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A Mouse Fibroblast Cell Model to Study Human Papillomavirus-Associated Tumorigenesis

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Cervical cancer represents the second most common cancer in women worldwide. About 90% of cervical cancer contain high-risk human papillomavirus (HPV) DNA, most often HPV type 16. Animal models and mostly laboratory mice are excellent for carrying out diverse immunological studies. We transfected a fibroblast cell line, 3T3-A31, with human papillomavirus type 16 genome to develop an *in vivo/in vitro* malignant transformant model. Isolated clones inoculated to immunocompetent mice displayed a tumorigenic phenotype. Small clusters of metastatic cells were found in the liver of animal 45 days after receiving the inoculum. Integrated viral DNA and expression of E7 viral oncogene from the high-risk HPV-16 were demonstrated both in transfectans and tumor-derived cells. The observed high-grade neovascularization was correlated with the upregulation of vascular endothelial growth factor (VEGF) mRNA on HPV-16 transformed fibroblast cells. These observations emphasize the association between papillomavirus expression and progression to malignancy. © 2000 Academic Press

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Human papillomaviruses (HPV) infect skin and mucous membranes epithelia and cause benign or malignant tumors in humans. Cervical cancer, of which 90–95% is HPV16-associated, represents the second most common cancer in women worldwide and the major cause of death in women from developing countries (1). An effective therapeutic HPV vaccine could have great impact in reducing morbidity and mortality from intraepithelial neoplasia, cervical and other anogenital cancers.

The evaluation of the therapeutic efficacy for vaccine candidates can only be carried out using animal models but certain obstacles still remain on this approach. Humans are the only natural host for the most relevant papillomavirus and HPV-associated tumors cannot be induced in other animals by direct infection. Some homologous papillomavirus animal systems have been described but they use species specific papillomavirus. The heterologous approach offers the advantage to transfer HPV to animals using syngeneic cells expressing the HPV genome. Heterologous model using laboratory mice are excellent for carrying out diverse immunological studies. Mouse models using immunodeficient mice has been reported but they are not directly useful for vaccine development (2) or other studies about immune responses in tumor bearing animals. We chose immunocompetent mice considering the importance of an intact immune system to evaluate any vaccine candidate or immune responses in tumor models.

Herein we described the *in vivo* behavior of established mouse fibroblasts transfected with a heterologous papillomavirus. Selected HPV transformants displayed a malignant phenotype as evidenced by the acquired tumorigenicity, metastatic capacity and angiogenic properties. A key mediator of angiogenesis, vascular endothelial growth factor (VEGF) (3), was analyzed in cells transformed by HPV-16 DNA. The observed malignant behavior of these HPV transformed cells in immunocompetent mice sustains its application as an appropriate animal model of HPV-associated tumors for testing immunotherapeutic tools.

MATERIALS AND METHODS

Cell culture and plasmids. The BALB/c fibroblast syngeneic cell line, 3T3-A31, was used for transfection experiments. This cell line does not contain HPV DNA sequences. CaSki cell derived from an HPV 16 DNA-positive cervical cancer, served as a positive control. Cell lines were maintained in DMEM supplemented with 10% fetal bovine serum. A 7.9-kb BamHI fragment containing the entire HPV-16 DNA was inserted into pcDNA-3 vector, which bears the CMV promoter and the neomycin resistance gene. The resulting plasmid was called pcDNA-16.

Transfection experiments. Subconfluent 3T3-A31 cells grown in 100 mm plastic Petri dishes were transfected with 7 μg of pcDNA-16 or pcDNA-3 using the lipofectin kit (Transfection Reagent DOTAP,

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Boehringer Mannheim) according to manufacturer's instructions. Parallel cultures were mock transfected. Six hours after transfection G418 (1 μg/ml) was added to select cells containing either control DNA (pcDNA-3) or HPV-16 sequences (pcDNA-16).

Cell growth assay. Cells (1×10^4) containing control or HPV-16 DNA were independently seeded in four replicate 35-mm dishes. Plates were incubated overnight at 37°C and the initial cell number was then determined. To estimate doubling times, growing cells were trypsinized and counted every day for 5 days.

Tumorigenicity assay. Female, pathogen-free BALB/c mice, 8–10 week old, were used for tumorigenicity assays. Transfected cells, freshly trypsinized and washed with PBS, were injected subcutaneously into BALB/c mice at various doses, including 0.5×10^6, 1×10^6, 2×10^6, 5×10^6 cells/mouse; each group contained ten animals. Mice were regularly examined for evidence of tumor formation. To isolate tumor cells, solid tumor specimens were minced, and mechanically disassociated with scissors. Tumor fragments were plated in p60 dishes containing RPMI 1640 supplemented with 10% fetal calf serum. One or two weeks later, when foci of anchored cells spreading from the tumor fragments were observed, medium was replaced by DMEM supplemented with 10% fetal calf serum. The resulting cell line was named T-16.

Histopathological analysis. The histopathological analysis was done at 20, 35 and 45 days after cell inoculation. Animals were sacrificed by cervical dislocation and processed to be histopathologically characterized. Samples from the tumor and different organs, including liver, spleen, lungs, kidneys, heart and pancreas, were harvested and fixed in neutral 10% formalin. Paraffin-embedded tissues samples were cut and histological analyses were performed on H&E-stained sections (4).

Nucleic acid analysis. Total RNA was prepared by the guanidinium thiocyanate–phenol–chloroform method (5). The RNA was electrophoresed on 1% agarose gels containing 6.3% formaldehyde and transferred onto Zeta-Probe nylon filters. Filters were then used for hybridization with α^{32}P-labeled HPV-16 full genome, mVEGF cDNA, and GAPDH cDNA. The expression levels of mRNA were quantified by the Molecular Analysis Software (Bio-Rad). High molecular mass DNA was extracted by the sodium dodecyl sulfate (SDS)–proteinase K–phenol–chloroform method (6). Twenty μg of genomic DNA were digested with BamHI and NcoI, electrophoresed through 0.8% agarose gels, and transferred onto a Zeta-Probe nylon filter. Prehybridization and hybridization with ^{32}P-labeled full-length viral DNA were performed according to manufacturer's instruction (7). One μg of total RNA was subjected to reverse transcription according to a previously established protocol (8). Then 10 μl of the reaction mixture were used for HPV-16 E7 specific polymerase chain reaction (PCR). Reverse transcriptase-untreated samples were used in parallel to evaluate possible DNA contamination of RNA preparations. PCR amplification was performed in a total volume of 50 μl. The following primers were employed in the amplification reaction: sense 5′-GCAACCAGAGACAACTGATCTCTAC-3′ and antisense 5′-GGTCTTCCAAAGTACGAATGTCTACG-3′ (annealing temperature: 55°C, 30 cycles). The reaction yields an amplification product of 199 base pairs corresponding to the HPV-16 E7 open reading frame (606–805 on HPV-16 genome). Aliquots of the reaction products were size fractionated by 2% agarose gel electrophoresis.

Immunological procedure. For immunoblotting, cellular lysates were prepared in the lysis buffer. The lysis buffer consisted of 250 mM NaCl, 50 mM HEPES (pH 7.0), 0.1% NP-40 and 1% Aprotinin. The lysates were clarified at 15,000*g* at 4°C for 10 min. Homogenate of tumor tissues from mice were also prepared in the lysis buffer and cleared by centrifugation as described. Protein concentrations were determined by using the Bio-Rad (Bio-Rad Laboratories) protein assay. Samples were fractionated on SDS-PAGE and transferred onto nitrocellulose membrane A mouse mAb was used to detect E7 (Santa Cruz Biotechnology, Inc.). The bound antibodies were visualized by using enhanced chemiluminescence (ECL; Amersham).

RESULTS

Isolation and growth properties of transfected cells. Fifteen days after cell culturing in the presence of G418, colonies observed in dishes containing pcDNA-16 and pcDNA-3 transfected cells were pooled and subcultured as independent cell lines. The established cells lines were termed 3T3-16 (HPV-16 DNA) and 3T3-C (pcDNA-3). To test the effect of HPV-16 DNA on *in vitro* growth, we examined each cell line growth rate. During the first 72 h the population doubling times were similar for 3T3-16 and 3T3-C cells (17 and 15 h, respectively). However, when cultures were confluent the population doubling time for 3T3-C increases, as expected for immortalized non-transformed cells, while 3T3-16 cells maintain the initial high growth rate. These results indicate that in contrast to 3T3-C, 3T3-16 cells have acquired the capacity to growth at high cellular density. This independence of contact inhibition is a characteristic feature of the transformed phenotype (Fig. 1A).

Tumorigenicity. Cells from the 5th passage after transfection were inoculated subcutaneously into immunocompetent BALB/c mice at various doses. Tumors developed at the injection sites at rates depending on the administered cell doses. In mice that received 5×10^6 3T3-16 cells (the highest dose), palpable tumors were established as early as seven days after inoculation. Latency periods were longer for animals receiving inocula of lower cell quantities. Results from two different experiments of tumor implant are summarized in Fig. 1B. No tumors were detected in mice receiving 3T3-C cells even after 150 days of observation. In the groups of animals with tumors, death was observed after 28 days of cell inoculation, in apparent relation with the administered quantity of cells.

Presence of HPV-16 DNA and HPV-16 E7 gene expression in transfected cells and growing tumors. Genomic DNA was isolated and subjected to Southern blot analysis. DNA samples from both, established transfected cells (3T3-16) and cells derived from tumors induced by HPV-16 DNA-containing cells (T-16), were digested with NcoI and BamHI to determine integration and potential rearrangement of viral DNA. If viral DNA was integrated NcoI digestion would produce two bands since only one NcoI digestion site is present in the HPV genome. BamHI digestion was expected to produce a 6.6-kb DNA fragment if no rearrangements of HPV DNA sequences had occurred. DNA from 3T3-16 and their derived tumor cells T-16, exhibited similar digestion patterns. Results of the Southern blot using as probe α^{32}P-labeled HPV-16 are shown in Fig. 2. Both cell lines contained integrated viral DNA and no rearrangements have occurred dur-

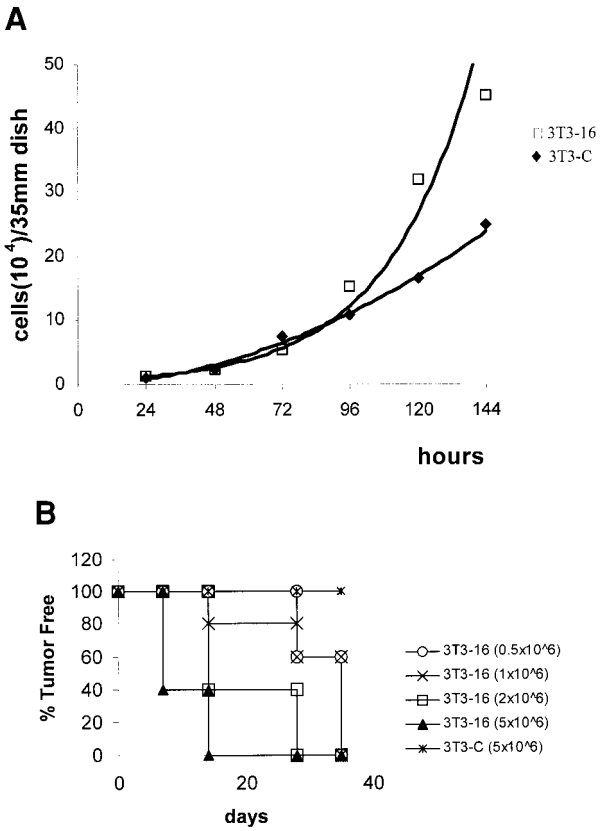

FIG. 1. (A) Proliferation of BALB/c 3T3-derived transfectants *in vitro.* Cells were cultivated in complete medium (DMEM supplemented with 10% FCS) in 35-mm dishes. Cells from duplicate dishes were harvested and counted on the indicated days. Tendency lines are represented. (B) Kinetics of tumor growth after 3T3-16 cells inoculation. 3T3-16 cells were injected into Balb/c mice s.c. at various doses: (○) 0.5×10^6, (×) 1×10^6, (□) 2×10^6, (▲) 5×10^6 cells per mouse. Results from animal inoculated with 3T3-C (5×10^6 cells) are also shown (*). Mice were examined weekly for evidence of tumor formation.

ing integration. Caski cells were used as control of HPV-containing cells and 3T3-A31 and 3T3-C as negative controls for the presence of HPV DNA sequences. To verify the expression of the integrated viral DNA, a RT-PCR analysis using specific primers for E7 open reading frame (ORF) was performed. RNA isolated from CaSki cells was used as positive control, since these cells express high levels of HPV-16 RNA. An amplified 200 bp cDNA fragment indicated the expression of the E7 ORF in HPV-transfected and tumor cells. The amplified cDNA fragment from β actin (660 bp) served as internal control of RNA integrity for every analyzed cell line (Fig. 3A). As expected no E7 PCR products were observed in cells non-containing HPV DNA sequences. E7 expression was 5.5-fold higher in tumor-derived cells than in parental HPV-transfected cells, when expression relative to the housekeeping gene was estimated. Additionally, E7 protein expression was studied by Western blot. E7

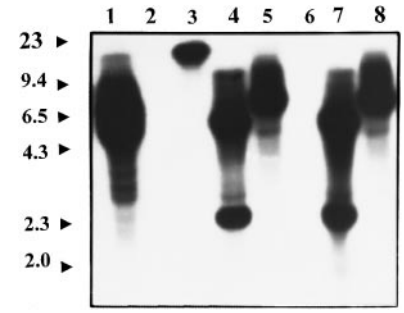

FIG. 2. Southern blot analysis of HPV-16 DNA in transfected and in tumor-derived cells. Genomic DNA was digested with NcoI (Lanes 1, 4, and 7) or BamHI (Lanes 5 and 8). Lane 1, CaSkI cells; lane 2, parental 3T3-A31 cells (nondigested); lane 3, 3T3-16-transfected cells (nondigested); lane 4, 3T3-16; lane 5, 3T3-16; lane 6, 3T3-C; lane 7, T-16; lane 8, T-16. Left, molecular sizes of standards (kb).

expression was observed both in 3T3-16 and tumor-derived cells. Figure 3B shows that E7 MAb recognizes protein bands of about 21 kDa in cellular extracts from 3T3-16, T-16 and Caski cells.

Histological examination. Optic microscopy revealed that malignant cells are differentiated, rounded in shape with giant and vesicular nucleus. Bipolar mitosis figures and apoptotic cells were abundant in the tumor; from 20 microscopic fields at random selected and observed with 400× magnitude, and counting approximately 7500 cells, averages of 4.2 mitotic cells/field and 3.1 apoptotic cells/field were obtained. Tumors, which were implanted on subcutaneous tissue, were characterized as locally invasive spreading to dermis, epidermis and striate muscle tissue. At the

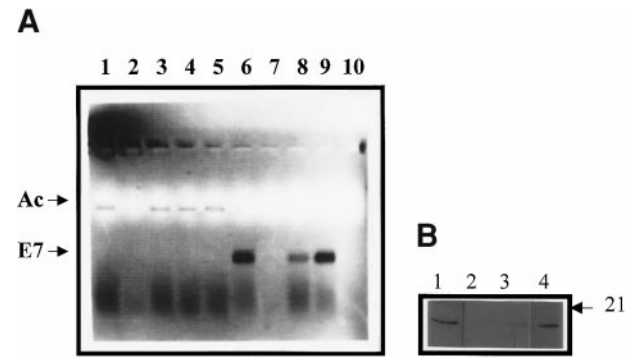

FIG. 3. Expression of E7 mRNA and protein in transfected and tumor cells. (A) Total RNA was isolated from each sample and analyzed by RT-PCR using specific primers for E7 protein (Lanes 6–10) and actin (Lanes 1–5). CaSki (Lanes 1 and 6); no RNA sample (Lanes 2 and 7); 3T3-16 (Lanes 3 and 8); tumor tissue from mice inoculated with 3T3-16 cells (Lanes 4 and 9); and 3T3-C (Lanes 5 and 10). A 200-bp amplified cDNA fragment, specific for E7, was detected in cells lines containing HPV-16 DNA. A 600-bp amplified cDNA of actin (Ac) is shown as a control of RNA integrity. (B) Western blot analysis of E7. Lane 1, CaSki cells; lane 2, 3T3-C cells; lane 3, 3T3-16 cells; lane 4, tumor cells. Right molecular size of estandar (kDa).

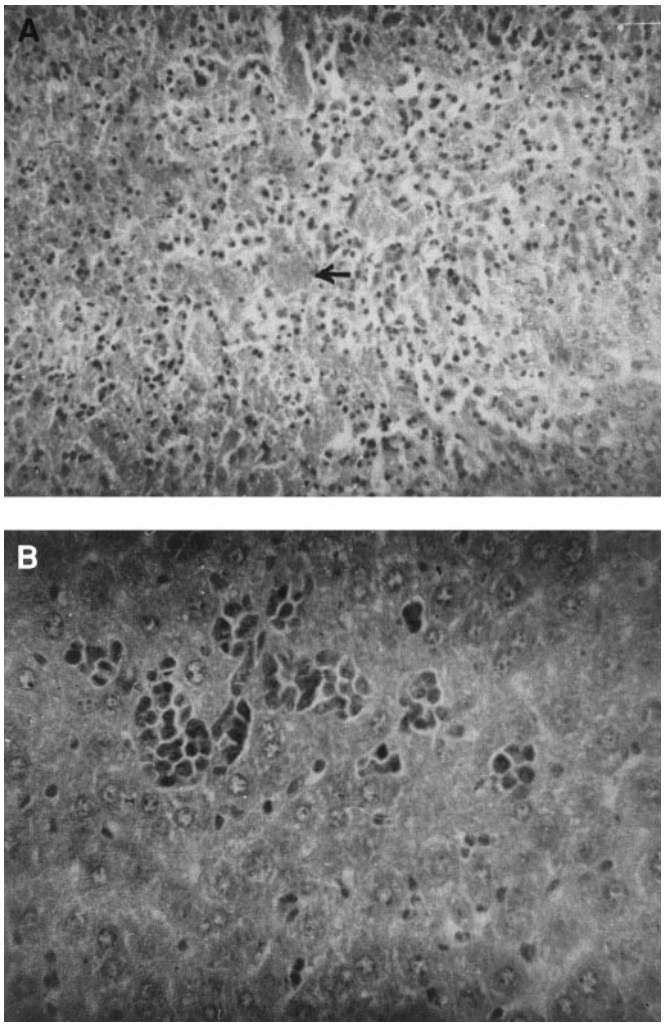

FIG. 4. Histological analysis of tissue samples from mice, receiving 2×10^6 3T3-16 cells. Animals were sacrificed 30 days after injection. Specimens were stained with H&E. (A) Tumor tissue containing increased density of visible blood filling capillaries demonstrates functional vascularization. (B) Section of liver showing microscopic metastatic clusters of tumor cells in the sinusoidal system.

central part of the tumor mass an extensive necrosis zone was observed, surrounded by vascular stroma distinguished by the increment in the number of new capillaries (Fig. 4A). Vascularization was notable in the tumors, being characterized by vascular thrombosis phenomenon and hemorrhagic angiomatoses zones. Macrophages were abundant surrounding the necrotic foci. Microscopic clusters of metastatic cells were found in the liver of those animals that received cell doses higher than 2×10^6 cells, 35 days after cells injection (Fig. 4B).

VEGF/VPF mRNA in transfected cells. To identify mechanisms underlying the increased vascularization of the tumors, we evaluated the transcription of VEGF on transfected cells by Northern blot. 3T3-16 cells showed 10-fold higher basal levels of VEGF/VPF mRNA compared with 3T3-C. CaSki cells were used as control of HPV-16 expression and the housekeeping gene GAPDH was used to normalize results of mRNA expression (Fig. 5).

DISCUSSION

The importance of the immunotherapeutic approach for the control and treatment of HPV-associated tumor diseases have received much attention in recent years. However, animal models to test immunotherapeutic agents are limited and generally based on other papillomavirus infection of susceptible hosts. The present study examines a heterologous model of HPV-associated tumors where host immunocompetence is preserved. Tumors are induced by inoculation of HPV-16 transformed syngeneic fibroblasts in BALB/c mice. Therefore, the antitumor specific immune responses triggered by a particular agent in this model would be relevant for its application in HPV natural hosts.

Since evidences argue that high levels of HPV expression are essential to induce and maintain the transformed phenotype (9), we cloned the entire HPV-16 DNA under a strong viral transcriptional signal, the CMV promoter, to induce the expression of the HPV sequences at levels high enough to cause transformation. The syngeneic fibroblast cell line 3T3A31 was selected because of its low background of spontaneous transformation and the capacity of high risk HPV oncoproteins of transforming this established mouse fibroblasts.

The HPV-16 DNA transfected cells (3T3-16) shown several differences respect to those transfected with the control vector (3T3-C), such as the smaller cellular size and the growth independence of contact inhibition. 3T3-16 cells, when injected subcutaneously, induced

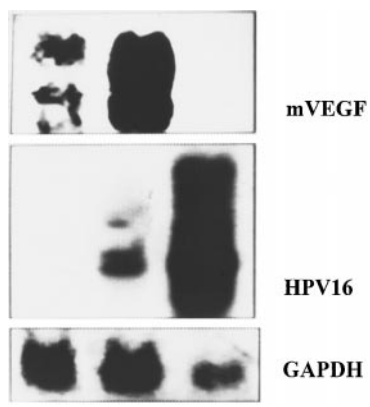

FIG. 5. Northern blot analysis for detecting murine VEGF transcripts in transfected cells. RNA was isolated from 3T3-C cells (Lane 1); 3T3-16 cells (Lane 2); and CaSki cells (Lane 3). A probe from the housekeeping gene GAPDH was use for normalizing transcript signals.

tumors in immunocompetent animals few days after inoculation. On the contrary, after several months of observation, there was no evidence of tumor formation when cells containing the control plasmid (3T3-C) were inoculated, even using gross cell inocula (5×10^6 cells/mouse).

The ability to produce tumors in singeneic immunocompetent animals was reported for HPV-transformed primary rodent cells co-expressing an activated *ras* gene (10). HPV-E7 was both necessary and sufficient for transformation of these epithelial cells, cooperating with *ras*. The tumorigenic behavior of HPV-16 transformed mouse fibroblasts was previously demonstrated in nude mice (9).

We were interested into further characterize tumors generated in immunocompetent mice by HPV-16 transformed cells. Histology demonstrated that tumors were differentiated, locally invasive and aggressive in their growth properties. Some features resemble the histopathology of human cervical tumors. Mitotic figures, common throughout the entire thickness of cervical lesions, consistent with marked increase in proliferation observed in human CIN III (11), were evidenced in all analyzed tumor sections. Moreover, the increased density and altered distribution of the vasculature in mouse tumors reproduce the angiogenic morphology of high-grade premalignant lesions and cervical cancer in human (12).

Micrometastatic clusters were only observed in the liver of those animals receiving more than 2×10^6 cells, subcutaneously. The fact that metastasis were not observed with lower cell inocula may be related with the use of a policlonal cell line to induce tumors and the period selected for histopathologic analysis. Other aspects, like the site of implantation, should be considered for future studies of the metastatic potential of 3T3-16 cells.

Since it is now well established that tumors depend on neovascularization for their continued growth, expansion and potential metastasis, we went on to determine the expression of a potent and highly specific angiogenic growth factor, VEGF/VPF in 3T3-16 cells. VEGF production by diverse tumor cells has been demonstrated (13–15). We found an upregulation of VEGF/VPF transcript in the HPV-16 transformed cell line. Recently, VEGF upregulation was demonstrated during cervical carcinogenesis in humans by in situ hybridization (16). Also, the tumorigenic conversion of HPV-immortalized human keratinocytes by chronic exposure to benzo(a)pyrene was related with high secretion of VEGF (17). Our data confirm this association between VEGF expression and malignant transformation with HPV. In this model, angiogenic processes, demonstrated by histological and molecular evidences, seem to be relevant for the biological behavior of the HPV-associated tumor in immunocompetent animals.

In accordance with previous reports, transformed and tumor-derived cells contained integrated viral DNA and expressed viral mRNA. Viral transcript levels were higher in the tumor derived cell line T-16 than in the parental 3T3-16 cells. The most likely explanation for this difference is that there was a selection in the tumor for cells expressing high levels of HPV. Several studies on HPV-16 positive cervical carcinoma cells and *in vitro* transformed cell lines have shown that viral oncoprotein expression is required to maintain the transformed phenotype, and that high levels of expression are essential for tumorigenicity (10, 18, 19).

In the present study, expression of E7 protein in transfectants and tumor-derived cells supports the close relation between overexpression of early genes and malignancy. Similar morphological transformation induced by "high risk" E7 proteins in immortalized rodent fibroblasts have been reported (10, 20–22). Although data herein presented, regarding PCR experiments, showed only overexpression of E7 sequences, both spliced and unspliced HPV-derived mRNAs have been detected in 3T3-16 cells in our lab (unpublished results). Therefore, the contribution of E6 protein expression to tumorigenicity in this model could not be discarded. Indeed, the requirement of E6 expression for tumorigenicity of HPV16 transformed mouse established cells lines have been previously demonstrated (23).

In conclusion, this experimental model comprises both basic features of malignant HPV-associated lesions and immune competence of the recipient. Hence, it should provide a valuable tool of investigating immune responses involved in HPV-associated tumor rejection and agents to modulate them. In addition, it could constitute a setting for analyzing the role of some *in vivo* basic events in HPV-induced tumor biology.

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