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Two Distinct Human Uterine Cervical Epithelial Cell Lines Established after Transfection with Human Papillomavirus 16 DNA

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We have established two distinct human cervical cell lines, NCC16 and NCE16, after transfecting human papillomavirus type 16 (HPV16) DNA into normal human ecto-cervical and endo-cervical epithelial cells, respectively. Both lines expressed HPV16 E6 and E7 as detected by reverse transcriptase-polymerase chain reaction and northern blot hybridization. These cells have been passaged for over 100 population doublings and express strong telomerase activity. Neither cell line was tumorigenic in athymic *nu/nu* mice. However, both NCC16 and NCE16 developed abnormally stratified architectures following implantation with a silicon membrane sheet in the back of athymic nude mice. The former cells were pathohistologically similar to carcinoma, while the latter produced Alcian-blue positive cells, suggesting the occurrence of metaplastic changes. These distinct cell lines offer a useful model system for the study of cervical carcinogenesis and of its regulatory mechanism after HPV infection in different regions of the uterine cervix.

Key words: HPV16 — Cervical epithelial cells — Immortalization — Telomerase

Evidence accumulated to date indicates that human papillomavirus type 16 (HPV16) plays an etiologic role in the development of cancers in the uterine cervix.[1,2] A close relationship between HPV infection and tumor development is implied by the persistence of certain HPV subgenome types, most notably type 16, which is recognized as a high risk factor for carcinoma. Since some of these HPVs show transforming activity in appropriately cultured cells and cell lines, including human epithelial cells,[3–7] the presence of transforming genes seems probable. Both E6 and E7 genes have been proven to collaborate in the tumorigenic transformation of rodent cells and to immortalize human epithelial cells.[8–11] Although uterine cervical epithelial cells are presumed to be the natural host cells for HPV infection, the mechanism of their involvement in the development of cervical carcinoma is largely unknown. Characterization of several HPV-immortalized cell lines indicates that their transformed phenotypes appear to deviate minimally from those of untransformed human epithelial cells.[12–16] However, further comparative studies of the effects of HPVs are necessary for the various presumptive natural host cells.

In this study, we conducted an experiment to elucidate further HPV-related carcinogenesis in the uterine cervix which results in the formation of a unique epithelial architecture in three histologically distinct tissue regions: squamous epithelium in the region of the ecto-cervix, simple columnar epithelium at the endo-cervix and a junction region in between, termed the transformation zone. It is generally acknowledged that uterine cervical squamous cell carcinomas arise in the transformation zone, which is believed to be a transit area from the margin of the columnar simple epithelium to the squamous epithelial region. In the endo-cervical epithelial region, reserve cells exist beneath the columnar epithelial cell layer. In contrast, keratinized epithelium differentiates from basal and supra-basal cells in the squamous epithelial region. Reserve cells that lie beneath the secretive columnar epithelium differentiate into squamous metaplastic cells in the transformation zone. We separately dissected endo-cervical and ecto-cervical epithelial tissue regions to isolate simple columnar and squamous basal cells in order to investigate HPV16-related carcinogenesis of the uterine cervix *in vitro*. We transfected HPV16 DNA into cultured epithelial cells prepared from two histologically distinct epithelial regions of normal uterine cervix and established cell lines which showed different phenotypes. These cell lines should provide a useful model for the study of HPV16-related carcinogenesis at both the molecular and cellular level.

MATERIALS AND METHODS

Preparation of primary cells Human uterine cervical epithelial cells were isolated from hysterectomy specimens which had been histologically diagnosed as normal with no evidence of infection. Squamous cell and columnar cell regions obviously distant from the squamocolumnar junction were isolated from the cervical specimens. Stromal tissues underneath the epithelium were removed while viewing under a phase contrast microscope. Epithelial tissues were minced into small pieces (approximately

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5–8 mm^3 in size) and washed several times in phosphate-buffered saline (PBS) containing antibiotics (Gentamicin). The minced tissue segments were placed in collagen IV-coated dishes with small aliquots of media (0.05 ml/cm^2): DMEM/MCDB153-medium for squamous epithelium and MCDB-medium for simple columnar epithelium. The DMEM/MCDB153-medium was a mixture of DMEM and MCDB153 (1/4 v/v) supplemented with a hormone mixture of hydrocortisone (0.2 μM), epidermal growth factor (EGF) (10 ng/ml), ethanolamine (5 μM), *O*-phosphoethanolamine (5 μM), transferrin (10 μg/ml), insulin (5 μg/ml) and 0.5% dialyzed bovine serum. The MCDB153-medium consisted of MCDB153-based medium containing the same supplements as above. Explant cultures were maintained for the first week in fresh media renewed every other day (1/2 the initial volume) and then for a further one or two weeks in fresh MCDB153-based medium renewed at similar intervals.

Cells and cell culture Outgrowing epithelial cells were dissociated by treatment with 0.25% trypsin (GIBCO-BRL, Inc., Gaithersburg, MD)-0.01% EDTA for 10 min, and single cell suspensions collected by filtration through an 85-mesh filter. The cells were processed to secondary cultures and used for the transformation experiments or stored in liquid nitrogen in MCDB153-based medium containing 10% dialyzed serum and 10% dimethyl sulfoxide until use.

Transfection of HPV16 DNA Endo-cervical epithelial cells (NCC) and ecto-cervical keratinocytes (NCE) were transfected with either recombinant full-length HPV16 DNA (pHPV16d) harboring the neomycin resistance gene (*neo* gene)[3,4] or full-length HPV16 DNA alone without the *neo* gene. The DNA was ethanol-precipitated and redissolved in transfection buffer (TB) containing 0.3 *M* glucose in 0.1×HEPES buffered saline (HBS) (1 m*M* KCl, 28 m*M* NaCl, 5 m*M* HEPES, pH 7.1). The cells were harvested by the use of trypsin-EDTA and pelleted in medium that contained 10% fetal bovine serum (FBS). The cells were washed twice with Ca^{2+} and Mg^{2+}-depleted PBS and once with TB. Then 1×10^6 NCC were resuspended in TB containing 15 μg of DNA and electroporated using a Baekon 2000 series-1 (Advanced-Gene-Transfer-System, Saratoga, CA) as described previously.[15,16]

RNA analysis Total cellular RNA was extracted with 4 *M* guanidine isothiocyanate as described previously.[4] After ethanol precipitation, RNA was redissolved in distilled water at a concentration of 1 mg/ml. Poly(A)-terminated mRNA was obtained by the standard method with oligo (dT)-cellulose column chromatography. The mRNA was electrophoresed and size-fractionated on 1.0% agarose gel containing formaldehyde, then alkaline-blotted on Hybond N+ nylon membrane (Amersham Internatl. plc, Buckinghamshire, U.K.) using 0.4 *M* sodium hydroxide. The RNA was hybridized with ^{32}P-labeled full-length HPV16 probe under stringent conditions.[4]

DNA analysis Total cellular DNA was extracted by means of the proteinase K-sodium dodecyl sulfate method as described previously.[3] The DNA was dissolved in TE-buffer and digested by restriction endonuclease. DNA fragments were electrophoresed on 0.8% agarose gel, blotted on Nylon membrane (Amersham), and hybridized with ^{32}P-labeled full-length HPV16 under stringent conditions.

Polymerase chain reaction (PCR) To detect HPV16 genome DNA in the transformants, 1 μg of extracted cellular DNA was subjected to polymerase chain reaction. The primer set was as follows; HPV16E6-5′: C-CGGATCCGAGAACTGCAATGTTTCAGGACCC, HPV16E6-3′: CCGGATCCATCGATGATTACAGTC-GG. Amplified DNA was electrophoresed on 0.8% agarose gel and stained with ethidium bromide.

Reverse transcriptase-linked PCR (RT-PCR) To detect the splicing site in NCC16 mRNA, we used reverse-transcriptase to synthesize the cDNA. This cDNA was amplified by polymerase chain reaction. The target of the PCR primer was the E6 gene. The amplified fragments were electrophoresed on 0.8% agarose gel and subjected to Southern hybridization using HPV16-E6 DNA fragment (475 bp).

Telomerase assay Telomerase activity was assayed using a highly sensitive detection method as described previously.[17,18]

Implantation of the cells in athymic nude mice Cells were trypsinized and seeded on silicone sheets (Dow Corning, Midland, MI). After 8 to 12 h, the silicone sheets with the cells were implanted into the back of 4- to 8-week-old female athymic mice (*nu/nu*), using a technique described previously.[19] The implanted cells were isolated after 8 and 14 days, fixed with 5% paraformaldehyde solution, and embedded in paraffin. The paraffin blocks were sectioned, then stained with hematoxylin and eosin.

RESULTS

Cellular characterization of squamous and simple epithelial cells *in vitro* Optimal conditions for the outgrowth of epithelial cells varied depending upon the media used for the explant cultures for ecto-cervical and endo-cervical tissue segments (see "Materials and Methods"). Outgrowth of epithelial cells from ecto-cervical tissues required relatively high concentrations of Ca^{2+} (0.5–1.0 m*M*), especially for the first 3 days (Fig. 1A; compare a and b). After the outgrowth and stable expansion of the epithelial sheet, usually requiring one week, the medium was replaced with MCDB153-based medium containing

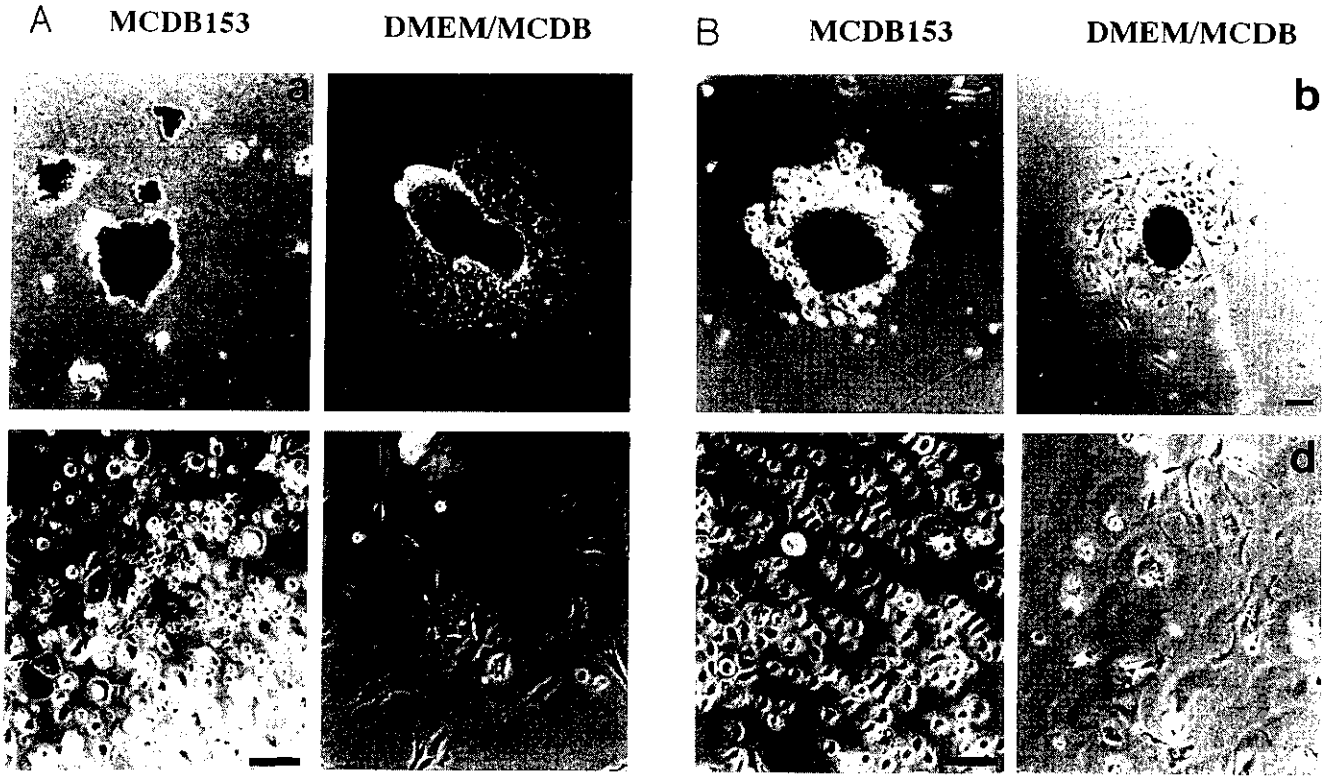

Fig. 1. Cultivation of two different epithelial cells from uterine cervix. A, Ecto-cervical keratinocytes in two different culture media. a: Explant cultures in MCDB153-based medium. b: Explant cultures with epithelial outgrowth in DMEM/MCDB153-based medium. c: Selective cultivation of ecto-cervical keratinocytes with MCDB153-based medium in secondary cultures prepared from outgrowing epithelial cells shown in panel b. d: DMEM/MCDB153-based medium was inefficient for the selective cultivation of ecto-cervical keratinocytes in secondary cultures prepared from cells shown in panel b. Note contaminating fibroblastic cells. B, Endo-cervical simple epithelial cells in media described above. a: Exoplant cultures in MCDB153-based medium and b: in DMEM/MCDB153-based medium. c: Selective cultivation of simple epithelial cells with MCDB153-based medium in secondary cultures prepared from outgrowing epithelial cells shown in panel a. d: These simple epithelial cells did not grow in DMEM/MCDB153-based medium. Bars indicate 50 μm.

low Ca^{2+} to maintain the actively growing keratinocyte population (Fig. 1A; compare c and d). The switch to media containing low Ca^{2+} (0.03–0.1 m*M*) minimizes the loss of actively growing cells blocking the stimulation of differentiation of keratinocytes. In contrast, MCDB153-based medium containing a low Ca^{2+} concentration optimized both outgrowth from the explant (Fig. 1B; compare a and b) and proliferation of epithelial cells in secondary cultures (Fig. 1B; compare c and d). These culture conditions allow for selective cultivation of actively proliferating epithelial basal cells and minimize the accumulation of terminally differentiated cells, in accordance with previously published results.[20, 21] After 2 to 4 weeks, approximately 5×10^6 cells were recovered from the explant culture of a single specimen.

To characterize further normal human NCE and NCC, we first examined the effects of EGF and hydrocortisone on cell growth. The result showed that the growth factor requirements of NCC differed from those of NCE. NCE strictly required EGF, but not hydrocortisone. Hydrocortisone had no effect on the cell growth of NCE (Fig. 2). NCC also showed EGF-dependent growth, but hydrocortisone was required for optimal growth (Fig. 2). Hydrocortisone alone had some positive effect on growth of NCC. Sex hormones such as estradiol and progesterone had virtually no effect on the growth of either cell type (data not shown).

No morphologic differences were evident between NCC and NCE even by Papanicolaou staining. Both lines showed epithelial gland-like cell morphologies in MCDB153 with 0.5% FBS (Fig. 3A, a and b). However, some NCC had vacuolated cytoplasms with cilia which stained Alcian blue-positive (Fig. 3A, a and c), indicating the production of mucin (Fig. 3A, c, arrow heads).

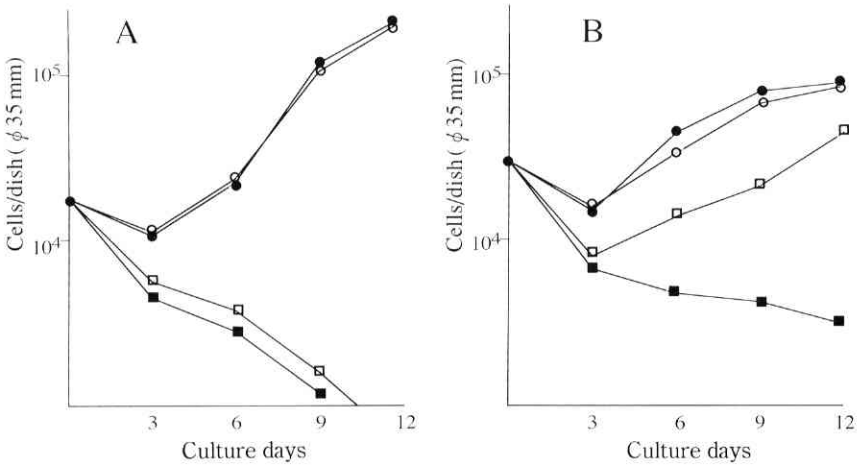

Fig. 2. Growth factor-requirement of squamous and simple epithelial cells. Cells prepared from outgrowing explant cultures were seeded in triplicate in MCDM153-based media w/o EGF and hydrocortisone. Viable cells were counted with a hematocytometer in the presence of 0.05% trypan-blue. Panel A: NCE, panel B: NCC. ● EGF (+) HC (+), ○ EGF (+) HC (−), □ EGF (−) HC (+), ■ EGF (−) HC (−).

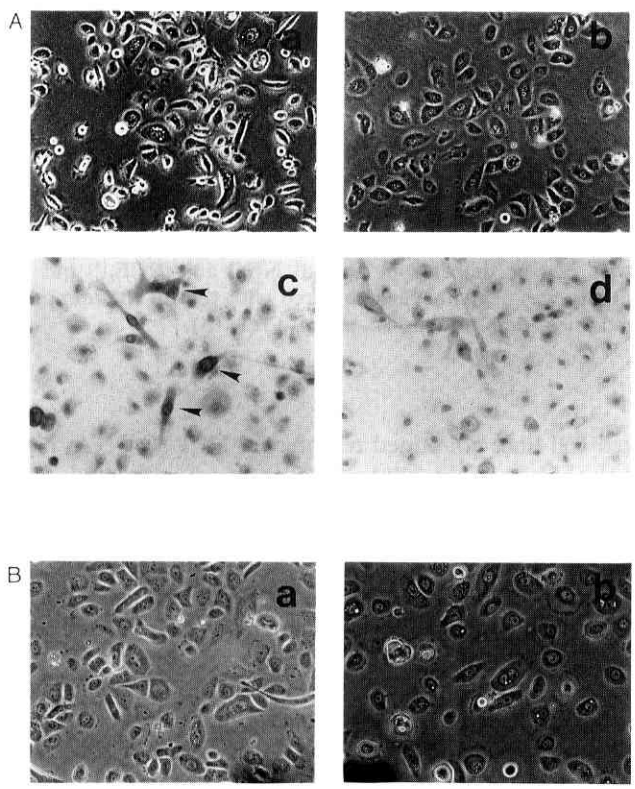

Fig. 3. A, Morphological characteristics of squamous and columnar epithelial cells of the uterine cervix. Morphologies of endo-cervical simple epithelial cells (a) and ecto-cervical keratinocytes (b) in MCDB153-based medium (secondary culture) under a phase-contrast microscope. The bar indicates 50 μm. Alcian-blue staining of mucin produced by cultured endo-cervical simple epithelial cells (indicated by arrowheads in panel c). Ecto-cervical keratinocytes were Alcian-blue negative (d). Cells were fixed in 100% methanol. B, Two cell lines, NCC16 (endo-cervical) and NCE16 (ecto-cervical) established after transfecting full-length HPV16 DNA.

Although some NCE also contained vacuolate cytoplasm, all were Alcian blue-negative (Fig. 3A, b and d). Thus, the isolated cells phenotypically reflect their tissue origins.

Establishment of immortalized cell lines Using these distinctive cell types, 5×10^5 cells were transfected with 15 μg of HPV16 DNA by electroporation as described in "Materials and Methods." The electroporated cells were seeded in duplicate at $1.0\times10^4/cm^2$ in 5 cm dishes. One day after plating, the cells were incubated for 3 days with the antibiotic G418 at a concentration of 100 μg/ml or selected for long-term survival (i.e., subjected to more than 100 population doublings). Both G418-treated and untreated plates were maintained in fresh medium changed twice a week. Three weeks after electroporation, several microcolonies appeared in the G418-treated dishes. These colonies were maintained for another 2 weeks. One of the well-developed macrocolonies (2 cm in size), consisting of homogeneously small cells, appeared to grow stably from the transfected NCC. The macrocolony was passaged by trypsinization. This line derived from NCC was propagated as a stable cell line (named NCC16) and has been maintained for over 200 population doublings to date. Unexpectedly, no stable transformant was isolated from the series of the transfected NCE which were selected by G418. Mock-transfected NCC and NCE which recieved pdMMTneo without HPV16 genomes all died off and no macrocolonies developed after G418 selection.

On the other hand, one stable transformant of NCE was obtained from the experiment in which NCE were transfected with full-length HPV16 DNA without the *neo* gene. These cells were serially passaged without G418-selection. Instead, the transformant was selected in the presence of 10% FBS, as described elsewhere.[10] The transfected cervical keratinocytes were split at a 1 : 5

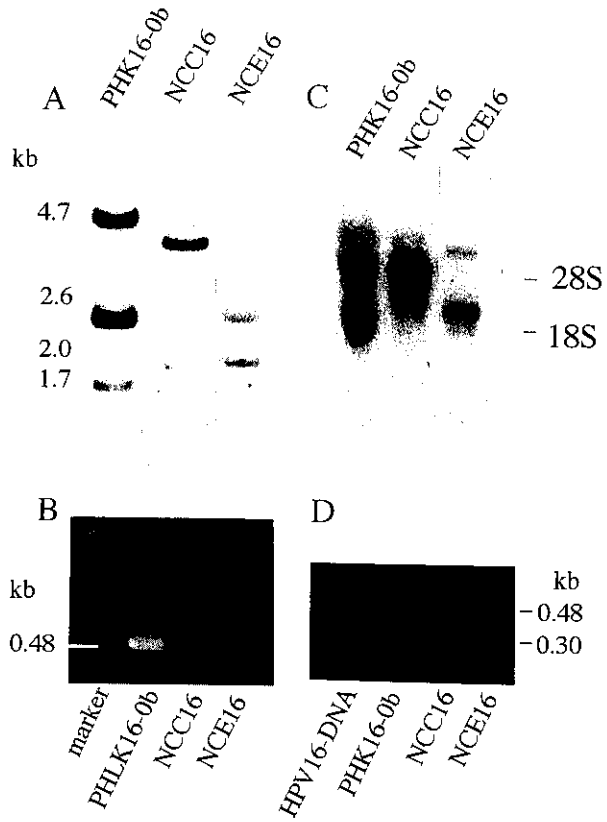

Fig. 4. HPV16 genome and its expression in the transformants, NCC16 and NCE16. A, Detection of HPV16 genomes in the established cell lines by Southern-blot hybridization. B, Detection of E6 region by PCR. C, Northern-blot hybridization of the transformants. The 1.8 kb mRNA is common in both cell lines. D, Detection of mRNA containing E6 region by RT-PCR. Lane HPV16-DNA indicates full-length HPV16 DNA used as positivecontrol. PHK16-0b is an HPV16-immortalized cell line of foreskin epidermal keratinocytes.[15,16]

Fig. 5. Telomerase activation in the transformants. Cellular extracts equivalent to 500 cells of NCC16 and NCE16 were assayed for telomerase activity as described.[18] Telomerase activity can be detected in normal NCC and NCE,[18] but the activity was negligible as compared with that of the immortalized lines (data not shown, see also ref. 18).

ratio at each passage. After several passages over 2 months, the cells had hardly propagated because of a decline of proliferative potential, and were maintained without splitting thereafter. A single macrocolony developed in the following 2–3 weeks and could be propagated to mass culture. All mock-transfected NCE cells died off after several passages and no colony appeared even after maintenance for 6 months. The morphologies of these stable transformants (NCC16 and NCE16) are shown in Fig. 3B.

HPV16 DNA and its expression in established cell lines

NCC and NCE lines originated from histologically normal uterine tissues. PCR using a sequence specific for the HPV16 E6 region confirmed that there was no contamination of endogenous HPV16 DNA in these cells (data not shown). To examine whether these immortalized cell lines contained transfected HPV16 DNA, high-molecular-weight cellular DNAs were extracted from NCC16 cells and digested with restriction enzymes, BamHI and PstI. Southern blot hybridization was carried out under stringent conditions. Specific DNA segments of the HPV16 genome were present, but not all PstI-specific HPV16 DNA segments were detected, suggesting that the integrated HPV16 DNA does not contain the entire HPV16 genome (Fig. 4A). To confirm the presence of HPV16 early genes, the PCR method was employed to detect the HPV16-E6 region of the transformants. The amplified DNA was the same size (0.48 kb) as the complete HPV16 E6 gene (Fig. 4B). Next, to examine viral gene expression, poly(A)-terminated mRNA was isolated and subjected to northern blot analysis using full-length HPV16 as a probe. An mRNA 4.5 kb in size was the predominant species in NCC16 cells (Fig. 4C). In contrast, two distinct mRNAs related to HPV16 were expressed in NCE16 line. Although the sizes of the mRNA species appeared to differ between the two cell lines, the 1.8 kb mRNA expressed in both cell lines is common among many HPV16-transformed cells.

This 1.8 kb mRNA is probably transcribed from the P97-major transcriptional promoter that predominantly produces transcript encoding E6 or E6*/E7, as described previously.[4, 15, 22–25] The larger mRNA species may be the product transcribed from an unknown upstream regulatory region in the LCR.[24] To characterize further the mRNA species in terms of E6 gene transcripts, the E6 coding sequences of the HPV16 mRNA of the transformants were examined by the RT-PCR method using the primers described above (see "Materials and Methods"). We found that the transformant only expressed E6* mRNA, because only a 0.3 kb fragment which is approximately 180 bases smaller than the E6 gene was amplified (Fig. 4D). The E6* mRNA is frequently found in HPV16-related cervical cancer cells[22] as well as in various HPV16-transformants.[24]

Telomerase activity To establish whether or not telomerase activation had occurred in these transformants, cellular extracts were assayed for telomerase activity as described previously.[17, 18] Strong telomerase activities were detected in both cell lines (Fig. 5). The telomerase activity was stably maintained during subpassages, demonstrating the immortality of these cell lines.

Tumorigenicity NCC16 cells did not grow in soft agar and were not tumorigenic in nude mice. However, when cells on a silicon membrane sheet were implanted into the dermis of the back of nude mice, spotty nodules were produced. Upon microscopic observation, the cells that constructed these nodular tissues were found to have prominent macronuclei with markedly irregular distribution of chromatin, including coarse chromatin clumping (data not shown).

Implantation into the back of athymic mouse Untransformed primary NCC and NCE were implanted underneath the epidermis of athymic mice. Four weeks after implantation, the implants were dissected out and fixed in 20% formaldehyde for histologic examination. The implanted NCC and NCE normally reconstituted simple and squamous epithelia, respectively (Fig. 6, A and C). NCE were stratified and keratinized. No dysplastic changes were observed in NCC. These findings were consistent with our previous observations.[19] We performed similar implantations of NCC16 and NCE16. In contrast to untransformed NCC and NCE, abnormal histologic architectures developed; NCC16 cells were stratified and exhibited nuclear atypical changes (Fig. 6B). Since NCC16 cells express cytokeratin 18, they are confirmed to originate from the endocervix (data not shown). NCE16 cells also stratified as expected, but exhibited severe keratinization compared to normal NCE (Fig. 6D). Thus, NCC16 and NCE16 cells have distinctive morphologic characteristics that reflect their origins, but developed abnormal epithelial architectures, probably due to viral functions.

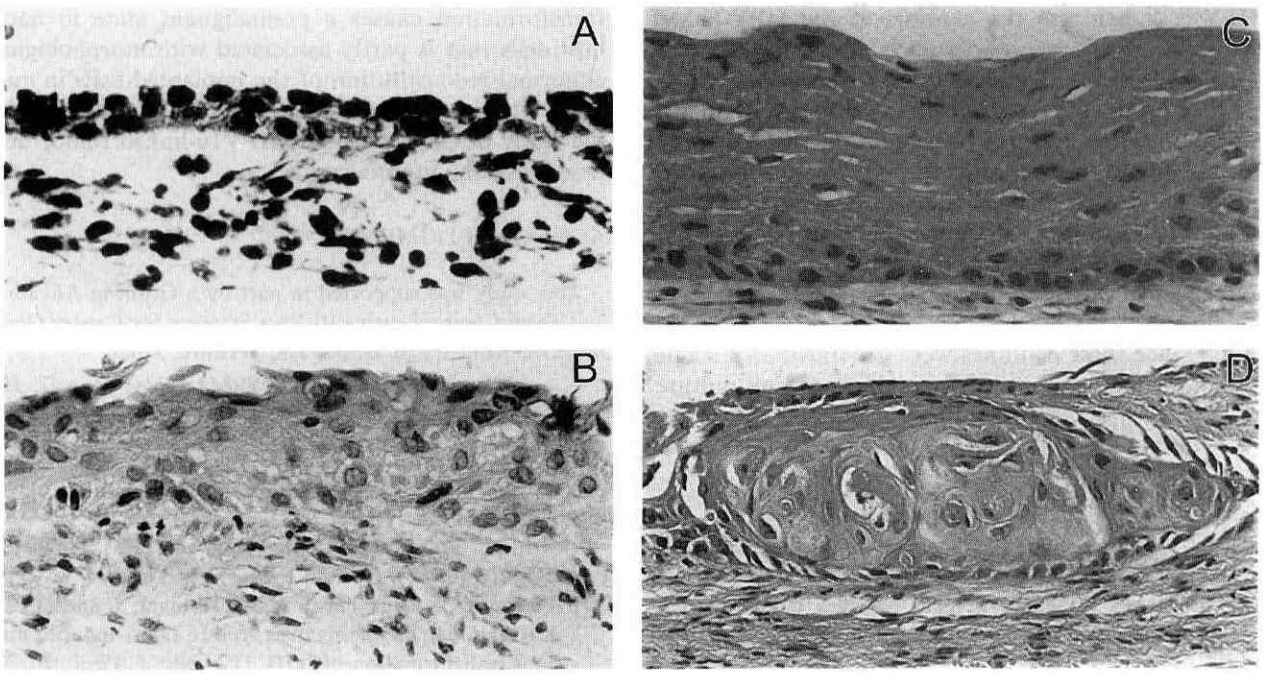

Fig. 6. Reconstitution of epithelial tissues *in vivo* by the transformants. A, Normal endo-cervical simple epithelial cells. B, NCC16. C, Normal ecto-cervical keratinocytes. D, NCE16. See the text for details. The bar indicates 50 μm.

DISCUSSION

We have characterized HPV16-immortalized human epithelial cell lines established after transfecting HPV16 DNA into uterine endocervical columnar and ectocervical squamous epithelial cells, respectively. These distinct immortalized cell lines should provide a useful model for the study of HPV-related carcinogenesis in humans. Since uterine cervical cancers appear most frequently in the transformation zone, which alters in shape depending on hormones, age, infection, and other physiological conditions, it is of interest to transform the cells in this area. However, in practice, it is difficult to dissect this area as it contains complex morphologic architecture. In this study, the aim was to obtain transformants that could be used as a model for the study of HPV-related carcinogenesis. To do this, it is necessary to use cells whose origin is defined. We therefore dissected epithelial tissue segments from ecto-cervical and endo-cervical regions distinct from the transit epithelial region. The tissue origins of the dissected regions were clearly distinguishable, since the endocervical region is highly mucous.

Previous studies have demonstrated that HPV16 can immortalize various human epithelial cells *in vitro*.[7, 10, 12, 13, 16] Although the previously established cell lines provide promising systems with which to analyze HPV-linked oncogenic potentials *in vitro*, the number of established cell lines is limited and very few of the cell lines have been characterized in terms of their cellular origin. Further, the relatively weak transforming activity of HPV[4, 7, 16] hampers the analysis of the HPV-linked transformation process, particularly in defined natural host cells. We demonstrated that the two cell lines established here originated from apparently different epithelial cells of the uterine cervix. The cellular characteristics of the two cell lines well reflect these origins. In this study, only one line was established from 5.0×10^5 cells of ecto- and endo-cervical normal epithelial cells, respectively. The transfection efficiency as measured by the use of a β-gal expression vector suggested that transformation was achieved at the level of one out of 50 to 100 transfectants. Since these cell lines were generated as a single macrocolony among one of several expanding culture dishes, the established transformants are thought to develop clonally over 3 months after transfection with HPV16 DNA. The reason for such a long latent period before initiation of growth remains to be clarified. The expanding macrocolonies can be serially passaged for over 200 population doublings, which would generally be considered indicative of immortalization. We confirmed the telomerase activation in these cell lines is directly correlated to the indefinite life span of the cells.[26–28] However, these cell lines were neither tumorigenic, in accordance with previous findings, when directly injected under the skin of athymic *nu/nu* mice, nor capable of growing in soft agar. These observations indicate that immortalization and tumorigenic transformation are distinct phases in HPV-related tumor development. When these immortalized cell lines were implanted with a supportive substratum (silicon membrane) into athymic *nu/nu* mice, NCC16 cells, unlike untransformed NCC, were stratified under the silicon substratum and exhibited deviant morphologic architecture similar to that of carcinoma. Similarly, NCE16 cells show an abnormal stratification, partly producing a cornified layer and a metaplastic change was detected by Alcian-blue staining, in accordance with the previous study.[19] NCE16 cells also undergo squamous cell differentiation and apoptosis when placed in suspension, which is characteristic of normal human keratinocytes, but not of cancer-derived cell lines such as SiHa and HeLa cells.[29] Therefore, the cellular characteristics of the two isolated HPV16-transformants further support the notion that HPV16-induced transformation causes a premalignant state in natural host cells that is partly associated with morphologically abnormal reconstitution of the implanted cells in *nu/nu* mice. These cell lines should provide a useful model for the study of early events of HPV16-linked tumor development in the uterine cervix.

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