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Papilloma Formation in Human Foreskin Xenografts after Inoculation of Human Papillomavirus Type 16 DNA

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A mouse model of high-risk human papillomavirus infection was developed in which human papillomavirus (HPV) type 16 DNA was inoculated into human foreskin grafted to the skin of severe combined immunodeficient (*scid*) mice. Grafted skin contained human epidermis and dermis and, like normal human skin, expressed involucrin in differentiating keratinocytes. HPV type 16 DNA, attached to gold particles, was delivered directly into human epidermal cells and induced exophytic papilloma with histologic features of papillomavirus infection, including koilocytosis and expression of papillomavirus capsid antigen. This model should be useful for determining in vivo the functions of viral genes and for developing strategies to prevent and treat HPV-associated disease. It may also be of value in developing animal models of other human skin diseases.

Infection of the anogenital tract with high-risk human papillomaviruses (HPVs) causes benign intraepithelial neoplasias that, after an extended latency period, can become dysplastic and eventually progress to invasive squamous cell and adenocarcinomas (reviewed in references 20, 45, and 60). HPVs have also been associated with some cancers of the head and neck (13). Anogenital infections are common in sexually active women and men, and a significant proportion are due to high-risk HPVs. Each year, about 500,000 such infections at the uterine cervix undergo malignant conversion, making cervical cancer the most common malignancy in women worldwide. About 90% of such tumors contain high-risk HPVs, with HPV type 16 (HPV-16) being most prevalent.

To determine the pathogenesis of virus-associated cancers and premalignant lesions and to develop effective strategies for their treatment, it is imperative to have a satisfactory animal model system (3, 4). However, direct inoculation of animals with HPV does not induce disease, and identification of the biological and biochemical properties of HPV genes and *cis*-acting elements has relied almost exclusively on in vitro systems (reviewed in references 14, 32, 39, 40, and 46). How properties identified in vitro relate to the induction and progression of actual disease remains largely unknown. In addition, the lack of an adequate system for papillomavirus replication has prevented isolation of whole viruses from clinical specimens and generation of mutant viruses. It has also impeded our understanding of vegetative papillomavirus replication.

Previously, a nonhuman animal model was developed for HPV-11 (19, 25, 27, 29), a low-risk genital virus not associated with cancer. Human foreskin chips were incubated in vitro with virus obtained from condylomata acuminata, an HPV-induced human lesion, and transplanted under the renal capsule of athymic mice, or, more recently, in the subcutis, peritoneum, or renal capsule of severe combined immunodeficient (*scid*) mice (2). Infected tissue developed into virus-producing condylomatous cysts, and this model has been useful for studying the natural history of HPV-11 infection, including virus production (26, 28, 50). The approach has also been adapted for HPV-1, a cause of plantar warts (30). Infectious mutants of HPV-11 (or HPV-1) have, however, not been described, and the functions of individual viral genes in this model have not been determined. The model may also not be applicable to high-risk HPVs because human lesions induced by high-risk HPVs appear to produce little virus (20, 49).

Another mouse model was developed by grafting human W12 keratinocytes, established from a natural HPV-16 infection, onto the backs of athymic mice (47, 48). W12 cells, in which HPV-16 DNA persisted in an episomal state, differentiated to form an epithelium with morphologic features of a low-grade HPV-16 lesion. Some grafts expressed viral capsid antigen and produced viral particles, an effect that correlated with the episomal nature of the viral genome. Because most cell lines derived from natural infections contain only integrated HPVs, the applicability of this approach for generating engrafted animals expressing a variety of HPV types appears to be limited. The model also does not lend itself to systematic analysis of viral mutants.

An animal system exists that permits experimental induction of benign papillomas upon intradermal inoculation of rabbits with molecular clones of the cottontail rabbit papillomavirus (CRPV) genome (9, 17, 42). With time, such lesions can progress to squamous cell carcinoma (5). In this model, CRPV DNA has been inoculated with a high-pressure jet injector method (9) or by manual scarification (17, 42). The CRPV DNA-rabbit system has enabled the identification of viral genes required for papilloma formation (9, 10, 12, 36, 42, 55). It has also been used to produce infectious virus stocks (8). Data obtained in the CRPV-rabbit system are likely to be relevant to the establishment of HPV gene functions but require extrapolation. To develop a model of HPV-16-induced disease that closely mimics natural human papillomavirus infection, we inoculated a molecular clone of HPV-16 into human foreskins grafted onto *scid* mice.

Human foreskins were obtained from newborn human males after routine circumcision through the Yale Skin Diseases Research Center. The underlying dermis was partially removed with scissors, and the full-thickness skin was cut into squares measuring approximately 10 by 10 mm. Each foreskin yielded

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two or three such squares. *scid* mice were selected as graft recipients on the basis of their lack of immunologically functional T and B cells. *scid* mice can receive both allogeneic and xenogeneic grafts, including human skin grafts, and have proved useful for developing several animal models of human disease (35, 37, 38).

Recipient mice were *Pneumocystis carinii*-free, 5- to 8-week-old Fox Chase C.B17 *scid*/*scid* mice (Taconic Farms Inc., Germantown, N.Y.). Foreskin samples were grafted onto the back of each recipient, typically within 4 h of the circumcision. The animals were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. Autologous mouse skin was clipped free of hair, and surgical sites were disinfected. By using an aseptic technique, the mouse skin was excised, the fascia was removed, and the muscle was superficially wounded. The human tissue was then placed on the site and held in place with 6.9-mm skin staples (3M Healthcare, St. Paul, Minn.). In the first week following engraftment, the human foreskin xenografts underwent mild necrosis and appeared darkened and encrusted, but the necrotic crusts were gradually lost, and after about 3 weeks, the grafts assumed the appearance of normal human skin.

Xenografts were hairless, and those derived from pigmented human skin retained their pigmentation, which contrasted with the albino skin of the recipient mice. Other evidence also indicated that the grafts consisted of normal human skin. Human involucrin was detected immunohistochemically in maturing suprabasal keratinocytes of the grafts (Fig. 1A), a pattern characteristic of the stratified squamous epithelia of normal human skin (41, 44). Mouse involucrin does not cross-react with the highly specific antibody we used (Biomedical Technologies, Inc., Stoughton, Mass.), and as expected, adjacent mouse skin was negative (Fig. 1A). It was also observed that grafts derived from melanotic individuals had abundant melanin distributed diffusely in basal keratinocytes (Fig. 1A).

Additionally, in situ hybridization to an oligonucleotide probe corresponding to the human Alu consensus (GM-009) (34) was used to characterize the grafts. Cells of mouse origin did not hybridize to the probe, consistent with previous reports (33). Grafts contained epidermal and dermal cells of human origin, and mouse epidermis overlaid human dermis in transitional areas (Fig. 1B). Nuclei of keratinocytes, melanocytes, sebaceous glands, apocrine sweat glands, and dermal fibroblasts in the grafts hybridized to the Alu probe (6).

For delivery of HPV DNA, we sought an alternative to the jet injector method used to inoculate rabbit skin with CRPV DNA (9) because the high-pressure injection was judged to be potentially traumatic for mice (data not shown). Instead, we used the method of biolistic particle bombardment (22, 23, 54, 56–58), wherein naked DNA is bound to inert gold particles that are accelerated to a high velocity for penetration of target tissue. Several genes have previously been expressed in mice, rats, pigs, and monkeys with this approach (1, 11, 15, 16, 18, 21, 43, 52, 59).

HPV-16 DNA delivered into the experimental group of xenografts was derived from the W12 human cervical keratinocyte cell line, which contains a biologically active HPV episome (47, 48). An HPV-16 genome had previously been cloned from the W12 cell line in plasmid vector pSP64 and was used as the source of HPV DNA for our studies. Genomic HPV DNA was cleaved from the vector by *Bam*HI digestion and purified by electrophoresis in an agarose gel. Linear HPV-16 genomes were then circularized by ligation (8).

DNA delivery was performed by particle acceleration with an *Accell* particle acceleration device (Agracetus Inc., Middleton, Wis.). Quantities of the recircularized, supercoiled

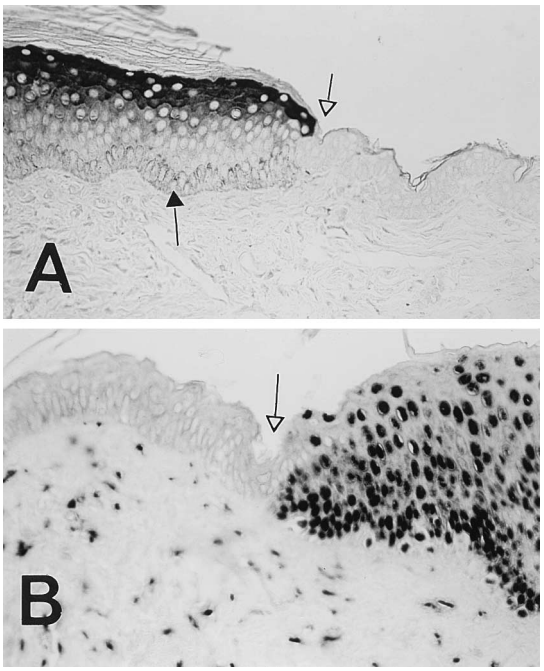

FIG. 1. (A) Immunohistochemical analysis demonstrates involucrin expression in a normal foreskin xenograft. Human involucrin is visible in maturing superficial cells above the basal layer in the xenograft. Mouse skin is negative for involucrin. The open arrow denotes the junction between human skin (left) and mouse skin (right). Note also the abundance of melanin (closed arrow) in the basal cell layer of this graft, which was derived from a melanotic donor. Immunohistochemical analysis was performed with polyclonal antiserum to human involucrin and commercial immunoperoxidase reagents (Biomedical Technologies). Sections were lightly counterstained with hematoxylin. (B) In situ hybridization differentiates human from mouse cells in xenograft sections. Nuclear staining identified human cells, which hybridized to the human Alu probe. The open arrow denotes the junction between epidermises of mouse (left) and human (right) origin. Note that the dermis underlying both epidermises is composed largely of human cells. In situ hybridization was performed on formalin-fixed sections that were deparaffinized and pretreated with Triton X-100 (0.1%, 3 min), proteinase K (1 μg/ml, 30 min, 37°C), and glycine (2 mg/ml, 5 min). An Alu oligonucleotide, GM-009, labeled with digoxigenin (33) was used as the probe. Hybridization was performed in 50% (vol/vol) formamide–600 mM NaCl–0.1% sodium PP_i–0.2% Ficoll–5 mM EDTA–150 μg of single-stranded salmon sperm DNA per ml–10% polyethylene glycol–5 pmol of the probe per 50 μl per section. Slides were placed at 94°C for 7 min and then incubated overnight at 37°C. They were washed in accordance with the following schedule: 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature (1 × 10 min), 1× SSC at 37°C (2 × 15 min), and 2× SSC at room temperature (4 × 5 min). The hybridized probe was then detected with antidigoxigenin antibody and alkaline phosphatase by using a commercial kit (Boehringer Mannheim, Indianapolis, Ind.).

HPV-16 genomic DNA were precipitated onto gold carrier particles by mixing 5 μg of DNA with 20 mg of gold powder (0.95-μm average diameter) in 0.2 ml of H_2O. Then, 800 μl of 0.25 M $CaCl_2$–12.5% polyethylene glycol 6000 was added to the mixture with continuous agitation, after which the coated gold carrier particles were permitted to precipitate. The supernatant was removed, and the carrier particles were washed gently with 1 ml of ethanol and resuspended in ethanol at 20 mg/ml in a glass-capped vial.

The DNA-coated carrier particles were then layered onto 324-mm^2 mylar sheets (1.8 cm on each side) at a density of 6.4 mg of gold (1.6 μg of DNA) per sheet. This was done by applying the ethanol suspension of the carrier particles to the carrier sheet and then allowing the gold to settle for several minutes, after which the ethanol meniscus was broken, the ethanol was removed by pipette, and the residual ethanol was allowed to evaporate. The mylar sheets were then dried under

TABLE 1. Papilloma formation in human foreskins grafted onto *scid* mice

Time of HPV-16 DNA inoculation	No. of animals	No. of grafts (foreskins)	No./total no. (%) of inoculated grafts		
			Long-term survival[a]	Macroscopic lesions[b]	Microscopic lesions[c]
Preengraftment	4	8 (4)	7/8 (88)	2/7 (29)	2/7 (29)
Postengraftment	4	8 (4)	4/8 (50)	0/4 (0)	0/4 (0)

[a] Eight weeks or longer after engraftment.
[b] Rough, warty feel and appearance.
[c] Histologic features of papillomavirus infection (see text).

a heat lamp. The DNA-coated gold particles on each mylar sheet were then placed in an *Accell* Gene Delivery System, which utilizes an adjustable electric spark discharge to accelerate the carrier particles to the target cells to be transfected. Meanwhile, the anesthetized engrafted animals were positioned below the accelerated particle apparatus, and gene delivery was performed by using a discharge voltage of 15 to 25 kV.

Naked HPV-16 DNA was inoculated directly into human foreskin xenografts on eight recipient mice. Of eight grafts on four mice that received DNA after engraftment and healing, seven survived and were monitored weekly for proliferative lesions (Table 1). Two of the seven developed signs of HPV infection. One developed a pronounced rough, warty feel and appearance with white exophytic papillary projections (Fig. 2). This graft also exhibited histologic features of papillomavirus infection, including pronounced epidermal hyperplasia, papillomatosis, acanthosis, parakeratosis, hypergranulosis, and koilocytosis (Fig. 3A). The nuclei of koilocytic cells were pyknotic and variably hyperchromatic. They were surrounded by a wide, clear zone of cytoplasmic degeneration and had prominent cellular borders (Fig. 3B). A second graft displayed a series of small multifocal macroscopic lesions with slightly roughened surfaces. The histologic features of this graft were also suggestive of papillomavirus infection and included epidermal hyperplasia, acanthosis, and hypergranulosis. These two grafts were derived from different foreskin donors and were grafted onto different mice. Each of the foreskins that gave rise to the lesions had been successfully grafted at two sites on a mouse. In each case, one of the two grafts appeared to be completely normal (Fig. 2) macroscopically and microscopically, demonstrating that there was no preexisting HPV infection in the target tissue and that penetration and/or expression of HPV DNA was not universal.

Paraffin sections of the larger lesion were studied further. The presence and type of HPV in the tissue were analyzed by using the PCR in conjunction with HPV-specific PCR primers MY09 and MY11 (7, 53). The PCR product was approximately 450 bp long, and it displayed *Bam*HI, *Pst*I, and *Hin*fI restriction patterns that are characteristic of HPV-16 and HPV-18 but not other HPV types. Hybridization of the PCR product to oligonucleotide probes that are type specific for HPV-16 or HPV-18 (53) demonstrated that the DNA was HPV-16 and not HPV-18 (Fig. 4). Immunohistochemistry for papillomavirus group-specific structural antigens was also performed (31). This analysis revealed the presence of papillomavirus capsid antigens in the nuclei of the cells of the intermediate epithelial layers (Fig. 5A). No viral capsid antigen was detected in the untransformed graft that derived from the same foreskin as its transformed counterpart (Fig. 5B).

We performed a second set of DNA inoculations in which HPV-16 DNA was inoculated directly into four human foreskin tissues before the tissues were grafted onto four recipient

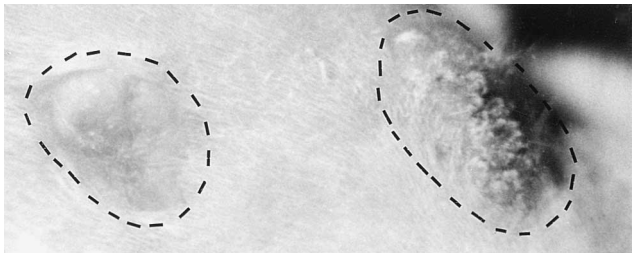

FIG. 2. Macroscopic papillomatous appearance of an HPV-16 DNA-infected xenograft. The exophytic, papillary character of the graft on the right indicates a papilloma. The graft on the left appears normal. Both grafts (circled by dashed lines) were derived from the same human foreskin and were inoculated at 20 kV. The photograph was taken 8 weeks after inoculation.

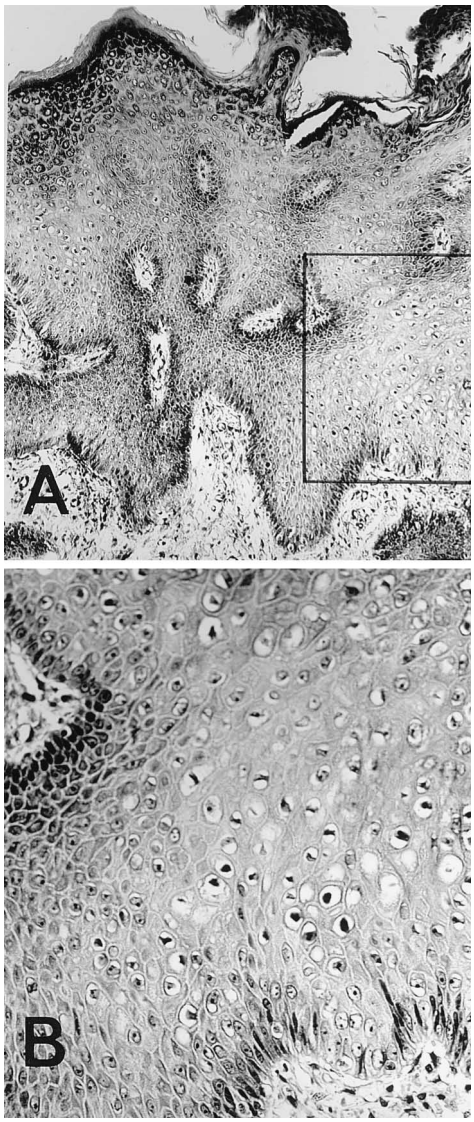

FIG. 3. Histologic examination reveals papilloma development in an HPV-16 DNA-infected xenograft. Papillomatous features include acanthosis, parakeratosis, and koilocytosis. The outlined portion of panel A is shown at a higher magnification in panel B. Note the abundance of koilocytes. Hematoxylin-eosin stain was used. Magnifications, ×10 (A) and ×25 (B).

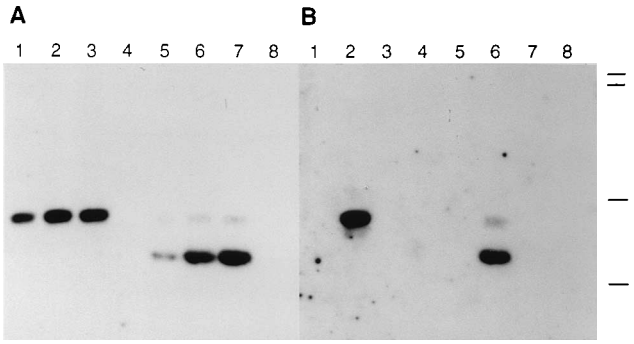

FIG. 4. HPV-16 DNA-induced experimental papilloma contains HPV-16 DNA. Restriction endonuclease analysis and Southern blot hybridization demonstrate that the papilloma shown in Fig. 2 and 3 contains HPV-16 DNA. DNA was extracted from paraffin sections of the lesion and amplified by PCR with papillomavirus L1 consensus primers MY09 and MY11. The 450-bp PCR product was cleaved with *Pst*I and *Hin*fI. Southern blots were prepared and hybridized to type-specific oligonucleotide probes for HPV-16 and HPV-18 that had been labeled with ^{32}P by using a random prime reaction kit (Bethesda Research Laboratories, Gaithersburg, Md.). PCR products from the following sources were loaded: experimental papilloma (lanes 1 and 5), a mixture of HPV-16 and HPV-18 plasmids (lanes 2 and 6), an HPV-16 plasmid (lanes 3 and 7), and a CRPV plasmid (lanes 4 and 8). DNAs were digested with *Hin*fI (lanes 1 to 4) or *Pst*I (lanes 5 to 8). The molecular size markers (from the top) on the right are 2,322, 2,027, 564, and 125 bp. The autoradiograms show hybridization to type-specific probes for HPV-16 (A) and HPV-18 (B).

mice. Four of eight grafts inoculated prior to engraftment survived (Table 1). None developed obvious HPV lesions.

Taken together, these analyses demonstrate that HPV-16 DNA can induce papillomatous transformation of established human foreskin xenografts. The full life cycle of virus replication may have been achieved, as suggested by the observation of capsid antigen expression in infected tissue. The results also show that human foreskin contains all of the species-specific elements required for HPV-16 DNA to induce clinical disease.

Seventy distinct HPVs have been cloned, of which many have been associated with human cancers (reviewed in references 13, 20, 45, and 60). Because the model system described herein does not require infectious virus, it should be applicable to several of the cloned isolates and permit the clinical effects of different HPVs, e.g., high risk versus low risk, to be compared in duplicate skin samples from the same host. Numerous site-directed mutations of HPV genes have been constructed and characterized in vitro. These and other mutations can be incorporated into a full-length viral genome and tested in the model to determine which gene functions cause actual disease. Identification of viral gene functions that are critical for disease induction or progression will then suggest avenues for prophylactic and therapeutic intervention. This model should also facilitate in vivo testing of antiviral and antitumor therapies prior to clinical trials (3, 4, 35).

In the analogous CRPV system, we and others have shown that the E6 and E7 transforming genes (9, 36, 55) and the E1 and E2 regulatory genes (55) are each required for papilloma formation in domestic rabbits and that the putative E5 gene is not (10, 36). We have also shown that papilloma formation does not require the retinoblastoma protein-binding function of the CRPV E7 protein (12) but does appear to require another, uncharacterized function located upstream of the pRB-binding domain (9, 55).

The model has other potential benefits. It could expedite the study of HPV replication and, as has been accomplished for CRPV with CRPV DNA in cottontail rabbits (8), might prove useful for producing virus stocks from molecular clones. The model may also allow investigation of the human immune responses to HPVs (3), since allografts of human lymphocytes and other components of the immune system can function in *scid* mice (24, 37). It remains to be seen whether the model will be practical for investigating HPV-induced malignant conversion (4, 38). Immunodeficiency in women, including those with human immunodeficiency virus infection, has been associated with a significantly increased risk of cervical cancer (51). CRPV-infected rabbit skin grafts on athymic mice can undergo malignant conversion within 4 months (25), and it is possible that latency could be even shorter in *scid* mice because of their severe immunodeficiency.

HPVs fall within a larger class of human epitheliotropic viruses, and it is envisioned that viral DNAs other than HPV DNA may be able to initiate infection of appropriate human

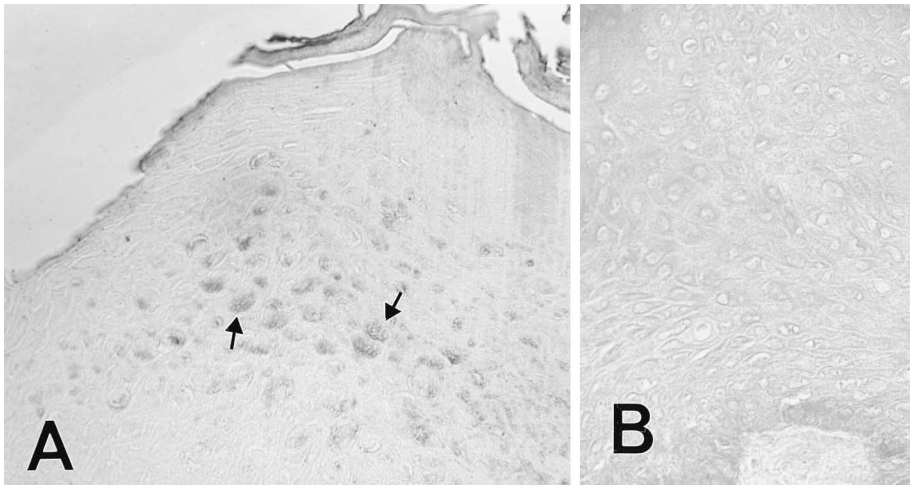

FIG. 5. Immunohistochemical analysis demonstrates papillomavirus capsid antigen in an HPV-16 DNA-transformed xenograft. Maturing epithelial cells of the transformed graft contain stained nuclei which are reactive with the antibody, indicating the presence of HPV capsid proteins. Two such cells are marked by arrows (A). The untransformed graft from the same foreskin was negative (B). Immunohistochemical analysis was performed by using the immunoperoxidase technique and a rabbit polyclonal antiserum that recognizes papillomavirus genus-specific structural antigens (Dakopatts, Accurate Chemical & Scientific Corp., Westbury, N.Y.). Sections were lightly counterstained light green.

skin xenografts. This approach may enable the development of much-needed animal models of other viruses that target human skin, such as Epstein-Barr and dengue viruses.

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