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Differential *In Vitro* Immortalization Capacity of Eleven, Probable High-Risk Human Papillomavirus Types

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Epidemiological studies identified 12 high-risk HPV (hrHPV) types and 8 probable/possible hrHPV types that display different cancer risks. Functional studies on transforming properties of hrHPV are mainly limited to HPV16 and -18, which induce immortalization of human foreskin keratinocytes (HFKs) by successive bypass of two proliferative life span barriers, senescence and crisis. Here, we systematically compared the *in vitro* immortalization capacities, as well as influences on p53, pRb, hTERT, growth behavior, and differentiation capacity, of nine hrHPV types (HPV16, -18, -31, -33, -35, -45, -51, -52, and -59), and two probable hrHPV types (HPV66 and -70). By retroviral transduction, the respective E6/E7 coding sequences were expressed in HFKs from two or three independent donors. Reduced p53 levels and low-level hTERT expression in early-passage cells, as seen in HPV16-, -31-, -33-, and -35-, and to a lesser extent HPV18-transduced HFKs, was associated with continuous growth and an increased immortalization capacity. Less frequent immortalization by HPV45 and -51 and immortalization by HPV66 and -70 was preceded by an intervening period of strongly reduced growth (crisis) without prior increase in hTERT expression. Immortalization by HPV59 was also preceded by a period crisis, despite the onset of low hTERT expression at early passage. HPV52 triggered an extended life span but failed to induce immortality. Variations in p53 and pRb levels were not correlated with differences in alternative E6/E7 mRNA splicing in all hrHPV-transduced HFKs. On collagen rafts, transductants showed disturbed differentiation reminiscent of precancerous lesions. In conclusion, *in vitro* oncogenic capacities differ between the established hrHPV types, and both some established and probable hrHPV types display weak or moderate immortalization potential.

Cervical cancer, the third most common cancer among women worldwide, is caused by a persistent infection with certain types of human papillomavirus (HPV) (1, 2). Most HPV infections are transient and are cleared within 1 to 2 years. However, a fraction of infections eventually give rise to either squamous cell carcinoma (SCC) or adenocarcinoma (AdCA) of the cervix. SCCs develop from so-called cervical intraepithelial neoplasia (CIN) precursor lesions. Low-grade CIN lesions (CIN1) mostly reflect a productive infection in which viral replication and virion production are linked to the differentiation program of the epithelium. High-grade CIN lesions (CIN2/3) mainly represent transforming infections and are characterized by deregulated expression of the early viral E6 and E7 genes in the proliferating basal cells of the epithelium (3, 4).

HPV types belonging to the alpha (α) genus can infect the cervical mucosa (5) and are classified into low-risk (lrHPV) and high-risk (hrHPV) HPV based on their association with malignancy (6). Infections with lrHPV types, e.g., HPV type 6 (HPV6) and HPV11, are associated with benign warts or low-grade lesions, whereas infections with hrHPV can give rise to high-grade CIN lesions and carcinoma. According to epidemiological and biological criteria, 12 HPV types have now been consistently classified as high risk (i.e., HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -59 [International Agency for Research on Cancer {IARC} group 1]). Another 8 types have been classified as probable or possible high risk (i.e., HPV26, -53, -66, -67, -68, -70, -73, and -82 [IARC group 2A/B]) (7). Due to the rare detection of the latter group of types in cervical cancers, there is very limited or no evidence of their carcinogenicity.

Longitudinal studies have revealed that even established hrHPV types confer different risks of CIN3 or cancer. Infections with the hrHPV types 16, 18, 31, 33, 35, 45, 52, and 58 comprise the highest long-term (>5-year) risk of CIN3 or higher lesions, whereas most of the other hrHPV types present an almost negligible long-term risk (8–10). Furthermore, a large, randomized, controlled prospective trial conducted in the Netherlands has revealed that among hrHPV-positive women with normal cytology or borderline to moderate dyskaryosis, those infected with hrHPV types 16, 18, 31, and 33 have an increased 18-month risk of CIN3 or cancer (8). Apart from type-specific differences in viral persistence (9), these and other data also point to the existence of different oncogenic properties of the various high-risk types.

Although some non-HPV16 and -18 types recently received some attention (11–14), functional studies on transforming properties of hrHPV are mainly limited to HPV16 and HPV18. We and others have previously demonstrated that HPV16 and HPV18 can induce immortalization of primary human foreskin keratinocytes (HFKs) by successive bypass of two proliferative life span barriers, i.e., senescence and crisis (reviewed in reference 15). Bypass of senescence relies primarily on the expression of the virus-encoded oncoproteins E6 and E7, which act synergistically by deregulating apoptosis and the cell cycle of the infected cell, respectively. E6 inactivates the tumor suppressor p53 gene, resulting in loss of p53-mediated apoptosis, as well as a number of other targets, such as BAK, Dlg, Magi-1, and hScrib (16). The retinoblastoma protein (pRb) and family members (e.g., p107 and p130) are inactivated

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by the viral oncogene E7 (reviewed in reference 17). Upon bypass of senescence, cells begin an extended though still limited life span. Subsequent immortalization may depend on the HPV type and/or, in part, also upon culture conditions and may be infrequent and preceded by an evident second proliferative life span barrier, crisis, resulting from extremely eroded chromosome ends (18, 19). In most instances, immortalization is characterized by activation of the telomere-lengthening enzyme telomerase, resulting from upregulated expression of the telomerase catalytic subunit hTERT (20, 21).

E6 and E7 are expressed from a bicistronic mRNA and, in the case of hrHPV, are subject to alternative splicing, leading to several truncated versions of E6 named E6*I to E6*IV and E7 (22–24). Whether splicing of E6 enhances the translation efficiency of E7 is still controversial. Whereas in some studies an increase in the efficiency of E7 translation was reported (25–27), in other studies, E7 is translated equally efficiently from spliced and unspliced transcripts (28, 29). Nevertheless, E6* has been found to function independently of E6 in the transformation process (30, 31).

To date, a limited number of comparative *in vitro* studies on the oncogenic capacity of high-risk and probable high-risk HPV types have been described, most of which lack a comprehensive comparison of multiple molecular and growth characteristics in combination with genetic background dependency.

This study aimed to analyze the *in vitro* immortalization capacities of the E6/E7 genes of 11 (probable) hrHPV types. HFKs isolated from independent donors were transduced with the E6/E7 open reading frame of hrHPV16, -18, -31, -33, -35, -45, -51, -52, and -59 and the probable hrHPV types 66 and 70. Low-risk HPV11 and an empty vector were included as negative controls. We closely monitored stably transduced HFKs for their p53, pRb, and $p16^{INK4A}$ status; their growth behavior in culture; and immortalization by analyzing hTERT expression and telomerase activation and their differentiation potentials on organotypic raft cultures.

MATERIALS AND METHODS

Generation of retroviral constructs. The E6 and E7 open reading frames of HPV11, -16, -18, -31, -33, -35, -45, -51, -52, -59, -66, and -70 were amplified using Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). PCR products containing an XhoI and a NotI restriction site and a Kozak sequence were first cloned into a pCR-Blunt II-TOPO vector (Invitrogen/Life Technologies, Carlsbad, CA). The E6/E7 fragments were subsequently inserted into the XhoI and NotI restriction sites of the retroviral vector LZRS-MS-IRES-NEO/pBR (LZRS) (kindly provided by J. Collard, NKI, Amsterdam, The Netherlands). All intermediate HPV E6/E7 pCR-Blunt II-Topo and LZRS constructs generated were confirmed by DNA sequence analysis and compared to the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html). Sequencing analysis of the E6E7 genes revealed no mutations for HPV11 (accession no. FR872717), HPV16 (accession no. K02718), HPV18 (accession no. EF202155), HPV31 (accession no. J04353), HPV33 (accession no. M12732), HPV35 (accession no. M74117), and HPV45 (accession no. X74479). In HPV51 E7 (accession no. M62877), one mutation (S66L) and two silent mutations (T79T and L91L) were observed. HPV52 (accession no. X74481) contains one silent mutation (L83L), one mutation in E6 (K93R), and two silent mutations in E7 (L67L and Q83Q), and HPV59 (accession no. X77858) has two silent mutations (S89S and D116D) in E6 and three mutations in E7 (L19G, L20F, and H95R). Two silent mutations (L43L and Thr146Thr) were observed in HPV66 E6 (accession no. M75123) and one mutation (N100D) in HPV70 E6 (accession no. U21941). HPV E6/E7 containing LZRS constructs were transfected into the 293T-based Phoenix amphotropic packaging cell line, and after selection with puromycin, recombinant helper retroviruses were retrieved as described previously (32).

Retroviral transduction and cell culture. All cells were grown at 37°C and 5% CO_2. HFKs were isolated from the foreskins of three independent donors as described previously (21). After two initial passages, HFKs were transduced with amphotropic retroviruses expressing the HPV E6/E7 oncogenes using 15 μg/ml Polybrene (Life Technologies, Breda, The Netherlands). Geneticin selection (80 μg/ml; Life Technologies) was performed 48 h after transduction. Primary HFKs and transductants were cultured in defined keratinocyte serum-free medium (SFM) (Life Technologies) containing 5 ng/ml epidermal growth factor (EGF) and 50 ng/ml bovine pituitary extract, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter L-glutamine (Life Technologies) and grown to subconfluence until passage. The splitting dates and dilutions were recorded to generate growth curves and proliferation characteristics. Exponentially growing cells were harvested every few passages, and the cell pellets were stored at −80°C. DNA was isolated by proteinase K digestion followed by UltraPure Phenol:Chloroform:Isoamyl Alcohol (Life Technologies) extraction (33). Total RNA was isolated using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions.

SiHa cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter L-glutamine (all from Life Technologies).

HPV DNA and RNA analysis. Throughout culturing, DNA was isolated from all transductants to verify the presence of the correct HPV type using HPV type-specific E7 primers. Using a quantitative PCR (qPCR) for the viral backbone targeting the LZRS sequence and beta-globin as a reference, the quantities of ectopic DNA were measured.

For E6 and E7 expression analysis, 500 ng of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and used for cDNA synthesis with specific reverse primers. For each sample, a reaction without reverse transcriptase (RT−) was run in parallel to control for DNA contamination. A quantitative RT-PCR for E7 was performed using a newly developed quantitative HPV detection method, which allowed the analysis of all HPV types under study, except for HPV70 (A. T. Hesselink, J. Berkhof, M. L. van der Salm, A. P. van Splunter, T. H. Geelen, F. J. van Kemenade, M. G. B. Bleeker, and D. A. M. Heideman, unpublished data). As a reference, we performed a qRT-PCR for snRNP, as described previously (34).

hTERT expression analysis and telomerase activity. For hTERT mRNA expression analysis, 500 ng of total RNA was treated with RQ1 RNase-free DNase (Promega), and qRT-PCR was performed as described previously (35).

Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISAPlus kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Beta-galactosidase staining. Senescent cells were detected by staining for acidic β-galactosidase (36). Cells were fixed in 3% formaldehyde in phosphate-buffered saline (PBS), washed with PBS, and stained with 40 mM citric acid-sodium phosphate buffer (pH 6), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM sodium chloride, 2 mM magnesium chloride, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37°C in the absence of CO_2.

Protein extraction and Western blotting. Total cellular protein extracts were derived from exponentially growing cells as described previously (37). Fifteen micrograms of protein lysates was electrophoresed on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Antibodies anti-p53 (clone D07; Dako, Glostrup, Denmark; 1:1,000), anti-pRb (clone 4H1; Cell Signaling Technology, Beverly, MA, USA; 1:1,000), β-actin (Cell Signaling Technology; 1:1,000), and anti-$p16^{INK4A}$ (G175-1239; BD Pharmingen; 1:1,000) were incubated in 3% dry milk in PBS containing 0.05% Tween 20 at 4°C overnight. For detection, membranes were

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incubated with the appropriate horseradish peroxidase-conjugated secondary rabbit anti-mouse (p0260; Dako; 1:1,000) and swine anti-rabbit (p0217; Dako; 1:1,000) antibody for 1 h, and protein levels were visualized with enhanced chemiluminescence (ECL) (GE Healthcare, Buckingham, United Kingdom).

Organotypic raft culture system and immunohistochemical staining. Organotypic raft cultures of untransduced HFKs and HPV E6/E7-transduced cells were performed as described previously (38). For all cultures, duplicate raft cultures were performed. Briefly, mouse fibroblast (J2 3T3) cells were seeded in a dermal equivalent. The raft culture medium contained DMEM-Ham's F-12 (3:1), 10% fetal calf serum (Life Technologies), hydrocortisone (0.4 μg/ml), 0.1 nM cholera toxin, transferrin (5 μg/ml), insulin (5 μg/ml) (all from Sigma), and human epidermal growth factor (0.5 ng/ml; Life Technologies). The rafts were harvested after 9 days, fixed in 10% saline-buffered formalin, paraffin embedded, and subjected to immunohistochemical staining.

Immunohistochemical staining was performed on 4-μm sections, which were deparaffinized and rehydrated. Following antigen retrieval with citrate buffer (pH 6; 800 W; 10 min), the slides were incubated for 30 min in 3% H_2O_2 in methanol and incubated overnight with MIB/Ki-67 (M7240; Dako; 1:40) and cytokeratin 10 (NCL-CK10; Novo Castra Laboratories; 1:200). For detection, the Envision horseradish peroxidase system (Dako) was used. Sections were counterstained with hematoxylin.

RESULTS

Generation of HPV E6/E7-transduced HFKs. To analyze the *in vitro* immortalization capacity of the hrHPV types 16, 18, 31, 33, 35, 45, 51, 52, and 59 and the probable hrHPV types 66 and 70, HFKs were transduced with recombinant retroviruses containing the E6/E7 open reading frames of the respective HPV types. HPV16, -18, -31, -33, -35, -45, and -66 were cloned from full-length HPV plasmids, which contained the prototype, as was confirmed by sequencing analysis. HPV52 was also amplified from a full-length HPV plasmid and contained a sequence variation in E6 (K93R). This HPV52 variant has frequently been detected in cervical cancers in Asia (39, 40). HPV51 and HPV59 E6/E7 were cloned from cervical scrapes, and HPV70 was cloned from a tumor sample. Sequencing analysis revealed nonsilent sequence variations in HPV51 E7 (S66L), HPV59 E7 (L19G, L20F, and H95R), and HPV70 E6 (N100D) relative to the GenBank prototype sequences (41, 42). LrHPV11 and the empty vector (LZRS) were included as negative controls. To control for genetic-background-dependent variations, HFKs isolated from at least two independent individuals were transduced. Cells isolated from the first donor, referred to as donor I, were transduced with all constructs. Cells from the second donor, donor II, were transduced with all constructs, except for HPV33, due to technical problems. Cells from a third donor (donor III) were transduced with HPV16, -33, -35, -45, -51, -52, -59, and -70.

At various passages throughout culturing, the presence of the correct HPV type was confirmed by type-specific DNA PCR analysis (data not shown). Moreover, analysis of viral DNA quantities in all transductants, using qPCR for the vector backbone, revealed relatively minor variations between cell lines, which were generally not donor related and most likely reflect neomycin selection (data not shown).

p53 and pRb status. To examine whether the E6 and E7 oncogenes of the different HPV types display different abilities to degrade p53 and pRb and to reactivate $p16^{INK4A}$, we first verified transgene expression by RT-PCR using HPV type-specific E6 and E7 primer sets. Both E6 and E7 were expressed in the various transductants of all three donors (Fig. 1A). Quantitative RT-PCR analysis for E7 verified comparable levels of viral oncogene expression between donors (Fig. 1B). Only in the case of HPV35 was a relatively low level of expression seen in donor I. HPV70 E7 expression could not be examined using this method.

Western blot results showing p53, pRb, and $p16^{INK4A}$ expression levels at early passage (~passage 8; 30 to 40 population doublings [PDs]) are shown in Fig. 1C. Although some slight variations between the different donors were evident, (probable) hrHPV-transduced cells showed generally decreased p53 levels to various degrees in comparison to untransduced and LZRS- and HPV11-transduced HFKs. In donor I, the lowest p53 levels were observed in HPV16, -31, -33, and -59 transductants, similar to donor III, in which p53 was markedly reduced in HPV16-, -33-, and -59-transduced HFKs (Fig. 1C). In donor II, less variation was evident, and all HPV16-, -18-, -31-, -33-, -45-, -51-, -59-, -66-, and -70-transduced HFKs showed decreased p53 levels.

pRb protein expression was also decreased to various degrees in all (probable) hrHPV transductants compared to controls (Fig. 1C). In some cell lines, an inverse correlation was seen between p53 and pRb degradation. The relatively low E7 expression in HPV35 transductants from donor I compared to donors II and III was not linked to reduced pRb degradation. The reduced pRb expression in donor I cells transduced with HPV11 and untransduced cells from donor II may reflect the onset of senescence in the cells.

To further validate HPV biological activity, we investigated $p16^{INK4A}$ protein levels. HrHPVE7 expression induces increased $p16^{INK4A}$ expression, being either or both pRb-dependent and -independent (43, 44). $p16^{INK4A}$ protein levels were strongly increased in all (probable) hrHPV-transduced HFKs (Fig. 1C). Elevated $p16^{INK4A}$ expression was also observed in HPV11 transductants from donor I and untransduced cells from donor II that had reduced pRb expression.

Splicing of the HPVE6 mRNA. Next, we aimed to determine whether variations in p53, pRb, and $p16^{INK4A}$ levels were related to differences in alternative splicing of the hrHPVE6E7 transcripts in favor of either more full-length E6 template or full-length E7 template. RT-PCR was performed on DNase-treated RNA samples using primers located upstream and downstream of the predicted donor-acceptor splice sites (Fig. 1D). In accordance with previous reports (12, 23), only a single band encoding full-length E6/E7 was observed in HPV11-transduced HFKs. The most prominent product seen in HPV16-, -18-, -31-, -33-, -35-, -45-, -51-, -52-, and -70-transduced HFKs represented an E6*I splice variant that was confirmed by sequencing. In HPV16-, -18-, -31-, -59-, and -66-transduced HFKs also, a full-length E6/E7 transcript was observed, which was most prominent in all HPV59 transductants and HPV66-transduced cells from donor I. Only in the case of HPV16 was an E6*II splice variant seen.

The presence of spliced E6*I transcripts in all (probable) hrHPV-transduced cell lines suggests that the observed variations in p53 and pRb degradation were not (entirely) due to alternative splicing of the E6/E7 transcripts.

Cell culture growth characteristics. To determine how the above-described characteristics correlated with immortalization, cells were closely monitored during culture for their potential to bypass the two proliferative life span barriers, i.e., senescence, which leads to an extended life span, and crisis, resulting in immortalization (Fig. 2A and B).

Based on their culture characteristics, distinct groups could be

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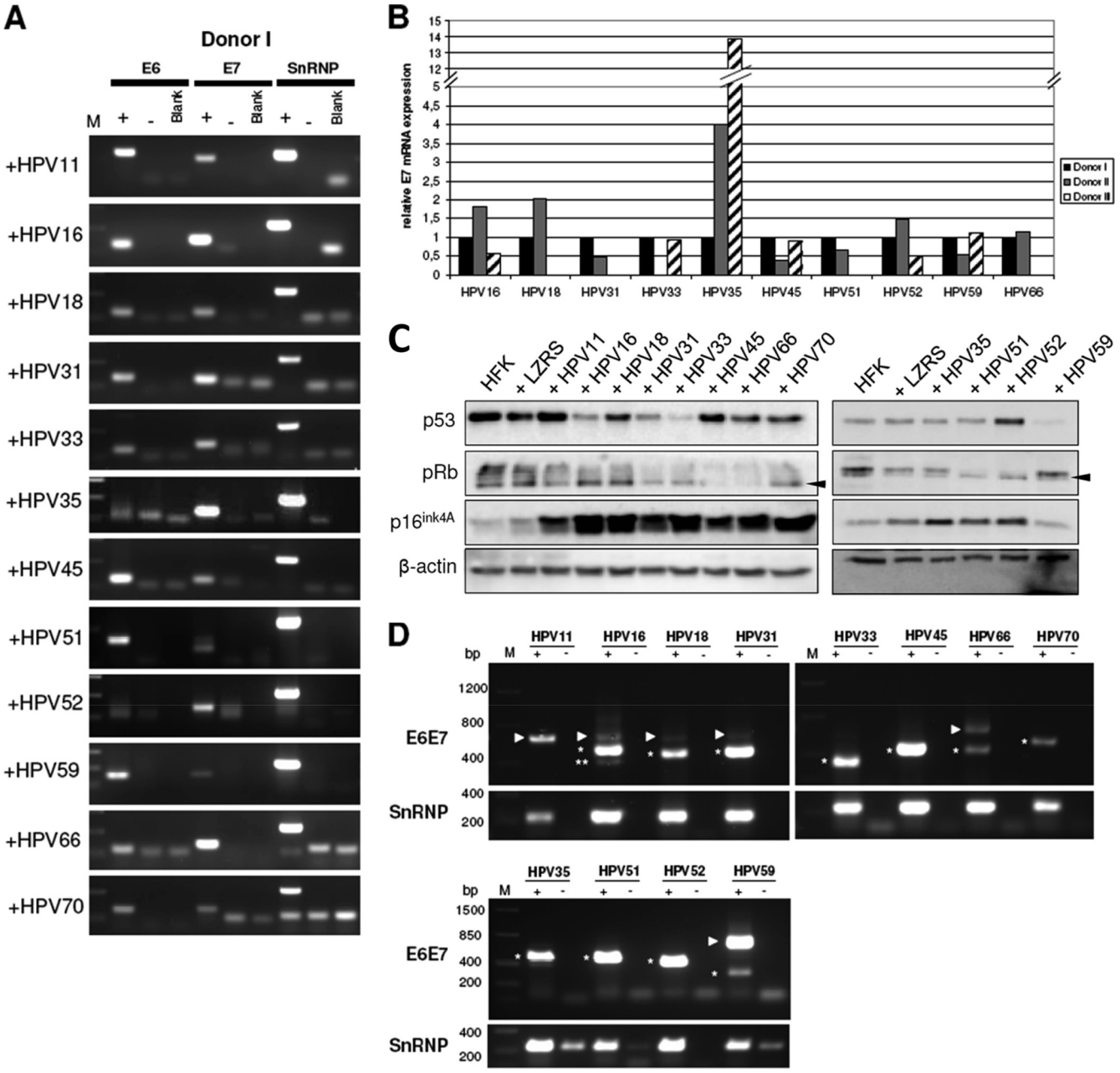

FIG 1 Functional expression of the viral oncogenes E6 and E7 in the various transductants. (A) E6 and E7 mRNA expression assessed by type-specific RT-PCR. RT-negative PCR (−), without reverse transcriptase, served as a negative control, and snRNP expression analysis was included as a housekeeping control. (B) Relative E7 mRNA expression determined by qRT-PCR. HPV51 donor III and HPV70 transductants could not be analyzed. mRNA expression was corrected for the housekeeping gene snRNP and is shown relative to donor I. (C) Steady-state protein levels of p53, pRb, and $p16^{INK4A}$ in preimmortal HFKs transduced with empty vector (LZRS) and HPV11, -16, -18, -31, -33, -35, -45, -51, -52, -59, -66, and -70, as determined by Western blotting. β-Actin expression served as a loading control. (D) E6/E7 mRNA products. Full-length transcripts are indicated by arrowheads, and spliced E6 mRNAs are indicated by asterisks (*, E6*I; **, E6*II). SnRNP served as a housekeeping control. mRNA and protein products of donor I are shown as representative.

discerned. The first group comprised untransduced and LZRS- and HPV11-transduced HFKs, which did not overcome senescence. In more detail, untransduced HFKs from donors I, II, and III entered senescence and eventually died after 38 PDs, 30 PDs, and 33 PDs, respectively (Fig. 2A and B). Cells transduced with empty vector (LZRS) senesced and died after 37, 39, and 40 PDs, respectively, and HPV11-transduced cells died after 43 PDs and 35 PDs for donors I and II, respectively. Morphologically, all untransduced HFKs and HFKs transduced with LZRS and HPV11 contained large, flattened cells, a senescence-related phenotype, upon approaching the last population doublings. Senescence was confirmed by staining for senescence-associated beta-galactosidase (SA-β-Gal) (Fig. 2C).

The second group consisted of HFKs transduced with HPV16, -18, -31, -33, and -35, which showed no signs of senescence or crisis throughout culturing and proliferated continuously in all

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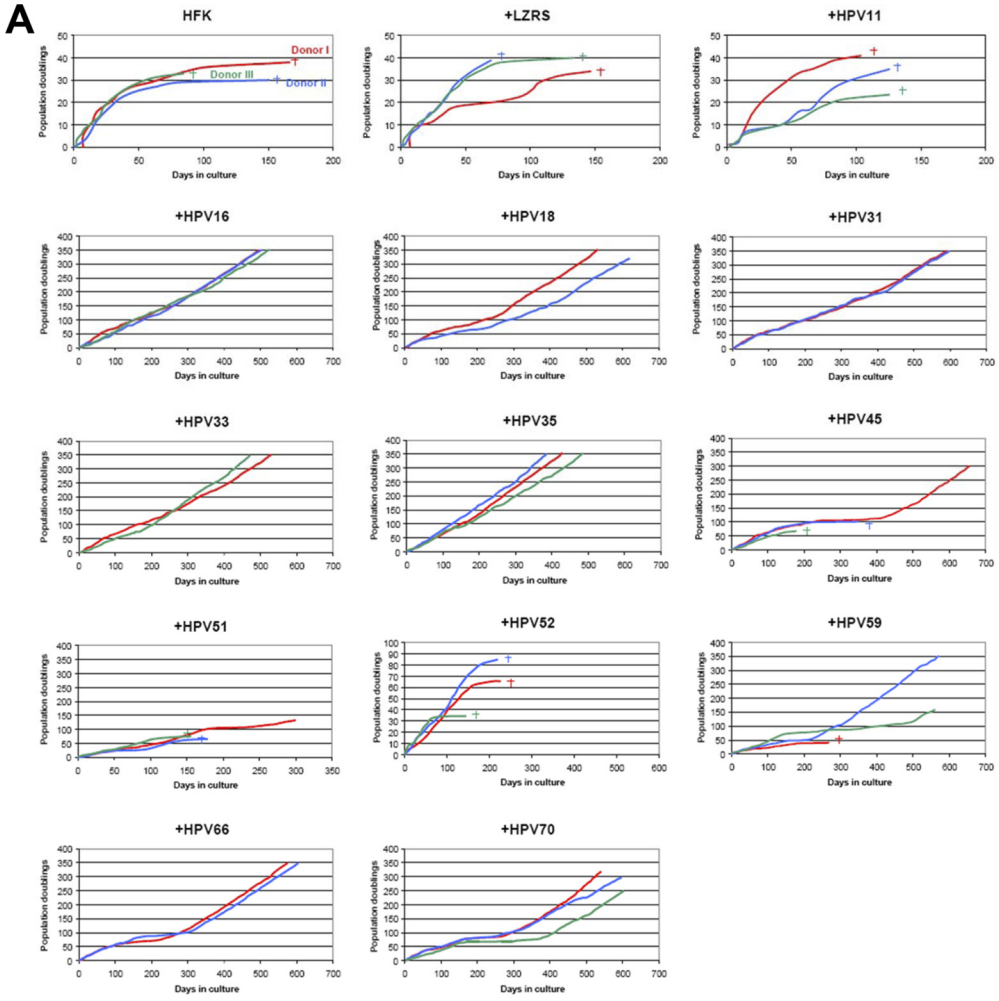

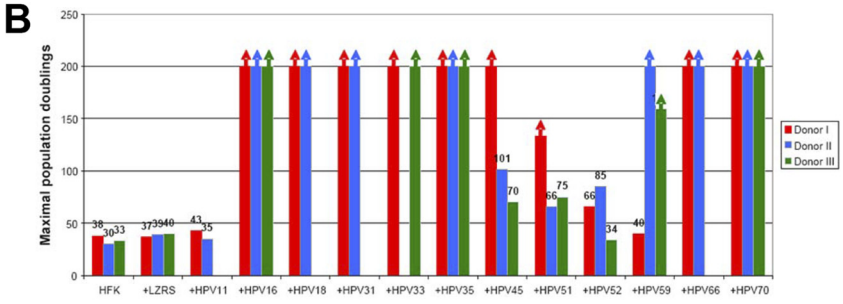

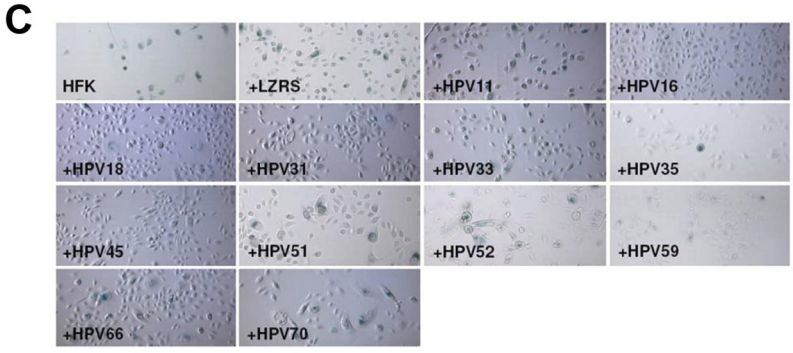

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three donors. Interestingly, these HPV types demonstrated the highest p53 degradation efficiencies. HPV18-transduced cells, which showed somewhat less efficient degradation of p53, encountered slight growth reduction for 61 days between 54 and 76 PDs in donor I and for 70 days between 58 and 69 PDs in donor II. During this period, a mixture of senescent cells and proliferating cells was observed.

HPV45-, -51-, -59-, -66-, and -70-transduced cells comprised the third group. Although these types revealed an abnormal growth pattern, their transductants endured a strong and long crisis period prior to immortalization. In HPV45-transduced HFKs from donor I, senescent cells were seen after 101 PDs, and after 189 days of crisis, islands of small proliferating cells emerged. In donors II and III, crisis was entered at 97 PDs and 60 PDs, and the cells eventually died. In HPV51-transduced HFKs, senescent-like cells appeared after 104 PDs, 62 PDs, and 70 PDs in donors I, II, and III, respectively. Whereas cells started to proliferate after 52 days in donor I, HPV51 transductants of donors II and III eventually died. The HPV59-transduced HFKs revealed senescent cells at 48 PDs and 72 PDs and escaped crisis after 64 and 202 days in donor II and donor III, respectively. In donor I, senescence was entered at 20 PDs, and the cells eventually died. In both HPV66-transduced donors, a strong crisis period was observed. The crisis period in donor I started at 53 PDs and ended after 130 days. Donor II cells transduced with HPV66 entered crisis after 87 PDs, which lasted 112 days. A similar growth pattern was observed in HFKs transduced with HPV70. Those from donors I and II ceased to proliferate after 81 PDs and 78 PDs and escaped crisis after 67 and 94 days, respectively. HPV70-transduced HFKs from donor III entered crisis at 71 PDs, which lasted longer (255 days) and from which a single colony of immortal cells emerged. Crisis-like cells show a senescent morphology, as verified by staining for senescence-associated beta-galactosidase.

The fourth group includes all HPV52-transduced HFKs with an extended life span that was followed by a long crisis period and eventual cell death. HPV52-transduced HFKs from donors I and II entered a strong crisis period after 62 PDs and 81 PDs, respectively. HPV52-transduced cells from donor III entered senescence after 32 PDs, and none of the HPV52-transduced HFKs bypassed crisis. Even upon repeated thawing and culturing of early-passage transductants, no outgrowth of immortal cells was seen.

Immortalization. To find support for the fact that all HPV16-, -31-, -33-, -35-, -66-, and -70-transduced HFKs, as well as one donor's HPV45- and -51- and two donor's HPV59-transduced HFKs that escaped from crisis, were indeed immortal, hTERT mRNA expression and telomerase activation were analyzed (Fig. 3). qRT-PCR analysis revealed no hTERT expression in untransduced HFKs and HFKs transduced with LZRS and HPV11. In cells without an intervening crisis period (HPV16-, -31-, and -33-transduced cells), but also HPV59-transduced cells (with a crisis period), generally low levels of hTERT mRNA were detected at early passage (<44 PDs). These levels progressively increased at 62 to 137 and >150 PDs, respectively. In other transductants with an intervening crisis period (with the exception of HPV52-transduced cells from donor I), i.e., HPV45, -51, -52, -66, and -70, as well as HPV18, transductants, no hTERT expression was observed at early passage (<44 PDs). hTERT expression, however, became apparent as soon as proliferating cells escaped from crisis (depending on the donor, 70 to 100 PDs) and was increased further at >150 PDs.

All immortalized cell cultures showing elevated hTERT mRNA expression tested positive for telomerase activation (Fig. 3, bottom). Weak telomerase activity was seen in early-passage cells from HPV51 donors II and III, HPV52 donor III, and HPV59 donor I, all of which did not become immortal.

Raft cultures of the transductants display dysplastic changes. Since immortalization of HFKs by full-length HPV16 and HPV18 has previously been associated with disturbed differentiation in organotypic raft cultures (38), the differentiation capacities of HPV16, -18, -31, -33, -35, -45, -59, -66, and -70 transductants were assessed at approximately equal passages (90 to 161 PDs) (Fig. 4). Raft cultures of untransduced HFKs closely resembled normal epithelium, showing distinct basal/parabasal, spinous, granular, and cornified layers. Immunohistochemical staining for Ki-67 revealed that proliferating cells were restricted to the basal/parabasal layer of the epithelium. The spinous and granular layers of the untransduced cells stained positive for the differentiation marker cytokeratin 10 (CK10).

Organotypic raft cultures of HPV16 (PD 104), -18 (PD 90), -31 (PD 103), -35 (PD 140), and -66 (PD 97) containing HFKs from donor I; HPV16 (PD 132), -31 (PD 101), -35 (PD 147), -59 (PD 92), -66 (PD 106), and -70 (PD 103) containing HFKs from donor II; and HPV16 (PD 117), -33 (PD 112), and -35 (PD 161) containing HFKs from donor III revealed disturbed differentiation, and no clearly distinct epithelial layers were seen, which was reminiscent of severe dysplasia. Only HPV33 (PD 108)-, -45 (PD 90)-, and -70 (PD 97)-transduced cells from donor I; HPV18 (PD 105)-transduced cells from donor II; and HPV59 (PD 93)-transduced cells from donor III showed abnormal differentiation resembling mild dysplasia. The dysplastic histology of all hrHPV-transduced cells was characterized by the presence of basal-like cells in almost all strata of the epithelium, as indicated by the presence of Ki-67-positive cells in upper cell layers and the absence of CK10 staining. Only the HPV70 (PD 97) transductant of donor I, the HPV18 (PD 105) and -35 (PD 147) transductants of donor II, and the HPV35 (PD 161) and -59 (PD 93) transductants of donor III stained (weakly) positive for CK10. Unfortunately, the HPV70-immortalized HFKs from donor III repeatedly failed to grow on organotypic rafts.

DISCUSSION

In the present study, we systematically compared the viral oncogenic E6/E7 regions of 9 hrHPV types (HPV16, -18, -31, -33, -35, -45, -51, -52, and -59) and 2 probable hrHPV types (HPV66 and

FIG 2 Growth characteristics of HPV-transduced HFKs and controls. HFKs were derived from independent individuals and are depicted in red (donor I), blue (donor II), and green (donor III). (A) HFKs and HFKs transduced with empty vector (LZRS) and HPV11 entered senescence and died. HPV52-transduced HFKs had an extended life span and died. A crisis period with reduced growth was observed in HPV45-, -51-, -59-, -66-, and -70-transduced cells prior to immortalization. HPV16-, -18-, -31-, -33-, and -35-transduced HFKs grew continuously in culture. (B) Maximal numbers of population doublings per individual transductant. The arrows pointing up indicate continuous growth. (C) Representative photographs of HPV-transduced cells and controls (donor I) stained for senescence-associated β-galactosidase. Over 90% of primary cells and cells transduced with LZRS and HPV11 stained blue. Only single hrHPV E6/E7-transduced keratinocytes stained blue. The same magnification (×10) was used for all photomicrographs.

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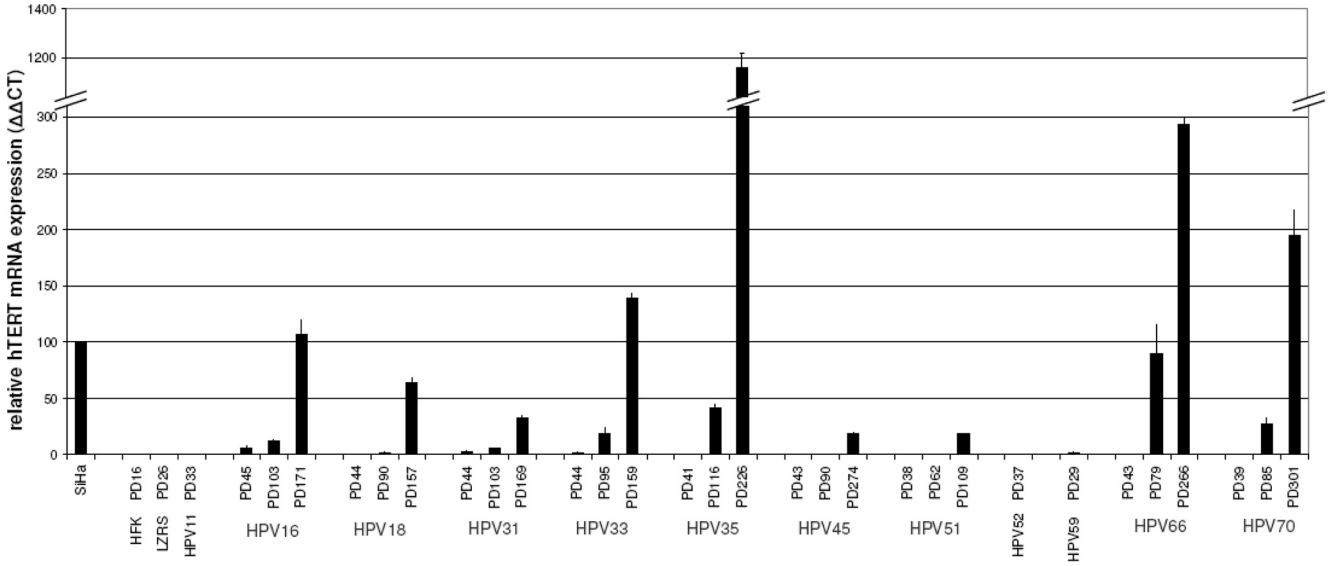

Telomerase

Donor	HFK	+LZRS	+11	+16	+18	+31	+33	+35	+45	+51	+52	+59	+66	+70
I	-	-	-	+	+	+	+	+	+	+	-	+/-	+	+
II	-	-	-	+	+	+	NA	+	-	+/-	-	+	+	+
III	-	-	NA	+	NA	NA	+	+	-	+/-	+/-	+	NA	+

FIG 3 (Top) Relative hTERT mRNA expression levels as determined by quantitative reverse transcriptase PCR in cells at a preimmortal stage (16 to 44 PDs), at 62 to 116 PDs (precrisis in the case of HPV45 and postcrisis in the cases of HPV51, -66, and -70 transductants), and at an immortal stage (157 to 301 PDs). Shown is a representative experiment performed in duplicate on donor I. The error bars indicate standard deviations. (Bottom) Telomerase activity as determined using the TeloTAGGG Telomerase PCR ELISAPlus kit (Roche, Mannheim, Germany). +, telomerase positive; +/−, weakly telomerase positive; −, telomerase negative; NA, not applicable.

-70) for their effects on known E6 and E7 targets, such as p53, pRb, $p16^{INK4A}$, and hTERT (17), as well as their immortalization capacities. Almost all (probable) hrHPV types, with the exception of HPV52 (alpha 9), were able to induce immortalization of HFKs, albeit with different efficiencies, as schematically represented in Fig. 5. E6/E7 of the closely related types HPV16, -31, -33, and -35 of the alpha 9 genus and, to a lesser extent, HPV18 (alpha 7 genus) displayed the highest growth-promoting activities early during the transformation process, with no signs of senescence or crisis throughout culture. This was generally accompanied by prominent E6 activity exemplified by relatively low p53 levels compared to other types and early onset of hTERT expression. HFKs transduced with HPV45, -59, and -70 (alpha 7); HPV51 (alpha 5); and HPV66 (alpha 6) encountered a long crisis period prior to immortalization. These types, with the exception of HPV59, showed no hTERT expression in preimmortal transductants. Growth characteristics were not related to the relatively small differences seen in transgene DNA copy numbers and expression levels. Overall, our Western blot results are in line with earlier reports showing p53 and pRb degradation by (probable) hrHPV types, but not by HPV11 (11–13, 45). Similar to other *in vitro* studies (11, 12), the p53 degradation capacities varied among the hrHPV types. However, type differences are not fully consistent across the different studies, most likely reflecting the different model systems and assays used. In a comparative study by Hiller et al. (11) using *in vitro*-translated E6, almost equal degradation efficiencies of p53 were found for HPV16, -18, -33, -35, -39, -45, -51, -52, -53, -56, -58, -66, -70, and -82. Using a more quantitative approach, HPV58 and -59 E6 proteins were found to be the most potent types, and HPV56 and -66 showed the least p53 degradation activity (12). HPV16, -18, -31, -33, and -35 had slightly lower p53 degradation capability than HPV45, -51, -52, -59, and -70 (12). The discrepancies with the present study may, at least in part, be explained by E6 being expressed from a bicistronic E6/E7 mRNA, which is subject to alternative splicing, potentially affecting E6 activity. HPV18 E6*I has been demonstrated to counteract full-length E6 in p53 degradation (30). In the study by Mesplede et al. (12) using HPV E6-transfected cells, splicing was observed in HPV16, -18, -31, -33, -35, -51, -66, and -70 E6-, but not in HPV45, -52, and -59 E6-transfected cells. In all our (probable) hrHPV-transduced cell lines, E6*I expression was detected. Full-length E6 was most prominent in HPV66 from donor I and all three HPV59 transductants, which was associated with more p53 degradation in HPV59-, but not in HPV66-transduced HFKs. These data suggest that differences in alternative splicing do not fully account for the variations in p53/pRb degradation and immortalization capacities. The latter most likely depend on potential type-dependent interactions with other cellular targets, as well (46).

E6-mediated activation of telomerase via hTERT promoter activation, as originally demonstrated for HPV16 (47–49), has recently also been demonstrated for other HPV types using luciferase assays and was found to be HPV type dependent. With respect

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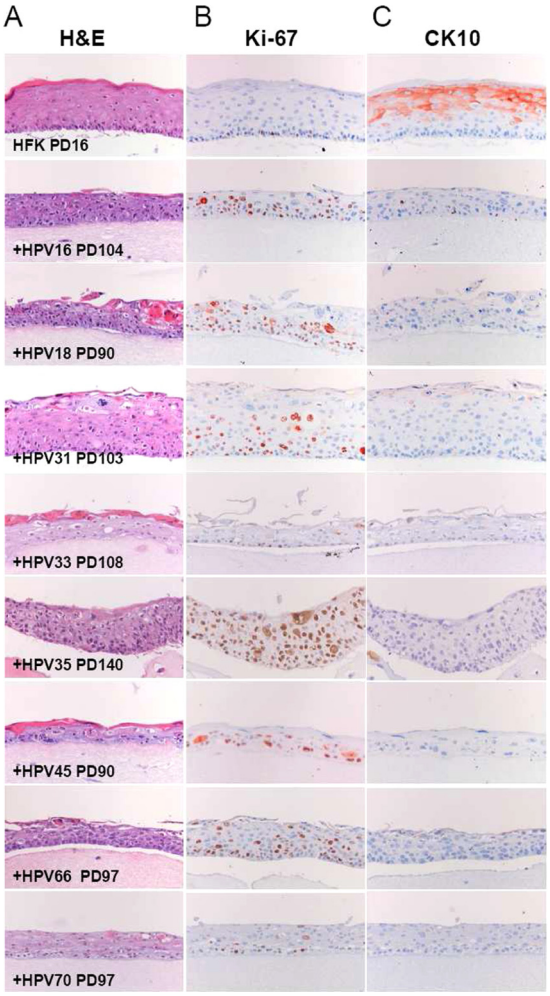

FIG 4 Organotypic raft cultures of untransduced and HPV16-, -18-, -31-, -33-, -35-, -45-, -66-, and -70-transduced HFKs. (A) H&E (hematoxylin and eosin) staining showing raft culture morphology. (B and C) Immunohistochemical staining on sections of the same cultures as in panel A were performed for the proliferation marker Ki-67 (B) and the differentiation marker CK10 (C). Raft cultures were performed in duplicate, and one raft per HPV type from donor I is shown as representative.

to the types studied by us, HPV16, -33, -35, -51, and -52 E6 proteins showed the highest hTERT promoter activation capacities, followed by HPV18 and -31. HPV11, -66, and -70 E6 proteins displayed no elevated hTERT activation, and HPV45 and -59 were not tested (50). In our study, hTERT expression was primarily found to be directly upregulated, albeit still at a relatively low level, in cell lines in which no intervening crisis period was observed, i.e., upon HPV16, -31, -33, or -35 transduction, as well as upon HPV59 transduction. This implies that upon stable transduction and in the presence of naturally occurring splice products, the E6 proteins of HPV16, -31, -33, -35, and -59 can induce low levels of hTERT transcription themselves, whereas the E6 proteins of HPV45, -51, -52, -66, and -70 may lack this ability. The major discrepancy between our results and the study by Van Doorslaer and Burk (50) concerns HPV51 and -52, which may be related to E6*I expression being dominant in the present study. The fact that in all cell lines hTERT expression levels increased upon passaging indicates that, besides HPV, additional events contribute to telomerase activation and immortalization, in line with previous observations (51–53). Recent studies indicate that telomerase-independent functions of hTERT also contribute to HPV-induced immortalization of HFKs; among others, they involve bypass of senescence by regulation of a gene set similar to that regulated by hrHPV E6 (54). How the increasing hTERT expression levels with passaging correlate with its telomeric and nontelomeric functions is currently unclear.

Whereas HPV16, -18, -31, -33, and -35 induced immortalization in all donors, immortalization by HPV45 was infrequent. Although this observation was surprising given its relatively high prevalence in cervical cancer worldwide (7), one may speculate that HPV45 is more effective in the induction of subsequent transformation events, such as anchorage-independent growth and colony formation, as was shown for HPV18 compared to HPV16 (55).

Infrequent induction of immortalization by HPV51, on the other hand, is in line with epidemiological data showing a strong decrease in prevalence with the progression of cervical disease (56).

HPV52 invariably induced an extended life span but failed to

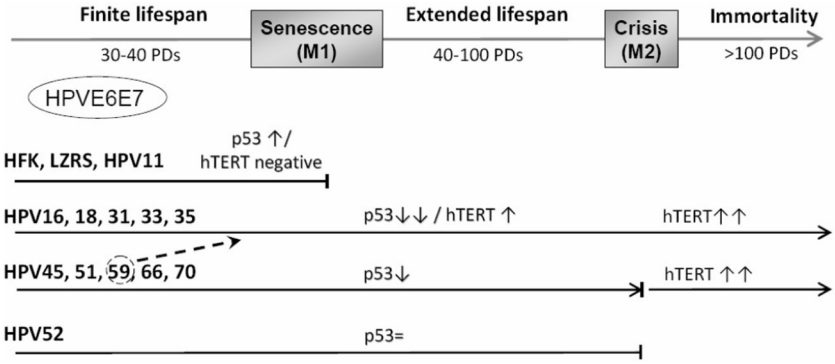

FIG 5 Schematic representation of the immortalization characteristics of untransduced and empty-vector (LZRS)- and HPV11-, -16-, -18-, -31-, -33-, -45-, -51-, -52-, -59-, -66-, and -70-transduced HFKs. Control transductants (HFKs, LZRS, and HPV11) were unable to degrade p53, showed no hTERT expression, entered senescence (M1), and died. Immortalization of HFKs transduced with HPV16, -31, -33, and -35 and, to a lesser extent, HPV18 was reached without an intervening crisis period (M2).Those cells generally showed strong p53 degradation and early onset of hTERT expression. HPV45-, -51-, -59-, -66-, and -70-transduced HFKs became immortal following a strong crisis period and, with the exception of HPV59, generally showed less efficient p53 degradation. hTERT expression was evident following escape from crisis. HPV52-transduced cells without obvious p53 degradation remained hTERT negative and had an extended life span but failed to induce immortalization.

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induce immortality. The E6 K93R variant in our construct is present in 98% of the HPV52-positive cervical cancers in Japan (39, 40, 56) and has been significantly associated with an increased CIN2/3 risk, suggesting that it represents a carcinogenic variant (57), yet the findings that p53 expression levels were unaltered and immortalization was unsuccessful in our hands indicate that this highly common variant is not as oncogenic as other hrHPV types, at least in a Caucasian genetic background.

HPV59, which is less prevalent in invasive cervical cancers (7), showed a better immortalization capacity. Despite three sequence variants in E7, the observed pRb degradation and $p16^{INK4A}$ upregulation indicates E7 activity. We cannot rule out the possibility that these variants render HPV59 more carcinogenic, as was shown for the Asian-American HPV16 E6 variant AAE6 (amino acid changes Q14H/H78Y/L83V) compared to the prototype (58, 59).

All donors transduced with hrHPV66 and HPV70 encountered a long crisis period, followed by the escape of single colonies of telomerase-positive cells. These findings are in accordance with earlier studies showing successful immortalization of primary keratinocytes by full-length HPV66 and HPV70 E6/E7 (11, 14). This study, however, is the first to systematically demonstrate that these probable hrHPV types display a reduced immortalization capacity compared to the established hrHPV types 16, 18, 31, 33, and 35. Unfortunately, no proper comparison to the various published studies can be made, given the fact that, besides varying culture conditions, most studies lack a comparative analysis between multiple HPV types and/or used a single keratinocyte isolate.

The reduced immortalization capacity of the hrHPV types 45, 51, 52, and 59 and the probable hrHPV types 66 and 70 may in part explain their rare detection in CIN3 lesions (15, 18), though the overall prevalence and potentially more efficient clearance by the immune system can also be important determinants. Diverse mechanisms of interference with immune surveillance and immune escape of HPV-induced lesions have been described, such as interference with interferon response, downregulation of major histocompatibility complex (MHC) class I and TAP-1, and control of Langerhans cell density (60–62). However, the potential existence of type-dependent mechanisms of immune escape and the role of the tumor microenvironment are still largely unexplored. Furthermore, we cannot rule out the possibility that the oncogenicity of HPV45, -51, -52, and -59 is epithelium dependent, although no such cell type dependency was demonstrated for HPV11, -16, -18, and -31 (55).

The finding that HPV16, -18, -31, -33, and -35 displayed the highest immortalization efficiency in a donor-independent manner corresponds to epidemiological studies in which HPV16, -18, -31, and -33 revealed the highest 18-month risk of developing into CIN3 or higher lesions and an increased prevalence in cervical cancers (7, 8, 56). The reduced immortalization efficiency of other types, like HPV45, -51, -52, -59, -66, and -70, reflected by a long crisis period and donor-dependent outgrowth of immortal cells, underlines the value of comparative analysis in multiple donors. Most studies so far have made use of a single HFK donor.

Shortly after immortalization, all hrHPV-transduced keratinocytes showed disturbed differentiation on organotypic raft cultures, and most were histologically classified as severe dysplasia, reminiscent of high-grade CIN, indicating that histomorphology is type independent, whereas molecular phenotypes may be type dependent (63).

These *in vitro* models not only enable a systematic comparison of the immortalization and transformation capacities of (probable) hrHPV types, but will also aid our understanding of the type-dependent molecular mechanisms of transformation.

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Differential *In Vitro* Immortalization Capacity of Eleven (Probable) High-Risk Human Papillomavirus Types

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