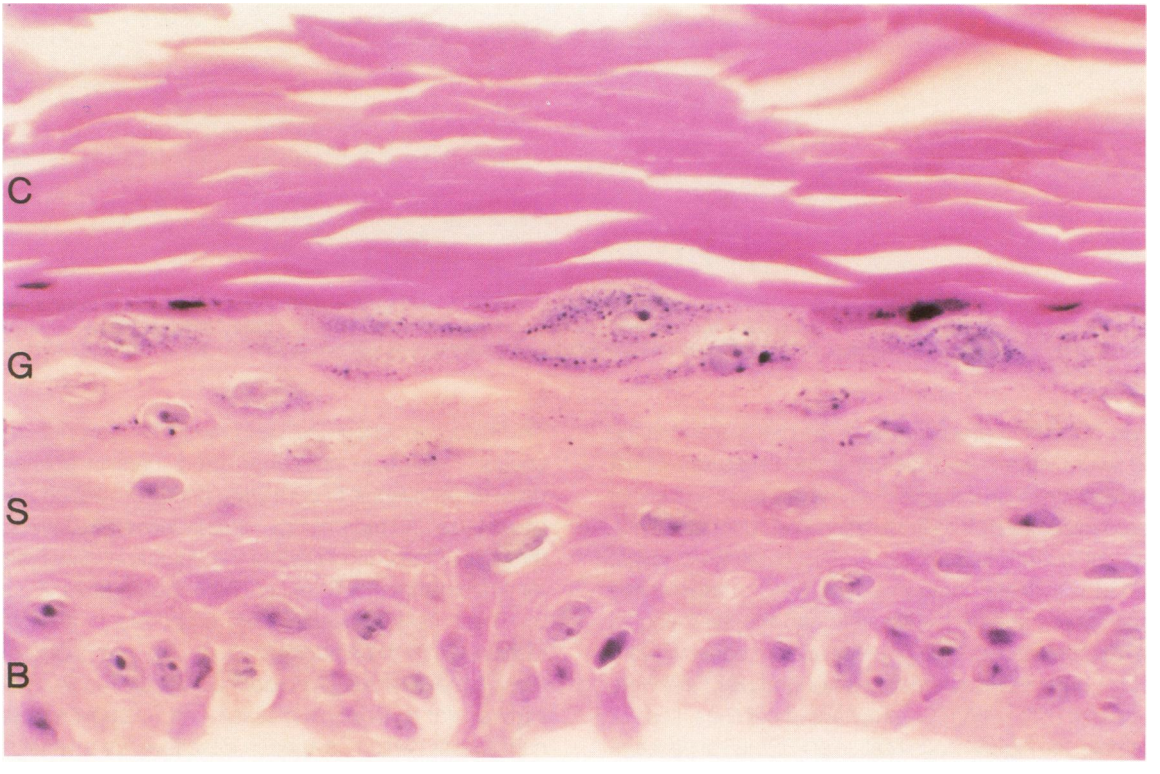

Figure 1. *Cross-section of formalin-fixed, paraffin-embedded organotypic cultures of normal foreskin keratinocytes stained with H&E staining. B, basal layer; S, spinous layer; G, granular layer; C, cornified layer (original magnification, 250×).*

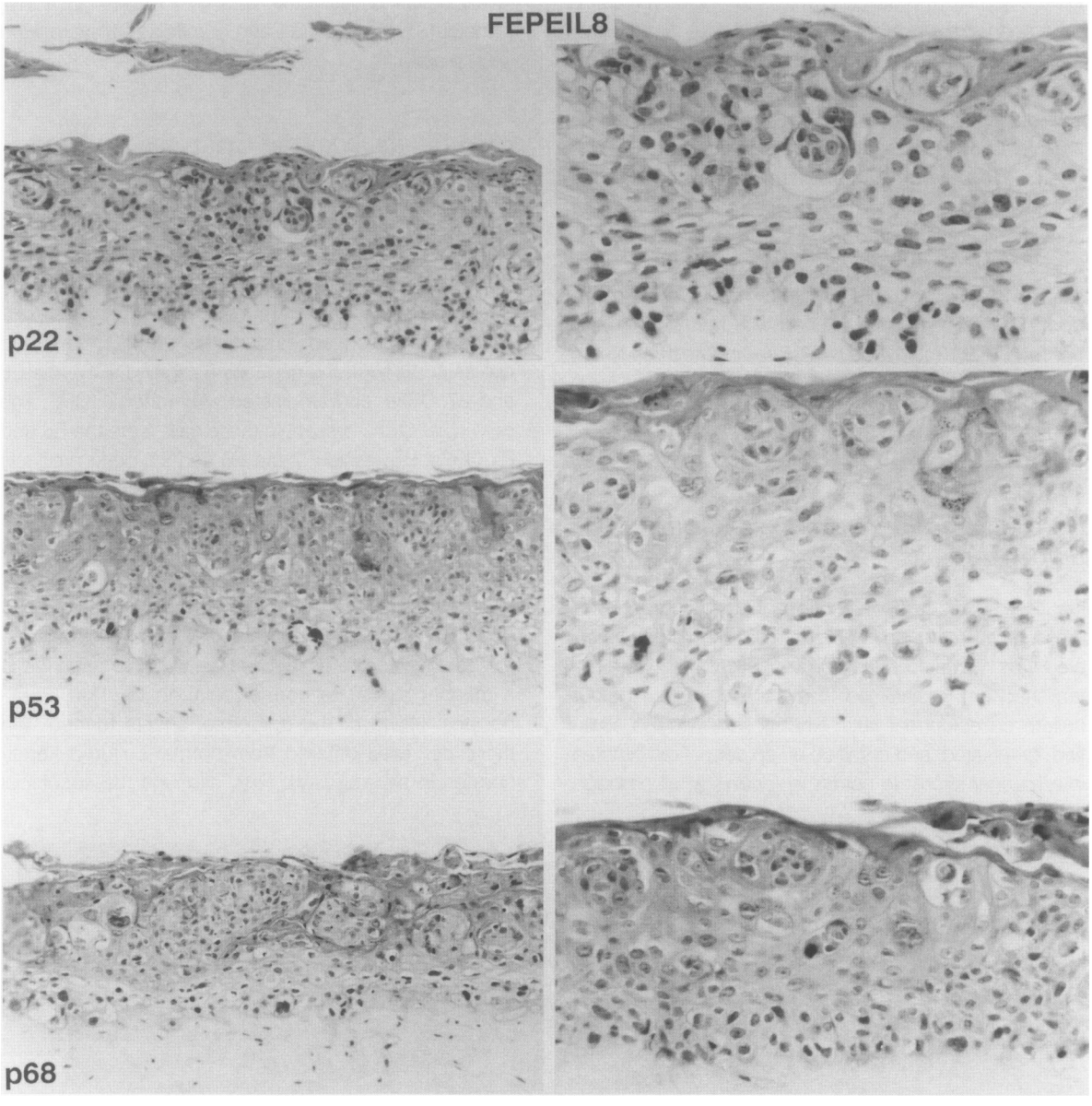

Figure 2. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the FEPEIL8 cell line at various passage levels stained with H&E. FEPEIL8 cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 22; middle row, passage 53; bottom row, passage 68 (original magnifications, 50× for the left column; 150× for the right column).*

layers of the normal epidermis, ie, the granular and cornified layers, appeared to be generated on epidermal raft cultures with these cell lines, these layers were somewhat disorganized relative to those seen in epidermal raft cultures of normal keratinocytes. The keratinized layers formed with these three cell lines were predominately parakeratotic and frequently protruded into the interior of the epidermal sheet. The granular layers frequently were absent but small scattered areas of granular cells were seen in all of the HPV-16–immortalized cell lines (see, for example, the higher magnification of FEPEIL8 at passage 53; Figure 2). Second dysplastic cells were present and extended throughout the living layers of the epidermal sheets. Relative to normal cultured foreskin epithelial cells, these dysplastic cells were poorly organized, variable in size and morphology, and underwent cell division throughout the living layers of the epidermal sheet (see, for example, the FEPEIL9 cell line at passage 74; Figure 3). These cells frequently were organized in a nested fashion within the epidermal sheet. The morphology of these three cell lines on epidermal raft cultures was strikingly similar to Bowen's disease and related premalignant lesions, which have been described for a variety of keratinized squamous epithelia.[49,50]

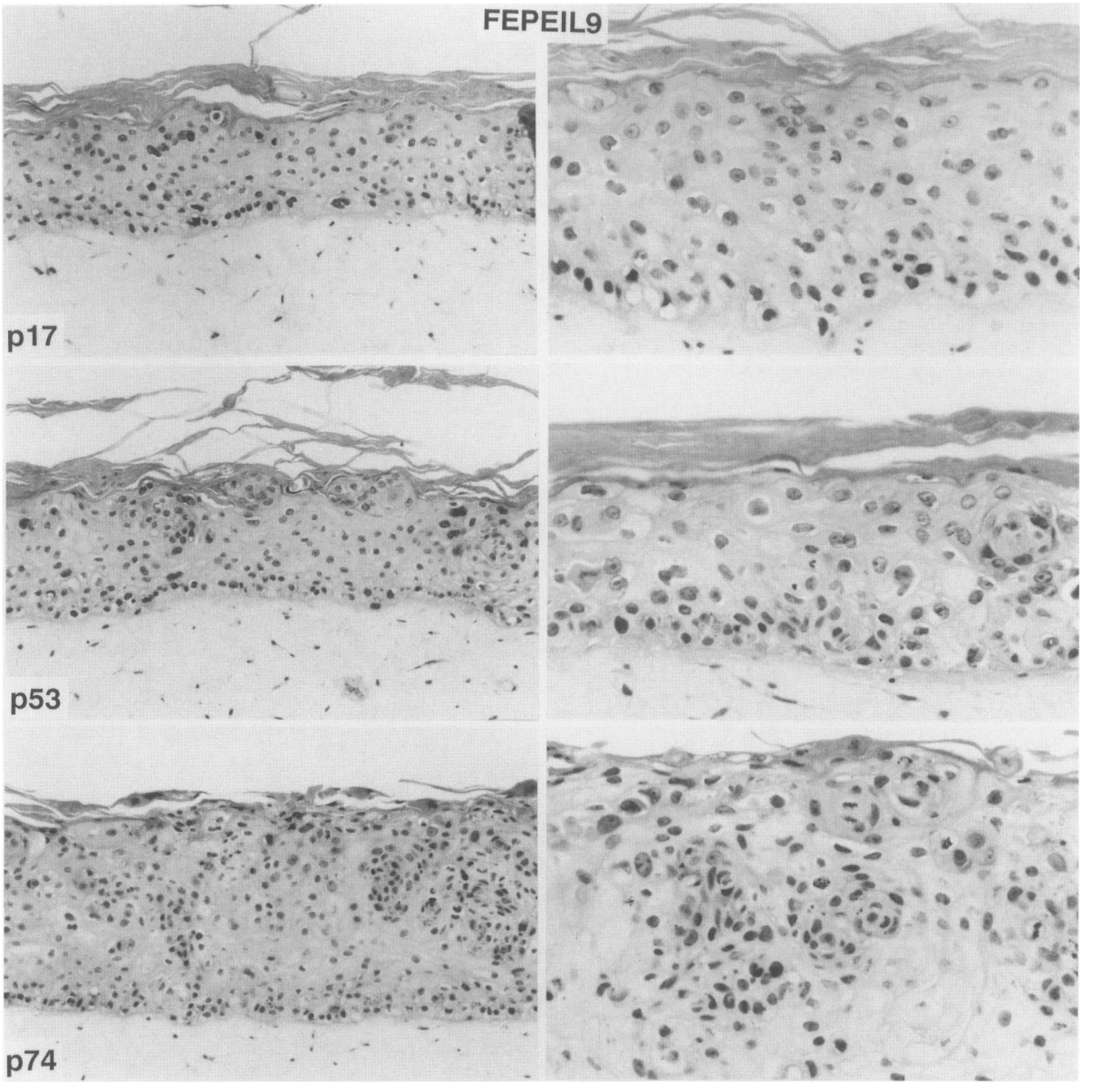

Figure 3. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the FEPEIL9 cell line at various passage levels stained with H&E. FEPEIL9 cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 17; middle row, passage 53; bottom row, passage 74 (original magnifications, 50× for the left column; 150× for the right column).*

HPV-18–Immortalized Cell Lines Cultured on Epidermal Rafts

Four HPV-18–immortalized cell lines were analyzed for epidermal morphology on raft cultures. The FEA, 185, and 1811 lines were generated after transfection of normal foreskin keratinocyte cultures[20,31] with a plasmid containing the entire HPV-18 genome interrupted within the E1 ORF by vector sequences. Therefore only the intact E6 and E7 ORFs were contiguous with the viral NCR, as was the case for the HPV-16 sequences used to generate the HPV-16–immortalized lines discussed above. The FEH cell line was generated after introduction of the complete HPV-18 genome, which had been excised from a vector plasmid and recircularized by ligation.[20]

Results with the 185, 1811, FEH, and FEA cell lines are shown in Figures 5 to 8. The 185 cell line (Figure 5) shared many properties with the HPV-16–immortalized cell lines discussed in the previous section. The 185 cell line stably retained the ability to form a superficial keratinized layer, although this keratinized layer was thin relative to that seen with the HPV-16–immortalized cell lines.

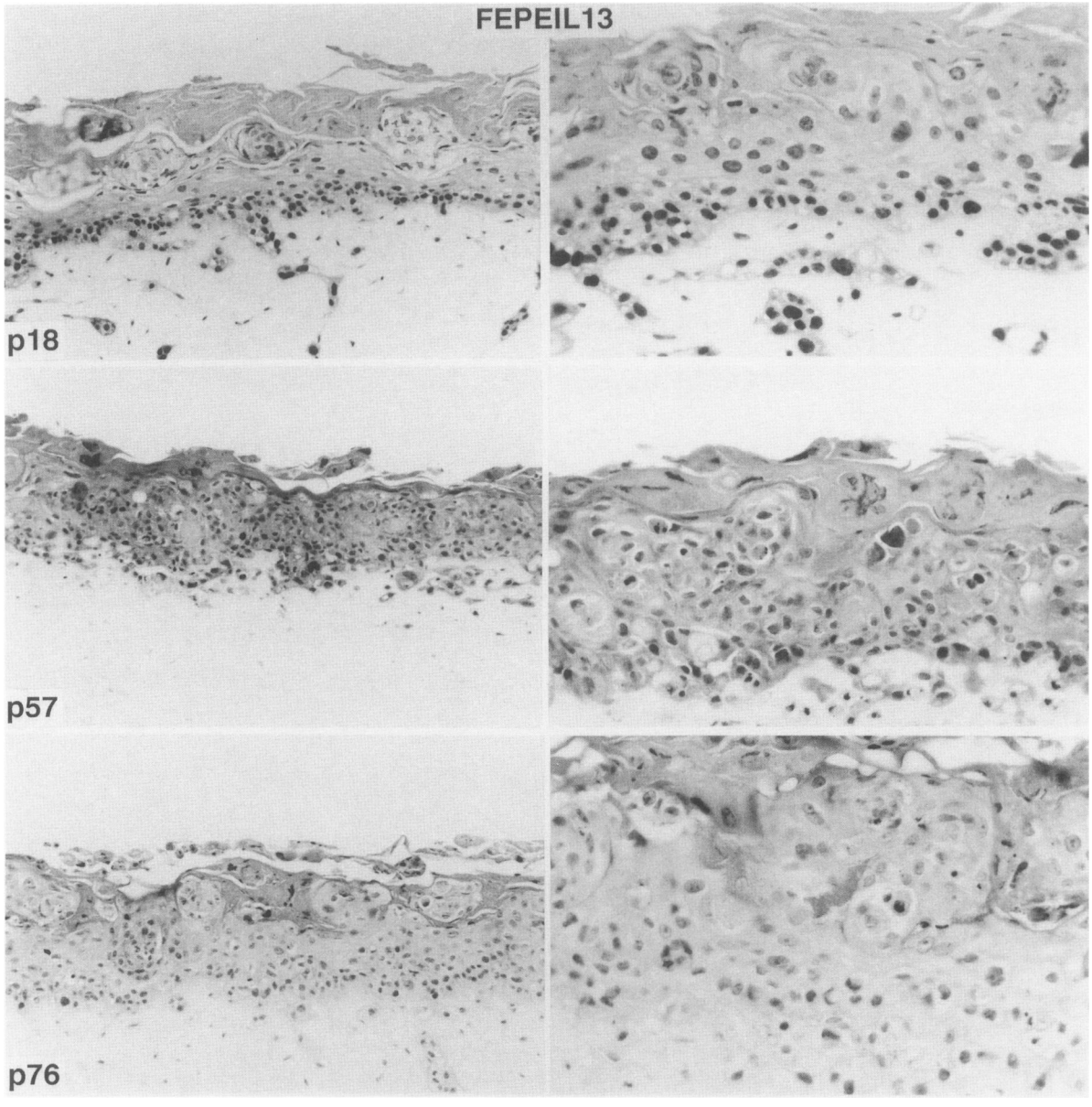

Figure 4. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the FEPEIL13 cell line at various passage levels stained with H&E. FEPEIL13 cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 18; middle row, passage 57; bottom row, passage 76 (original magnifications, 50× for the left column; 150× for the right column).*

The 185 cell line also displayed a loss of normal internal organization and the presence of dysplastic cells throughout the living layers of the epithelial sheet. The nested organization of cells internally within the epidermal sheet was particularly striking. The 1811 cell line (Figure 6) at low passage levels was similar to the HPV-immortalized cell lines discussed previously in that it was keratinized and dysplastic. The 1811 cell line differed from the majority of HPV-immortalized cell lines in that it underwent changes with passage in culture that resulted in a total loss of terminal squamous differentiation. At passage 37 the cells retained some squamous character, but keratinization was severely reduced. At passage 80 no keratinization was present, the cells had lost all morphologically apparent squamous character, and they appeared as uniformly small cells with high nuclear to cytoplasmic ratios (Figure 6). Concurrent with the loss of terminal squamous differentiation, the 1811 cell line became tumorigenic in nude mice (Hurlin et al, manuscript submitted for publication).

The other two HPV-18–immortalized cell lines, the FEH and FEA cell lines, differed from all the other HPV-immortalized cell lines in their patterns of keratinization (Figures 7 and 8). At early passage levels, these cell lines

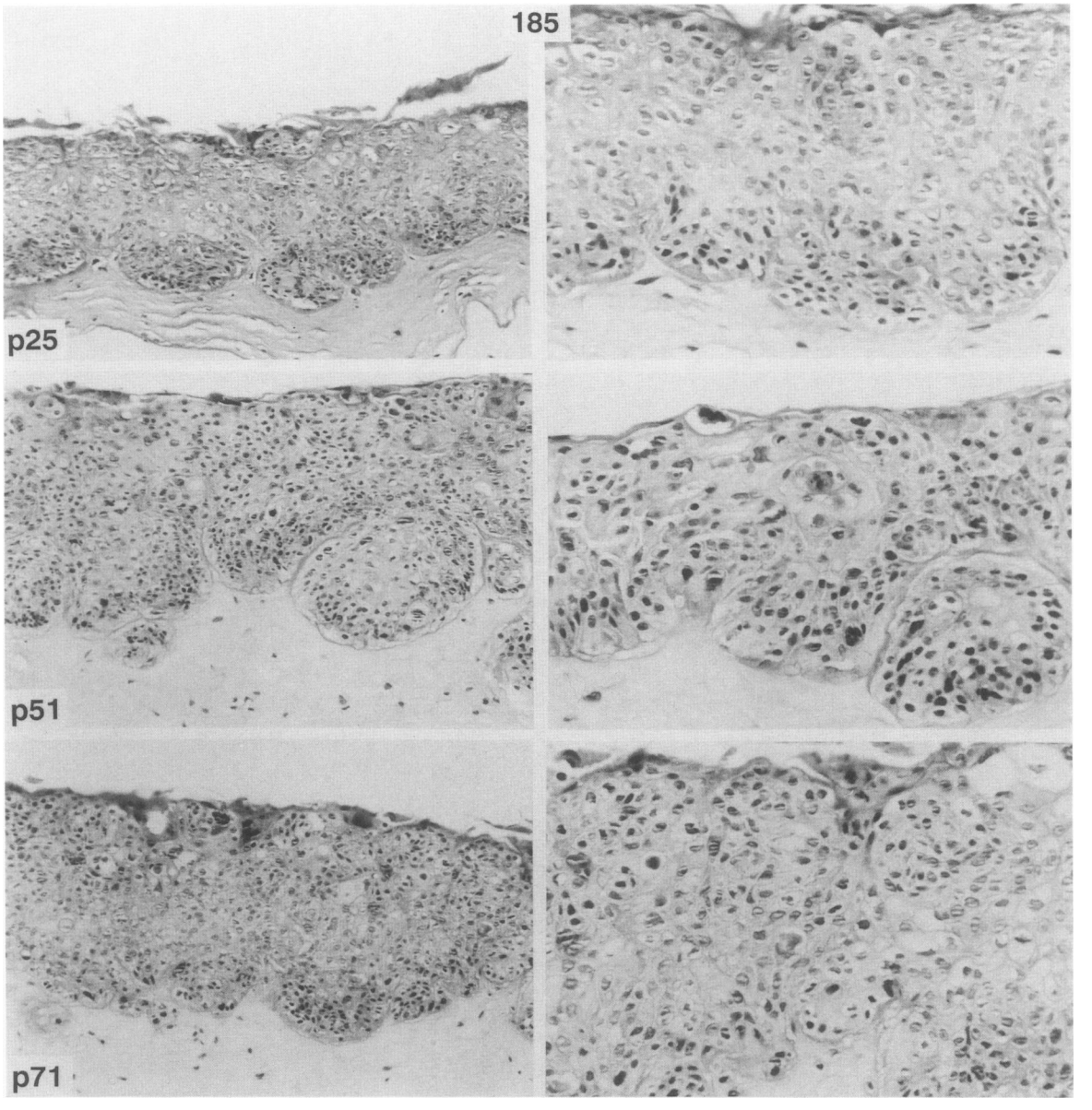

Figure 5. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the 185 cell line at various passage levels, stained with H&E. One hundred eighty-five cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 25; middle row, passage 51; bottom row, passage 71 (original magnifications, 50× for the left column; 150× for the right column).*

displayed extensive areas of internal keratinization or dyskeratosis similar to that seen in well-differentiated and moderately differentiated squamous cell carcinomas originating from a variety of squamous epithelia.[50] The internal keratinization ranged in appearance from small, poorly defined, acellular eosinophilic areas to somewhat better-defined small horn pearls. Surface keratinization in these two cell lines usually was minimal and frequently disrupted by areas of nonkeratinized, nucleated cells present on the surface of the epidermal sheets. The FEH cell line remained stable in terms of its highly disorganized squamous morphology with passage in culture. The FEA cell line, however, lost both the ability to keratinize and all internal squamous morphology at late passage in culture. In contrast to the 1811 cell line, which also lost the capacity for terminal squamous differentiation with passage (Figure 6), the FEA cell line remained nontumorigenic in nude mice.

Discussion

Seven different HPV-immortalized human neonatal foreskin keratinocyte cell lines were analyzed for growth and differentiation potential on epidermal raft cultures at a variety of passage levels. The three HPV-16–immortalized

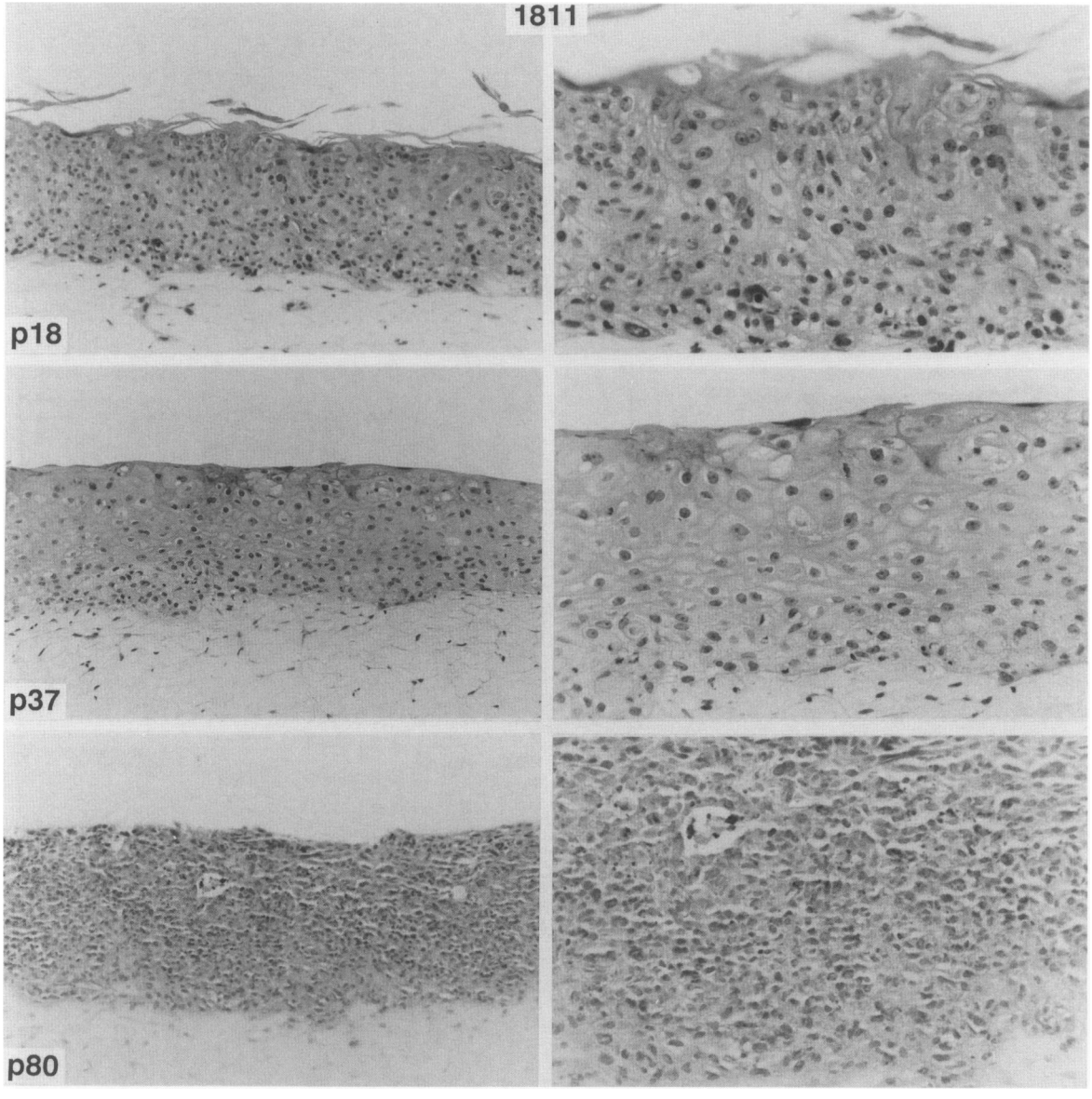

Figure 6. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the 1811 cell line at various passage levels, stained with H&E. Eighteen hundred eleven cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 18; middle row, passage 37; bottom row, passage 80 (original magnifications, left column, 50×; right column, 150×).*

cell lines analyzed, FEPEIL8, FEPEIL9, and FEPEIL13, retained the ability to keratinize on epidermal rafts. However the epidermal sheets were disorganized and poorly differentiated relative to similar cultures of normal keratinocytes. The appearance of these cell lines on epidermal rafts is strikingly similar to histologic sections of Bowenoid types of intraepithelial neoplasia.[50] In this regard, it is noteworthy that among squamous cell carcinomas derived from keratinizing vulvar epithelium, those with a Bowenoid morphology have a much greater association with HPV than other morphologic types of vulvar squamous cell carcinoma (CP Crum, oral personal communication, June 1990).

The four HPV-18–immortalized cell lines analyzed, although morphologically similar to squamous cell carcinomas grown on epidermal raft cultures, presented a more complex picture than that seen for the three HPV-16–immortalized cell lines. First the morphology of the HPV-18 cell lines on epidermal raft cultures was more variable than that seen for the three HPV-16–immortalized cell lines. Two of the HPV-18–immortalized cell lines (185 and 1811) retained the ability to form a superficial keratinized layer, at least at relatively early passage levels, and in this respect were morphologically similar to the three HPV-16–immortalized cell lines. The other two HPV-18–immortalized cell lines, FEA

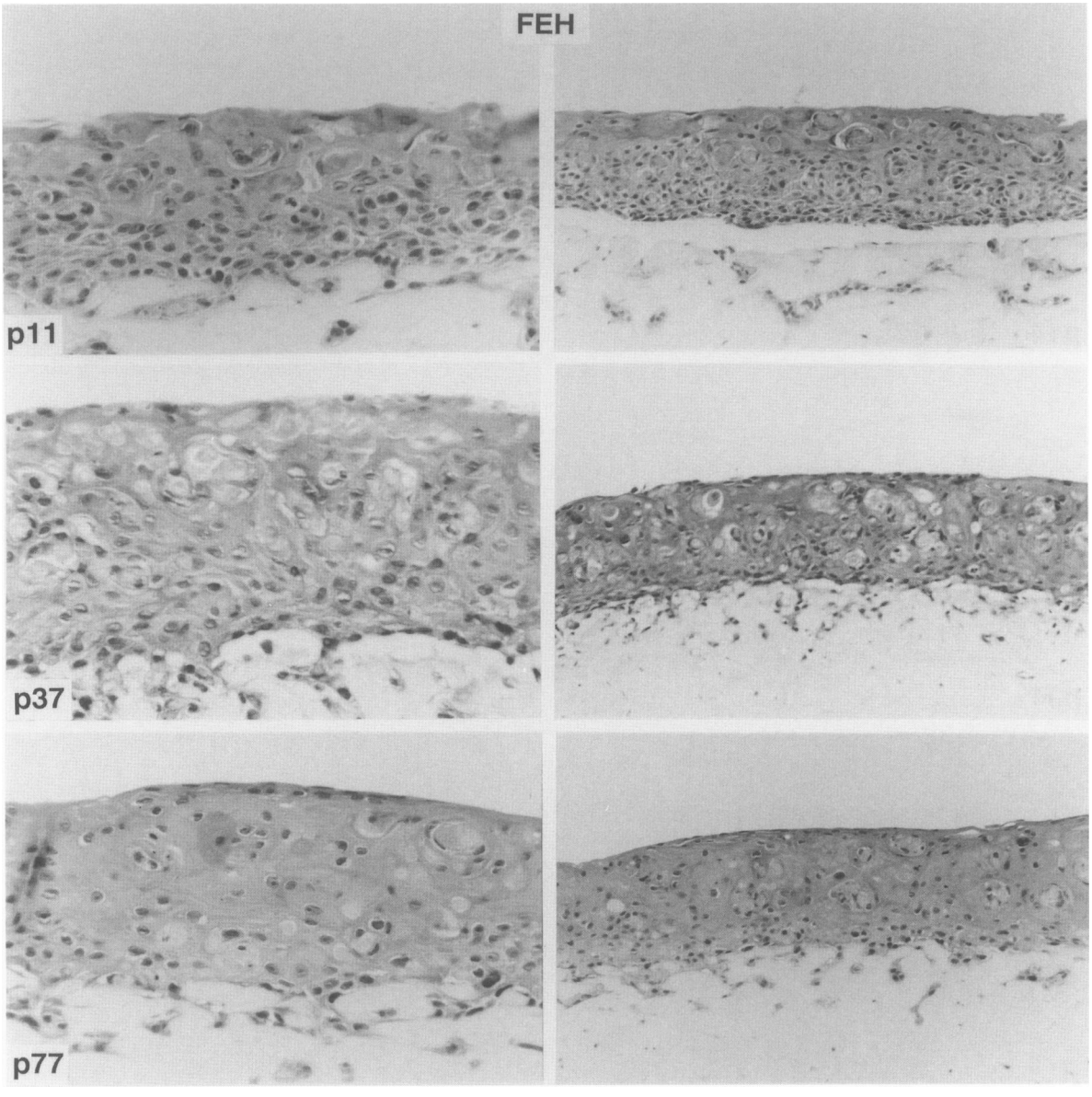

Figure 7. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the FEH cell line at various passage levels, stained with H&E. FEH cells cultured on organotypic cultures after previous passage in monolayer culture at the following passage levels are shown: top row, passage 11; middle row, passage 37; bottom row, passage 77 (original magnifications; left column, 150×; right column, 50×)*

and FEH, however, had patterns of keratinization that were more highly disorganized and that occurred internally within the epidermal sheet. This type of dyskeratosis has been described for moderately to well-differentiated squamous cell carcinomas, as well as for organotypic cultures of cell lines derived from such carcinomas.[45,50] Why these two HPV-18–immortalized cell lines should have a morphology on epidermal rafts that is somewhat different from that seen with all other HPV-immortalized lines analyzed is unclear. It is interesting that the two internally keratinizing cell lines were generated from one lot of normal secondary foreskin keratinocytes while the other HPV-immortalized cell lines were generated from a second lot of keratinocytes derived from a different donor. It is unlikely that differences in plasmid sequences used to generate the various cell lines could account for differences in morphology on epidermal rafts because the same construct was used to generate the internally keratinizing FEA cell line and the superficially keratinizing 185 and 1811 cell lines.

A second complexity noted with the HPV-18–immortalized cell lines concerns changes in epidermal morphology seen on rafts that occur after long-term passage in monolayer culture. Two of the HPV-18–immortalized cell lines (1811 and FEA) underwent changes during long-term subculture that resulted in loss

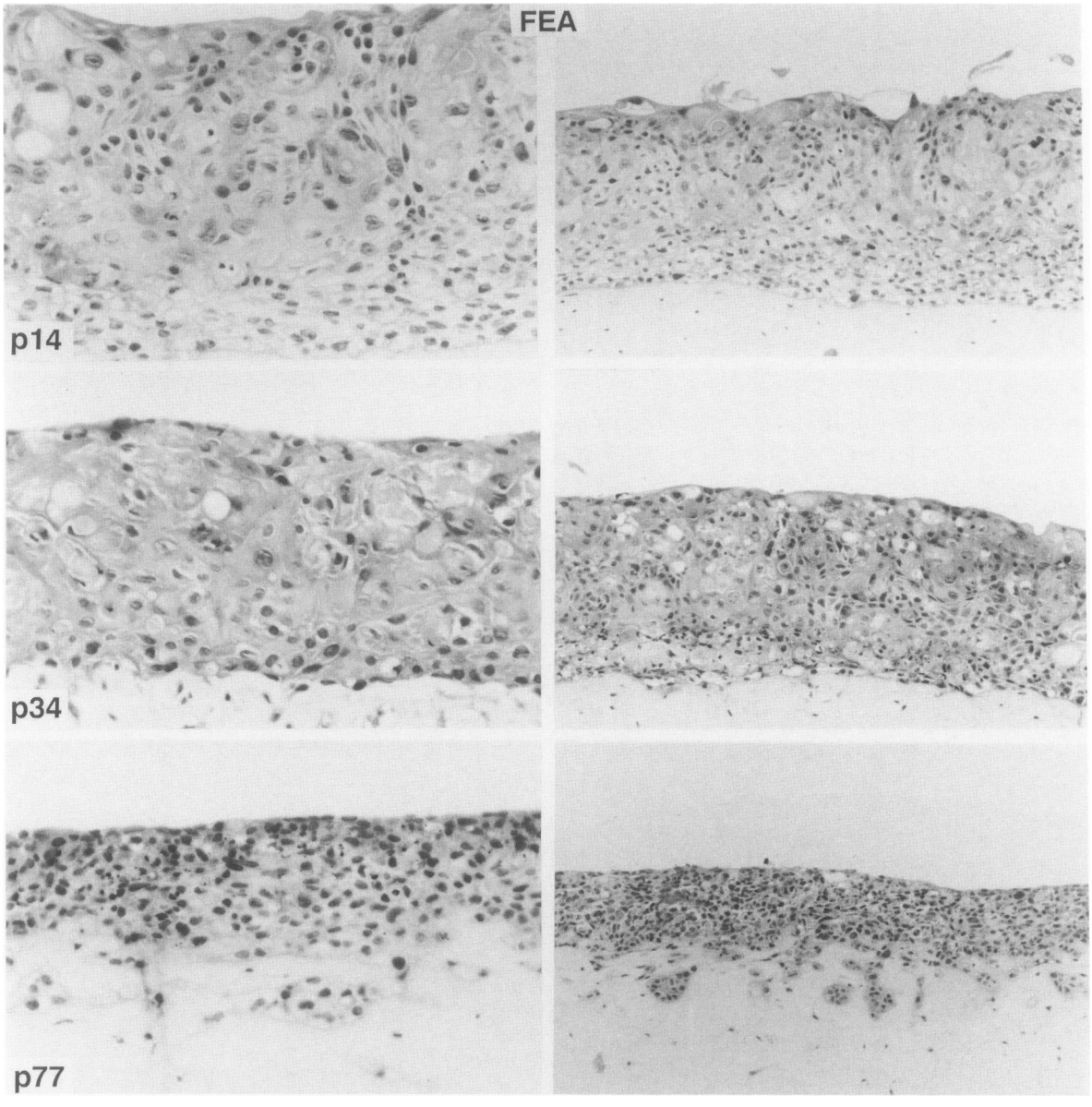

Figure 8. *Cross-sections of formalin-fixed, paraffin-embedded organotypic cultures of the FEA cell line at various passage levels, stained with H&E. FEA cells cultured on organotypic cultures after previous passage in monolayer culture to the following passage levels are shown: top row, passage 14; middle row, passage 34; bottom row, passage 77 (original magnifications; left column, 150×; right column, 50×).*

of terminal keratinization and of all squamous maturation in the living layers of the epidermal sheet. The significance of the loss of squamous differentiation potential is unclear and controversial. Our results suggest that loss of terminal squamous differentiation does not occur as a requisite consequence of immortalization with HPV because most HPV-immortalized cell lines retain the ability to keratinize and are morphologically stable in culture. The loss of terminal squamous differentiation potential seen in a few HPV-immortalized cell lines is not limited to HPV-18–immortalized cell lines because a fourth HPV-16–immortalized cell line in our laboratory recently underwent similar changes with passage in culture (RA Blanton, unpublished data). Finally loss of terminal squamous differentiation does not correlate with tumorigenicity in nude mice because only one of the three differentiation-defective cell lines, the 1811 cell line, makes tumors in nude mice (Hurlin, et al, manuscript submitted for publication). Recently we determined that all cell lines that lose the ability to keratinize also simultaneously cease to express one or more major squamous keratin proteins (Blanton et al., manuscript in preparation).

Our results concerning loss of terminal differentiation differ from those published by others,[51,52] who have

demonstrated that both normal foreskin keratinocytes and an established squamous carcinoma cell line rapidly lose all differentiated characteristics on epidermal rafts after introduction of HPV-16 or HPV-18 sequences. There are a variety of explanations for this discrepancy, but a strong possibility concerns the use of serum in the routine maintenance of HPV-immortalized lines. Several reports suggest that although HPV-16–immortalized keratinocyte cell lines initially retain the ability to terminally differentiate,[22,26,27] subpopulations of differentiation-resistant, nontumorigenic cells can be selected for after induction of differentiation with serum.[24,26–28] Maintenance of HPV-immortalized lines in serum-containing medium therefore represents a continuous selection process for this subpopulation. Generation of differentiation-resistant cells within a population of immortalized keratinocytes does not appear to be a universal property of all immortal keratinocyte lines because several non–HPV-containing squamous cell carcinoma lines[45,46,53] as well as a spontaneously immortalized foreskin keratinocyte line[54] have been shown to maintain stable differentiation functions after long-term culture in serum. The generation of serum-resistant populations therefore may be a consequence of specific interactions between HPV and the cell. Further study will be required to determine what role differentiation-resistant subpopulations of cells may play in the morphogenesis of neoplastic epidermis or in malignant progression. In any case, the results reported here with the FEPEIL8, FEPEIL9, FEPEIL13, and 185 cell lines demonstrate that HPV-16– and HPV-18–immortalized lines can, when routinely maintained under serum-free conditions, stably retain terminal squamous differentiation properties with passage in culture, and that immortalization with HPV *per se* does not obviate the rapid loss of all differentiation functions.

All of the HPV-16– and HPV-18–immortalized cell lines were analyzed on epidermal rafts at the earliest and latest passage levels available. An inherent and important limitation to these studies involves the fact that primary keratinocytes die in culture at lower passage levels than those at which we have analyzed the HPV-immortalized cell lines. Consequentially even the earliest morphologic changes reported here could be due to secondary selection of cells in culture with altered growth or differentiation properties. Experiments are in progress to directly assess the morphologic alterations that occur in organotypic cultures immediately after expression of E6 and E7.

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