厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

ヒトパピローマウイルスを標的とする発がん予防の研究

平成２２年度～２５年度　総合研究報告書

Ⅲ　研究成果の刊行物・別冊
その３

研究代表者　温川　恭至

平成２６（２０１４）年　５月

Ⅲ　研究成果の刊行物・別冊

その３

平成２４年度

JVI
Journals.ASM.org

The E1 Protein of Human Papillomavirus Type 16 Is Dispensable for Maintenance Replication of the Viral Genome

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Papillomavirus genomes are thought to be amplified to about 100 copies per cell soon after infection, maintained constant at this level in basal cells, and amplified for viral production upon keratinocyte differentiation. To determine the requirement for E1 in viral DNA replication at different stages, an E1-defective mutant of the human papillomavirus 16 (HPV16) genome featuring a translation termination mutation in the E1 gene was used. The ability of the mutant HPV16 genome to replicate as nuclear episomes was monitored with or without exogenous expression of E1. Unlike the wild-type genome, the E1-defective HPV16 genome became established in human keratinocytes only as episomes in the presence of exogenous E1 expression. Once established, it could replicate with the same efficiency as the wild-type genome, even after the exogenous E1 was removed. However, upon calcium-induced keratinocyte differentiation, once again amplification was dependent on exogenous E1. These results demonstrate that the E1 protein is dispensable for maintenance replication but not for initial and productive replication of HPV16.

Papillomaviruses (PVs) are small, double-stranded DNA viruses that infect stratified squamous epithelium. Human PVs (HPVs) are very important causative agents for various lesions, ranging from verrucas to cancer. Among them, a subset of HPVs, the so-called high-risk types such as type 16 and 18, are associated with more than 90% of all cervical carcinomas as primary etiological factors (45). PVs establish long-term persistent infections in squamous epithelium, and the viral life cycle is tightly linked with the differentiation state of the host keratinocytes (7).

PV genome is replicated and amplified in three different stages: establishment, maintenance, and productive stages of the life cycle. In the establishment stage, soon after infection of the basal layer keratinocytes, a single or a few initial copies of the viral genome amplify and establish residence as multicopy circular extrachromosomal elements (episomes) in the nucleus. In the maintenance stage, the viral genomes in each affected cell replicate approximately once in a cell cycle in proliferating basal layer keratinocytes. Then, in the productive stage, they are exponentially amplified in terminally differentiating keratinocytes and packaged into progeny virions. It is important to understand the molecular mechanisms underlying this triphasic model for development of new therapies against HPV-infected lesions, such as cervical intraepithelial neoplasias, which can progress to cervical cancer.

The regulation of viral DNA replication is thought to differ in these three distinct stages of the viral life cycle. Studies of lesions experimentally induced by rabbit oral papillomavirus (ROPV) infection showed that the genome copy number of ROPV is low in the basal layer and increases up to four orders of magnitude during the terminal differentiation of host keratinocytes (21). This corresponds to more than 13 rounds of continuous replication of the viral genome in the productive stage.

Most of our knowledge of PV replication is derived from short-term replication assays to identify the components required for replication of the viral genome. These transient replication assays suggest that both viral proteins E1, a DNA helicase, and E2, a transcriptional activator and auxiliary replication factor, as well as *cis*-acting elements, including the E1-binding site (E1BS), several E2-binding sites (E2BS), and an AT-rich region in the long control region (LCR), are all essential for efficient PV DNA replication (4, 6, 20, 23, 30, 35, 37, 38). These studies appear to have analyzed the molecular mechanisms of viral replication in the productive stage, though they did not necessarily examine the three stages separately.

In this context, it is of interest that the HPV genome lacking LCR can replicate in the absence of both E1 and E2 proteins in transient replication assays (1, 16, 29), and a temperature-sensitive (TS) E1 mutant of bovine papillomavirus type 1 (BPV1) can be maintained in mouse C127 cells at a nonpermissive temperature as efficiently as the wild-type BPV1 (17). These reports suggest that E1 might be dispensable for maintenance replication.

To test this hypothesis directly, we here used an E1-defective mutant HPV16 genome featuring a translation termination mutation in E1. We established human dermal keratinocytes (HDKs) containing the E1-defective HPV16 genome with the help of exogenous E1 expression and then removed the exogenous E1 expression cassette with the FLP/FRT system (36). Similar to the wild-type genome, the E1-defective HPV16 genome was maintained for numerous cell generations without exogenous E1 expression. However, unlike the wild-type genomes, the E1-defective HPV16 genome failed to amplify upon differentiation of host cells, with rescue dependent on reexpression of exogenous E1. These results indicate that HPV16 requires E1 protein for the

Received 1 October 2011 Accepted 3 January 2012
Published ahead of print 11 January 2012
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doi:10.1128/JVI.06450-11

TABLE 1 Primers for PCR and RT-PCR

Name	nt position	Sequence (5′→3′)	Product size
For detecting excision of HPV16 DNA[a]			
For total HPV DNA, HPV16 E7	660	GGAGGAGGATGAAATAGATGGTC	136
	795	AGTACGAATGTCTACGTGTGTGC	
For excised-circular HPV DNA, HPV16 LCR	7432	AGGCCCATTTTGTAGCTTC	271
	7702	CCTAACAGCGGTATGTAAGG (+loxP 305)	
For detecting mRNAs			
HA-E1	7	CCTTATGACGTGCCAGATTACGC	144
	150	GTCATTTTCGTTCTCATCGTCTGAGATG	
E1^E4	603	TTTGCAACCAGAGACAACTGAT	933
	4014	AGAGGCTGCTGTTATCCACAAT	
36B4[b]	655	TCGACAATGGCAGCATCTAC	223
	877	GCCTTGACCTTTTCAGCAAG	
For real-time quantitative PCR to quantify HPV16 DNA			
HPV16 L2	4474	CCCACAGCTACAGATACACTTGCT	143
	4616	GGAATGGAAGGTACAGATGTTGGTGC	

[a] GenBank accession number for HPV16 is K02718.
[b] GenBank accession number for 36B4 is M17885.

establishment and the productive stages but not for the maintenance stage of viral genome replication. The results have important implications for the development of E1 inhibitors as anti-HPV drugs.

MATERIALS AND METHODS

Cell culture. Human dermal keratinocytes (HDKs) were purchased from Cell Applications (San Diego, CA). HDKs were immortalized with TERT, a mutant form of CDK4 and cyclin D1 (HDK-K4DT) by lentivirus-mediated gene transfer as described below. The cells were maintained in low-calcium serum-free keratinocyte growth medium (Epilife, Invitrogen, Carlsbad, CA) unless otherwise described. To induce keratinocyte differentiation, cells were exposed to Epilife basal medium supplemented with 1.8 mM $CaCl_2$ for 7 days or more. W12 (20863) cells were obtained from Paul F. Lambert (McArdle Laboratory for Cancer Research, Madison, WI) and cultured on mitomycin-treated Swiss mouse 3T3 cells in F medium as previously described (13).

Plasmid construction. In pCMV-loxP-HPV16-loxP-puro, the full-length HPV16 genome linearized at the SphI site in the long control region and flanked by *loxP* recombination sites (18) was inserted between a CMV promoter and a puromycin resistance gene so that the HPV16 genome was located in reverse orientation to the CMV promoter to avoid CMV-driven expression of HPV16 genes. In pCMV-loxP-E1-defective HPV16-loxP-puro, an in-frame stop codon was created at nucleotides (nt) 892 to 894 just downstream of the E1 start codon at nucleotide 865 by site-directed mutagenesis. The segment encoding Cre recombinase with nuclear localization signal in AxCANCre (14) was cloned into pcDNA3 (Invitrogen) to generate pcDNA3-NCre. Detailed methods for the construction of pCMV-loxP-HPV16-loxP-puro, pCMV-loxP-E1-defective-HPV16-loxP-puro, and pcDNA3-NCre are available upon request.

DNA transfection. HDK-K4DT cells were seeded at a density of 2×10^5 cells onto six-well plates (BD Biosciences, Franklin Lakes, NJ) containing 2 ml of Epilife and incubated overnight and then cotransfected with 1 μg of pcDNA3-NCre and 3 μg of pCMV-loxP-HPV16-loxP-puro (wild-type or E1-defective strains) using FuGENE HD (Roche). One day after transfection, cells were selected by 1 μg/ml of puromycin for 2 days.

Vector construction and retroviral infection. Construction of lentiviral vectors, CSII-CMV-TERT, CSII-CMV-cyclin D1, and CSII-CMV-$CDK4^{R24C}$, were described previously (33). CSII-CMV-TetON-ADV contains the TetON-ADV segment from pTet-On Advanced Vector (Clontech, Mountain View, CA). To yield improved E1 gene expression in mammalian cells, the codon-optimized HPV16 E1 gene with an N-terminal hemagglutinin (HA) tag (HA16E1) was synthesized (GenScript, Piscataway, NJ). CSII-TRE-Tight-HA16E1 contains the HA16E1 gene under the control of the tetracycline responsive promoter from pTRE-Tight (Clonetech). pCMSCV-FRT-HA-E1-TKneo consists of the CMV/LTR fusion promoter, the Ψ packaging signal, a mutant (f72) FLP recognition target (5′FRT) (24), the HA16E1 gene, the PKG promoter, and the herpes simplex virus thymidine kinase (HSV-TK) fused to the neomycin-resistant gene (*neo*), 3′FRT, and 3′LTR, as shown in Fig. 3A. Cells infected with this retrovirus were positively or negatively selected in the presence of 50 μg/ml of G418 or 10 μg/ml of ganciclovir, respectively. The nucleotide sequence of the HA16E1 and the detailed methods for the construction of pCMSCV-FRT-HA16E1-TKneo-FRT, CSII-CMV-TetON-ADV, and CSII-TRE-Tight-HA16E1 are available upon request. The production of recombinant retroviruses and lentiviruses was accomplished as described previously (27, 33).

AdV. The thermostable FLP mutant (FLPe)-expressing adenovirus vector (AdV) (AxCAFLPe), a kind gift from Izumi Saito (The Institute of Medical Science, The University of Tokyo), was prepared as described previously (3, 36). Cells were infected with AxCAFLPe at a 5-particle titer multiplicity of infection.

Western analysis. Western blotting was conducted as described previously (26). Antibodies against HA (16B12; Covance, Princeton, NJ), involucrin (SY5; Sigma-Aldrich, St. Louis, MO), vinculin (Sigma-Aldrich), and loricrin (Covance) were used as probes, and horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), or anti-goat (sc-2033; Santa Cruz, Santa Cruz, CA) immunoglobulins were employed as secondary antibodies.

PCR and DNA blot hybridization. Total genomic DNA was isolated by a standard SDS-proteinase K method, and an aliquot (100 ng) was examined by PCR amplification for Cre-mediated HPV DNA excision. Primer sets used for detecting total HPV16 DNA or recombined HPV16 are shown in Table 1. The DNA was amplified by 30 cycles of PCR using Takara *Taq* DNA polymerase (Takara, Japan) according to the supplier's instructions, with annealing at 60°C and elongation at 72°C for 30 s. PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide. For Southern blot analyses, digested DNA was separated on a 0.75% agarose gel, soaked in 0.25 M HCl for 15 min, and alkaline transferred onto nylon membranes (Boehringer Mannheim, Mannheim, Germany). The membranes were prehybridized in Hybrisol I

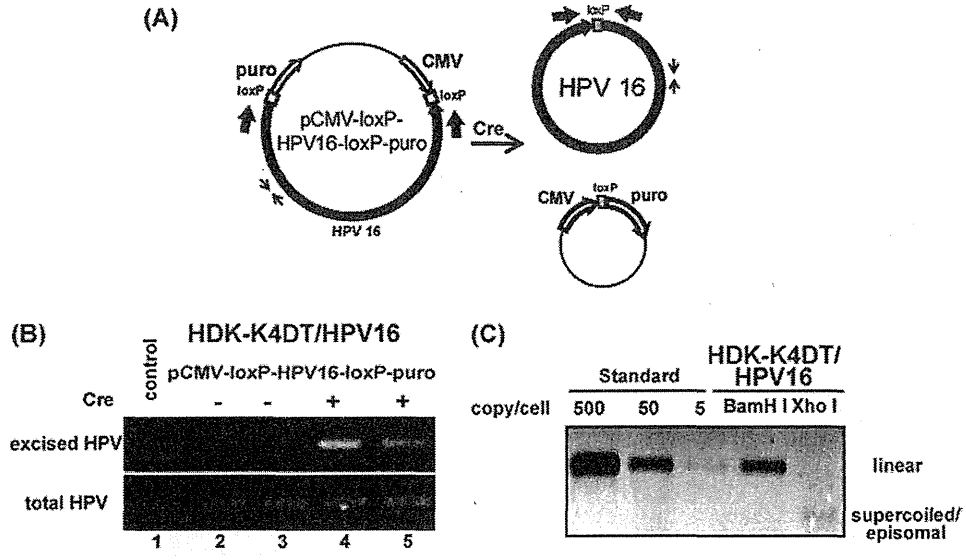

FIG 1 HPV genome excision and establishment of the cell line containing the HPV16 genome. (A) Schematic representation describing the parental pCMV-loxP-HPV16-loxP-puro, the Cre recombinase-excised HPV16 genome, and the pCMV-puro plasmid. PCR primers (thin and thick arrows) to detect total and excised HPV DNA, respectively, are indicated. (B) HDK-K4DT cells were cotransfected with the pCMV-loxP-HPV16-loxP-puro plasmid with (lanes 4 and 5) or without (lanes 2 and 3) the NCre expression plasmid. Total DNA was extracted from the cells 2 days after the transfection without puromycin selection. Representative pictures of an ethidium bromide-stained agarose gel with PCR products indicating total HPV16 DNA (bottom) and excised circular HPV16 DNA (top), containing a surplus of 34 bp of *loxP* DNA, are shown. (C) Southern blot hybridization for the HPV genome in HDK-K4DT cultures. DpnI and BamHI- or XhoI-digested total DNA isolated from HDK-K4DT at 3 weeks after transfection with pCMV-loxP-HPV16-loxP-puro plasmid and the NCre expression plasmid is shown. Digestion with BamHI, which cuts the HPV16 genome once, produced results of the expected size for the HPV16 genome. Digestion with XhoI, which does not cut the HPV16 genome, showed supercoiled plasmid of HPV16 genome. The BamHI-linearized HPV16 plasmid was used for length and copy number standards.

(Millipore, Billerica, MA) for 1 h at 42°C. A biotin-labeled probe of the entire HPV16 genome prepared with the NEBlot Phototope kit (New England BioLabs, Ipswich, MA) was applied for hybridization, and the hybridized DNA was visualized with a Phototope-Star detection kit (New England BioLabs) following the protocol provided by the manufacturer. The LAS3000 charge-coupled device (CCD) imaging system (Fujifilm Co. Ltd., Japan) was employed for detection and quantification.

RNA extraction and RT-PCR analyses. For detection of mRNAs, total RNA was purified with RNeasy (Qiagen, Valencia, CA) and reverse transcribed to generate cDNAs by the ThermoScript reverse transcription (RT)-PCR system (Invitrogen) using random hexamers according to the supplier's instructions. Primers used for the E1^E4 spliced transcript, exogenous codon optimized E1, and human acidic ribosomal phosphoprotein P0 (36B4) are shown in Table 1. The thermocycling profile for amplifying E1^E4, E1, and 36B4 cDNAs was 1 min at 95°C; 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s (or 1 min for E1^E4 cDNA); and 4 min of extension at 72°C. PCR products were separated in a 1.5 or 0.9% agarose gel and visualized with ethidium bromide.

Quantitative real-time PCR for genomic DNA. Reactions were prepared in a volume of 10 μl containing 1× quantitative PCR (qPCR) master mix of KAPA SYBR FAST qPCR kits (Kapa Biosystems, Woburn, MA) and 300 nM each primer. PCR was performed using StepOnePlus (Applied Biosystems) with 10 s of denaturation at 95°C followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Serial dilutions of linearized HPV16 genome from pUC-HPV16 plasmid DNA by BamHI digestion were used as controls to measure the amounts of HPV16 genomic DNA. All real-time PCRs were run in triplicate. Total DNA was digested with DpnI before PCR amplification of the HPV16 genome with a primer set amplifying a product containing two DpnI sites (Table 1). HPV16 DNA copy number was expressed as copies per cell assuming that the total human genomic DNA is 6.6 pg/diploid cell. The rate of HPV16 genome retention (RR) was calculated as follows: RR = (copy number of viral genomes at the end/copy number of viral genomes at the beginning) 1/PD, where population doubling (PD) of cells was calculated as follows: PD = log(number of cells obtained/initial number of cells)/log2.

RESULTS

Establishment of keratinocytes containing the HPV16 genome. Primary human keratinocytes are often used for establishment of HPV-containing cell lines. However, they have a finite life span, and cells harboring HPV genomes tend to preferentially grow in culture. To minimize this effect and to obtain reproducible results, we first immortalized human dermal keratinocytes (HDKs) with TERT, a mutant form of CDK4 and cyclin D1 (HDK-K4DT). HDK-K4DT formed fully stratified squamous epithelium in an organotypic raft culture (data not shown). Then, we established keratinocytes harboring HPV genomes as episomes. HDK-K4DT cells were transfected with pCMV-loxP-HPV16-loxP-puro, designed to generate a circular 7.9-Kb HPV16 genome and a circular pCMV-puro expression cassette (Fig. 1A). With the Cre expression plasmid, about 5 to 10% of the transfected cells survived after short-term puromycin selection, whereas only a few cells survived without Cre recombinase (data not shown). At 2 days posttransfection without puromycin selection, total DNA was analyzed by PCR using two sets of primers, one for total HPV16 DNA and the other specific for the circularized HPV16 genome (Fig. 1A). In cells cotransfected with Cre, PCR products corresponding to the recombined circular HPV16, whose size should be bigger by 34 bp due to an inserted *loxP* sequence, were observed (Fig. 1B, lanes 4 and 5), whereas no circularized HPV16 genome was detected in cells without Cre expression (Fig. 1B, lanes 2 and 3). Southern blot analysis of total DNA extracted from cells at 21 days posttransfection suggested that the established HDK-K4DT cells harbored

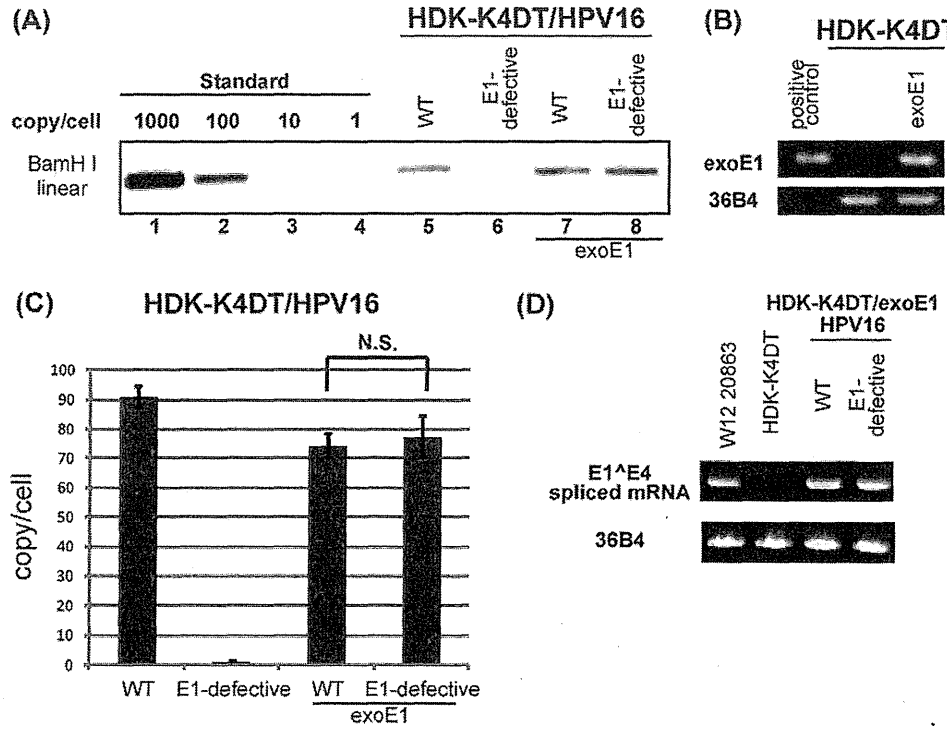

FIG 2 Requirement for E1 protein in the establishment stage. (A) Southern blot hybridization of the HPV genome in HDK-K4DT cultures. Parental (lanes 5 and 6) and exogenous E1 expressing HDK-K4DT cells (lanes 7 and 8) were transfected with the wild-type (WT) or the E1-defective pCMV-loxP-HPV16-loxP-puro plasmid and the NCre expression plasmid. Total DNA was extracted at 21 days posttransfection. DpnI- and BamHI-digested total DNA from parental or exogenous E1 expressing HDK-K4DT cultures was analyzed. The BamHI-linearized HPV16 plasmid was used for length and copy number standards (lanes 1 to 4). (B) mRNAs for exogenous E1 of HDK1-K4DT cells transduced with the retroviral vector expressing HA16E1 were analyzed. Total RNAs isolated from cells with or without the retroviral transduction were subjected to reverse transcription (RT)-PCR with a primer set specific to codon-optimized E1. 36B4 mRNA was also detected as an internal control. The PCR products of exogenous E1 (top) and 36B4 (bottom) visualized in ethidium bromide-stained agarose gels are shown. pCMSCV-FRT-HA16E1-TKneo-FRT was used as a positive control. (C) The copy number of the HPV16 genomes at 14 days posttransfection in each HDK-K4DT cell lines was determined by real-time PCR. The deviations of three independent sets of transfectants are shown as error bars. N.S., not significant. (D) Expression of the E1^E4 spliced mRNAs in E1-expressing HDK-K4DT cells harboring the wild type or the E1-defective HPV16 genomes at 35 days posttransfection. Total RNAs were subjected to RT-PCR with an E1^E4-specific primer set (Table 1). RNAs from parental HDK-K4DT cells and W12 cells were used as controls. PCR products of E1^E4 (top) and 36B4 (bottom) are shown, as described for panel B.

more than 50 viral genome copies per cell (Fig. 1C). Repeated transfection experiments confirmed the reproducibility of this technique. At 60 days posttransfection, after nine serial passages at a ratio of 1:8, we still detected 10 to 20 copies of the viral genomes per cell (data not shown). The rate of HPV16 genome retention was calculated as 90% per cell division.

E1 protein is required for establishment of HPV16 genomes as episomes in HDK-K4DT cells. To study the role of E1 in each stage of the viral life cycle, we prepared an E1-defective mutant HPV16 genome containing an E1 translation termination mutation at nt 892 to 894 to abrogate E1 protein expression. Unlike the wild-type HPV16 genome, the HPV16 E1-defective genome failed to establish in HDK-K4DT cells as episomes (Fig. 2A, lanes 5 and 6). However, in HDK-K4DT cells expressing exogenous E1 from the retrovirus, MSCV-FRT-HA16E1-TKneo-FRT, the E1-defective HPV16 genome could establish as episomes as efficiently as the wild type HPV16 genome (Fig. 2A, lanes 7 and 8). We confirmed the expression of exogenous E1 driven by the LTR promoter by RT-PCR using primers specific for exogenous E1 (Fig. 2B), though the E1 protein was undetectable by Western blot analysis. The copy numbers of the E1-defective HPV16 genome were comparable to that of the wild-type HPV16 genome in the presence of exogenous E1 expression (Fig. 2C). The translation termination mutation inserted in the downstream of the splice donor site for E1^E4 (nt 880) did not disrupt normal E1^E4 splicing (Fig. 2D). These data indicate that the E1 protein is required for initial replication and/or maintenance of the viral genome.

E1 protein is dispensable for maintenance replication of the viral genome. In order to assess the requirement of E1 protein for maintenance replication of the viral genome, we used the HDK-K4DT cells harboring the E1-defective HPV16 genomes established with exogenous E1 expression from the integrated MSCV-FRT-HA16E1-TKneo-FRT retrovirus. Upon infection of FLPe-expressing AdV, the exogenous E1 expression cassette as well as the TKneo gene was excised by FLP at an efficiency of around 50% (Fig. 3A). Then we isolated HDK-K4DT cells which no longer expressed exogenous E1 by ganciclovir selection (Fig. 3B). After several passages of cells at a ratio of 1:8, the copy number of HPV16 genomes was determined by real-time PCR. We detected 70 to 100 copies of the wild-type or the E1-defective HPV16 genomes per cell just before the AdV infection. Copy numbers of both the wild-type and the E1-defective HPV16 genomes gradually decreased during the passages (Fig. 3C). However, about 10 to 20 copies of the E1-defective HPV16 genomes

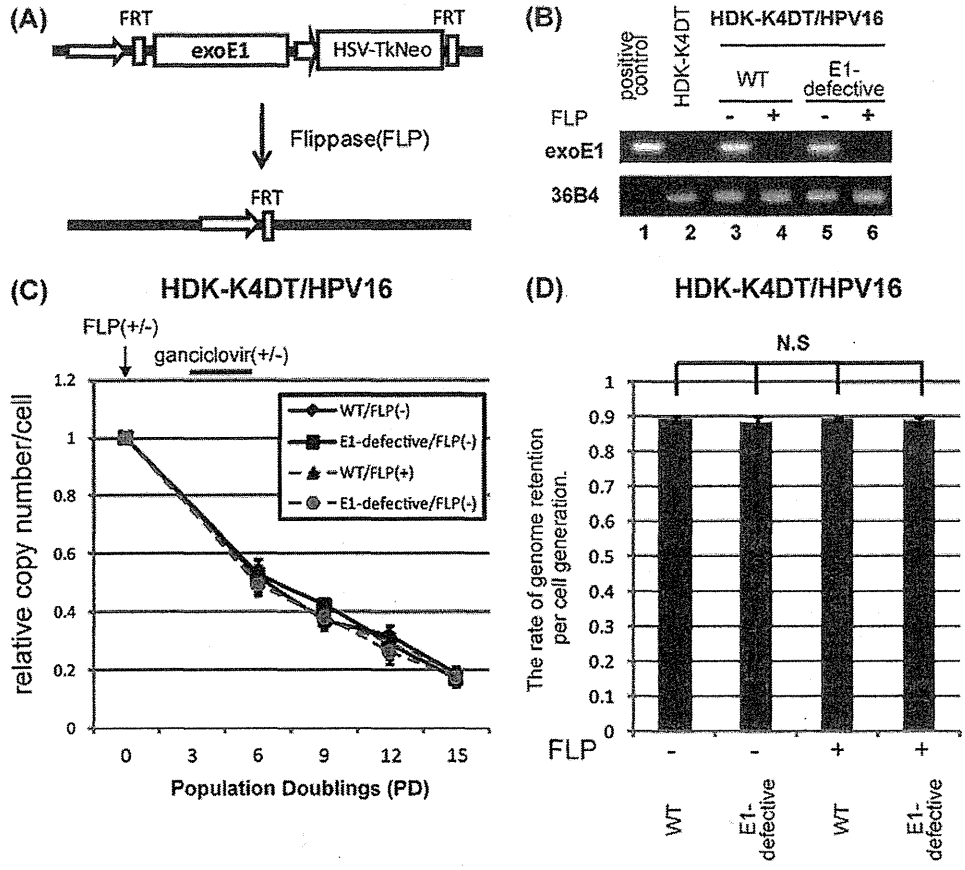

FIG 3 E1 protein is not essential for maintenance replication of the viral genome. (A) Schematic representation of the viral vector, MSCV-FRT-HA16E1-TKneo-FRT, expressing exogenous E1. The HA-tagged HPV16 E1 gene and the TKneo fusion gene are flanked by FLP recognition target (FRT) sites. When a thermostable FLP-expressing AdV (AxCAFLPe) is infected, the DNA in between the FRT sites is excised so that exogenous E1 as well as TKneo expression is terminated. Cells still expressing E1 and HSV-TK can be negatively selected with ganciclovir. (B) mRNAs containing exogenous E1 message were analyzed to ensure complete removal of E1 expression. Total RNAs isolated from HDK-K4DT/HPV16 cell lines at 21 days postinfection with (lanes 4 and 6) or without (lanes 3 and 5) AxCAFLPe were subjected to RT-PCR with a primer set specific to codon-optimized E1. Representative pictures of an ethidium bromide-stained agarose gel with PCR products indicating exogenous E1 (top) and 36B4 (an internal control; bottom) are shown. pCMSCV-FRT-HA16E1-TKneo-FRT was used as a positive control. (C) HDK-K4DT cells containing the wild type (WT) or the E1-defective HPV16 genome were established in the presence of exogenous E1 expression. Two weeks after transfection, aliquots of cells were infected with AxCAFLPe (FLP +) at a multiplicity of infection of 5, followed by selection with 10 μg/ml of ganciclovir for 1 week. After the selection, cells were cultured for 4 passages at a ratio of 1:8 to examine the retention rate of the wild-type or the E1-defective genome in the presence or the absence of exogenous E1. HDK-K4DT/HPV16 cells which were not infected with AxCAFLPe (FLP−) were also cultured for 5 passages at a ratio of 1:8. Total DNA was extracted just before the infection, at every passage and at the end of the culture. The copy number of HPV16 genomes was measured by real-time PCR and normalized to the total amount of DNA. The graph shows time courses of copy number change during the 5 passages after the infection. The copy number at the each time point is shown as a ratio to the copy number just before the FLP-expressing adenovirus infection (PD0). The end of the ganciclovir selection corresponds to PD6. Means and standard errors of the means are shown. (D) The graph shows the rates of HPV genome retention per cell division. The retention rates were calculated as described in Materials and Methods. Means from three independent experiments and standard deviations are shown as error bars. N.S., not significant, compared with each other.

per cell still remained after several passages of cells, even in the absence of exogenous E1 expression. No difference in the rate of genome retention was observed between the E1-defective and the wild-type HPV16 genomes. The rates were calculated as approximately 90% per cell division, irrespective of exogenous E1 expression, and proved quite constant in three independent experiments (Fig. 3D). These data indicate that the E1 protein is dispensable for maintenance replication of the viral genome.

E1 protein is required for viral genome amplification upon differentiation. To confirm that E1 protein is required for the productive stage of viral replication, differentiation-dependent viral genome amplification was examined with the same series of the cells established for the previous section (Fig. 3). HDK-K4DT cells harboring the wild-type or the E1-defective HPV16 genomes in the presence or the absence of exogenous E1 expression were exposed to a high calcium concentration. Induction of differentiation was confirmed by expression of keratinocyte differentiation markers, involucrin and loricrin (Fig. 4A). Southern blot analyses of DNA extracted from sister cultures showed the wild-type HPV16 genomes to be amplified episomally upon differentiation in the absence of exogenous E1 expression (Fig. 4B, lanes 1 to 2). However, the E1-defective HPV16 genomes were amplified only in the presence of exogenous E1 expression (Fig. 4B, lanes 3 to 6). Reintroduction of E1 with lentiviruses, CSII-CMV-tetON and CSII-TRE-Tight-HA16E1, to the cells whose exogenous E1 cassette had been excised by FLP rescued the E1-defective HPV16 genome amplification upon differentiation only when the E1 expression was induced by doxycycline (Fig. 4B, lanes 7 and 8),

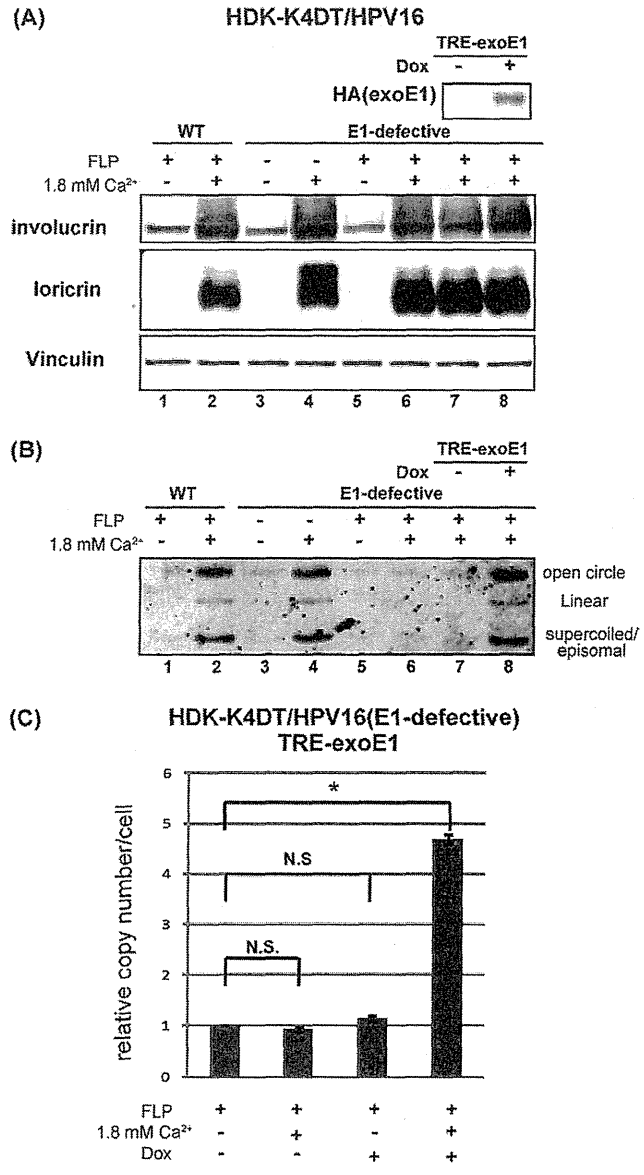

FIG 4 E1 protein is required for viral genome amplification upon differentiation. (A) HDK-K4DT cells harboring the wild-type (lanes 1 and 2) or the E1-defective (lanes 3 to 8) HPV16 genomes in the presence (FLP−, lanes 3 and 4) or the absence (FLP+, lanes 1 and 2, 5 to 8) of exogenous E1 expression were seeded at 2×10^5 cells per well (6-well plate) in Epilife complete growth medium, and then cells were exposed to 1.8 mM calcium (lanes 2, 4, 6, 7, 8) to induce keratinocyte differentiation. Total cell lysates were made, and total DNAs were extracted from the sister cultures just before and 10 days after calcium exposure. The expression of involucrin and loricrin, keratinocyte differentiation markers, was analyzed by Western blotting. Expression of reintroduced exogenous E1 controlled by doxycycline was detected by anti-HA antibody (lanes 7 and 8). Vinculin was detected as a loading control. (B) Southern blot hybridization for episomal HPV16 genomes in HDK-K4DT cells. XhoI-digested total DNA from each HDK-K4DT culture was loaded. A representative image of three independent experiments is shown. (C) The copy number of the E1-defective HPV16 genomes in HDK-K4DT cells in the indicated condition was determined by real-time PCR. Three replicates are shown and standard deviations are shown as error bars. N.S., not significant. The single asterisk indicates P values of <0.05.

whereas induction of E1 alone without high calcium failed to rescue the genome amplification (Fig. 4C). These data indicate that E1 protein is required for viral genome amplification upon differentiation. Since the same series of cells used for Fig. 3 were used in these experiments, the results confirmed that the E1-defective HPV16 genomes were episomally maintained in the absence of E1 expression.

DISCUSSION

In this study, we demonstrated that E1 is dispensable for maintenance replication of the HPV16 genome but required for replication in the establishment and productive stages. Taking the previous study using a TS E1 mutant of BPV1 (17) into account, it is likely that E1 is dispensable for the maintenance replication of other PVs, too.

Thus, HPVs have at least two replication modes and control the copy number of the viral genome depending on the situation. Such a strategy of the virus would clearly be beneficial for persistent infection and continuous virus production. In the maintenance phase, minimal expression of viral proteins in host cells with low copy numbers of viral genomes would allow HPV to evade cellular immune surveillance. Moreover, as recent studies indicate, a high level of E1 expression in basal-layer cells could activate an ATM-dependent damage response and cause growth suppression (10, 32).

It is reasonable to speculate that the E1-independent maintenance replication employs the cellular replication machinery to support viral genome maintenance under S phase control. Mechanisms of viral genome DNA replication control by host cell factors have been well studied for the Epstein-Barr virus (EBV). The EBV genome DNA is replicated once per S phase in the latent phase of infection (19, 44). In this phase, EBV employs replication licensing proteins, MCMs and ORC (19, 22), which assemble on the latent origin of replication of EBV, OriP. A low copy number for the viral genome may be an appropriate common strategy for episomal viruses to sustain latent infection. Although it is not known whether and how MCMs and ORC are involved in HPV DNA replication, E1- and E2-independent *cis*-replicating elements may reside outside the LCR and possibly in the late region (L2-L1 open reading frames [ORFs]) of the HPV16 genome (28, 29).

The observed rate of HPV16 genome retention was about 90% per cell generation, which is comparable to the reported rate for the EBV genome (25). With this retention rate, the viral genomes of 100 copies per cell can be maintained for 2 to 3 months, corresponding to approximately 40 cell divisions under our culture conditions. In the stratified squamous epithelium, stem cells self-renew by dividing infrequently and generate a population of cells that undergo limited but more frequent divisions before giving rise to nonproliferative, terminally differentiating cells. In the natural life cycle of HPVs, the virus must infect epithelial stem cells (8, 34), which would divide much less frequently than cultured cells do. Therefore, the viral genome in the stem cells *in vivo* would be able to persist for much longer period than in cultured cells, even if the genome retention rate is the same as that found in our study.

In maintenance replication, HPV may still employ two different modes of replication. Hoffmann et al. showed that HPV16 DNA replicates once per S phase in W12 cells, while HPV31 DNA replicates via a random-choice mechanism with some multiple

rounds of the viral genome replication per S phase in CIN612-9E cells, and that forced expression of E1 in W12 cells converted HPV16 DNA replication to random-choice replication (12). Interestingly, when HPV16 or HPV31 DNAs are separately introduced into NIKS cells, they both replicate randomly (12). Thus, it is likely that the difference between W12 and CIN612-9E cells depends on expression levels of E1. It is possible that occasional or low-level expression of auxiliary E1 hinders the copy number loss for an even longer period of maintenance, as indicated by previous studies (18, 39). Theoretically, E1 protein could be supplied from the infected virion and/or by *de novo* synthesis from the infected viral genome in the establishment stage. In this regard, it is not clear whether our experimental system recapitulates the actual establishment stage of the HPV life cycle, since E1 can be supplied only by *de novo* synthesis. At present, little is known about the underlying mechanisms of the establishment stage and the mechanism(s) of switching to the subsequent maintenance stage. Clearly, it needs to be examined whether the E1 protein is included in infectious virions and how the E1 expression is regulated in the three different stages.

PV E1 protein forms double hexamers at the replication origin in the LCR with the help of E2 (2, 11, 43) and unwinds DNA through helicase activity ahead of replication forks powered by the hydrolysis of ATP (31). Since E1 is the only viral protein with enzymatic activities, it is an attractive target for development of novel therapeutic agents to treat HPV-associated benign lesions where the whole viral life cycle is completed. Indeed, some candidate small molecules have been reported to inhibit the E1 function (5, 9, 15, 40–42). However, their identification and evaluation was done using biochemical assays or surrogate cell-based assays, and a true antiviral activity has yet to be tested. Based on our present study, the effectiveness of E1 inhibitors as antiviral drugs may be restricted, since they cannot inhibit E1-independent HPV replication in long-living basal cells. Inhibition of E1 protein function could prevent amplification of the viral genome in the establishment and productive stages. Thus, it may prevent HPV infection and reduce pathogenesis, including papilloma formation and virion production. In the case of cervical intraepithelial neoplasias (CINs), continuous inhibition of E1 might reverse low-grade lesions to apparently healthy mucosa, but interruption of the inhibition might lead to recurrence of the lesions. More importantly, it may not be able to eliminate the HPV genomes replicating in undifferentiated basal cells, which are thought to be the histogenetic origin of cervical cancer. Thus, E1 inhibition might not be able to prevent CIN lesions from progressing into cancer.

In summary, we have established an experimental system which can evaluate the requirement of any viral gene of interest in the viral life cycle by supplying and deleting exogenous expression of the gene and demonstrated that E1 is entirely dispensable for maintenance replication of the HPV16 genome in human keratinocytes. Thus, inhibition of E1 may not be able to eliminate the viral genome from the basal cell layer. The rationale for development of E1 inhibitors as anti-HPV drugs may be more restricted than formerly envisaged. Further studies will be required to elucidate the roles of cellular replication factors and the *cis* elements of the HPV genome in E1-independent maintenance replication.

ACKNOWLEDGMENTS

We express our appreciation to Takako Ishiyama for expert technical assistance. We are grateful to John H. Lee (University of Iowa Hospitals and Clinics) for the loxP-HPV16-loxP construct, Izumu Saito (University of Tokyo and RIKEN RDB) for adenovirus vectors, and Hiroyuki Miyoshi (RIKEN BRC) for lentivirus vectors and packaging constructs.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health Labor and Welfare to T.K., and for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to N.E., T.N., and T.K.

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Carcinogenesis Advance Access published March 1, 2012
Carcinogenesis vol.0 no.0 pp.1–8, 2012
doi:10.1093/carcin/bgs104
Advance Access publication February 15, 2012

A critical role of MYC for transformation of human cells by HPV16 E6E7 and oncogenic HRAS

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Human papillomaviruses (HPVs) are the primary causal agents for development of cervical cancer, and deregulated expression of two viral oncogenes E6 and E7 is considered to contribute to disease initiation. Recently, we have demonstrated that transduction of oncogenic HRAS ($HRAS^{G12V}$) and MYC together with HPV16 E6E7 is sufficient for tumorigenic transformation of normal human cervical keratinocytes (HCKs). Here, we show that transduction of $HRAS^{G12V}$ on the background of E6E7 expression causes accumulation of MYC protein and tumorigenic transformation of not only normal HCKs but also other normal primary human cells, including tongue keratinocytes and bronchial epithelial cells as well as hTERT-immortalized foreskin fibroblasts. Subcutaneous transplantation of as few as 200 HCKs expressing E6E7 and $HRAS^{G12V}$ resulted in tumor formation within 2 months. Dissecting RAS signaling pathways, constitutively active forms of AKT1 or MEK1 did not result in tumor formation with E6E7, but tumorigenic transformation was induced with addition of MYC. Increased MYC expression endowed resistance to calcium- and serum-induced terminal differentiation and activated the mammalian target of rapamycin (mTOR) pathway. An mTOR inhibitor (Rapamycin) and MYC inhibition a level not affecting proliferation in culture both markedly suppressed tumor formation by HCKs expressing E6E7 and $HRAS^{G12V}$. These results suggest that a single mutation of HRAS could be oncogenic in the background of deregulated expression of E6E7 and MYC plays a critical role in cooperation with the RAS signaling pathways in tumorigenesis. Thus inhibition of MYC and/or the downstream mTOR pathway could be a therapeutic strategy not only for the MYC-altered but also RAS-activated cancers.

Introduction

A subset of human papillomaviruses (HPVs), the so called high-risk types such as type 16 and 18, are associated with >90% of all cervical carcinomas as primary causal agents (1), with deregulated expression of the HPV viral oncogenes E6 and E7 as the main contributors to an etiology (2). However, epidemiological studies and experimental data indicate that the viral presence is not enough to induce cervical cancer and additional genetic and epigenetic events (to alter the cellular factors) are presumably required (3). To address this, we have established an *in vitro* model for cervical cancer with normal human cervical keratinocytes (HCKs) focusing on sequential transduction of defined genetic elements (4) and succeeded in the creation of highly potent cancer initiating cells by introduction of c-MYC (MYC) and oncogenic $HRAS^{G12V}$ (HRAS) on a background of HPV16 E6 and E7 expression (4). However, since the cells having been cultivated in the differentiating medium were used for the assays, we could not exclude the possibility that genetic and/or epigenetic alterations during the selection might be critical for the transformation. In the present study, by directly examining transformed phenotype of the cells without such selection, we could demonstrate that oncogenic HRAS, without overexpression of exogenous MYC, is sufficient for tumorigenic transformation of normal human cells expressing E6 and E7. Nonetheless, endogenous MYC stabilized by HRAS was revealed to be a critical player in tumor-initiating potential.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; ES, embryonic stem cell; HBEC, human bronchial epithelial cell; HCK, human cervical keratinocyte; HFF, human foreskin fibroblast; HPV, human papillomavirus; KGM, keratinocyte growth medium; mTOR, mammalian target of rapamycin.

[†]These authors contributed equally to this work.

Materials and methods

Cell culture and cell lines

Normal HCKs were obtained with written consent from patients who underwent abdominal surgery for a gynecological disease other than cervical cancer. HCK1, HCK4 and HCK8 cells derived from different donors were maintained in low-calcium serum-free keratinocyte growth medium (KGM) (Epilife-KG2 KURABO Industries, Ltd, Osaka, Japan) unless otherwise described. HCK1T cells were established by transduction of *hTERT* into HCK1 cells (4). These HCK cells were then further transduced with HPV16 *E6E7* followed by the oncogene(s) of interest. Normal human bronchial epithelial cells (HBECs) were purchased from Cell Applications (San Diego) and cultivated in KGM. Normal human foreskin fibroblasts (HFFs) purchased from BioWhittaker (Walkersville) were immortalized by transduction of *hTERT*. HFFs and cervical cancer cell lines, SiHa, CaSki and HeLa and C33A, were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum. The source, authentication and methods of maintenance of the cell lines are described in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Vector construction and retroviral infection

Construction of the retroviral expression vectors, pCLXSN-16E6E7, pCLXSH-hTERT, pCMSCVpuro-MYC, pCLMSCV-puro-BCL2, pCMSCVpuro-myr-AKT1, pCMSCVbsd-$HRAS^{G12V}$, pCMSCVbsd and pCMSCVpuro-MEK1DD was as described previously (4,5). OmoMYC, a human version of the dominant-interfering MYC mutant (6), which encodes the C-terminal 92 amino acids of MYC with four amino acid substitutions (E410T, E417I, R423Q and R424N) and MYC^{T58A} were made by *in vitro* mutagenesis and cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter in CSII-EF-RfA (a gift from Hiroyuki Miyoshi, RIKEN, BioResource Center) was replaced with the tetracycline-responsive promoter from pTRE-Tight (Clonetech). CSII-TRE-Tight-16E6E7-2A-MYC^{T58A}-2A-$HRAS^{G12V}$ was constructed by inserting the 16E6E7, MYC^{T58A} and $HRAS^{G12V}$ segments separated by the sequences encoding the autonomous 'self-cleaving' 2A peptides derived from foot-and-mouse disease virus (7) into CSII-TRE-Tight-RfA. CSII-TRE-Tight-MYCmiR-1, -2 and -3 were constructed by inserting the micro RNA sequence based on the BLOCK-iT Pol II miR RNAi system (Invitrogen) into CSII-TRE-Tight-RfA. The target sequence for MYCmiR-1, -2 and -3 are 5′-TAGTC-GAGGTCATAGTTCCTG-3′, 5′-ATGAAACTCTGGTTCACCATG-3′ and 5′-TTGACATTCTCCTCGGTGTCC-3′, respectively. The production of recombinant viruses and selection of infected HCKs were detailed earlier (4,5).

Western analysis

Western blotting was conducted as described previously (4). Antibodies used were listed in the Supplementary Material and Methods, available at *Carcinogenesis* Online.

Colony formation in soft agar medium

Cells were seeded at 5×10^4 cells per 35 mm dish (BD-Falcon 3046) in an appropriate medium. Colonies over 50 μm in diameter were counted after 3 weeks as described previously (4).

Clonogenic assay

Aliquots of 500 cells were seeded on 35 mm dishes under sparse conditions. After cultivation for 2 weeks, the cells were stained with Giemsa's dye, and the number of colonies was counted.

Tumorigenesis in nude mice

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. A 100 μl volume of cells in a 1:1 mixture of Matrigel (BD Biosciences) was subcutaneously injected into female BALB/c nude mice (Clea Japan). The expression of human involucrin in all tumors was determined by western blots with antibodies against human involucrin that do not react with mouse epidermis to confirm that the tumors were derived from implanted HCKs (data not shown).

Quantitative reverse transcription–PCR analysis

Quantitative reverse transcription–PCR was performed as described previously (8). Amplified products were detected with a TaqMan Gene Expression Assay (Applied Biosystems). The expression level of the MYC gene was then normalized to RNA content for each sample using beta-2-microglobulin messenger RNA as a control.

Results

Oncogenic HRAS is sufficient for tumor initiation with normal human cells expressing HPV16 E6E7

Previously, we demonstrated that introduction of HPV16 *E6* and *E7* (E6E7), *H-RAS*^{G12V} (HRAS) and *c-MYC* (MYC) to normal HCKs transduced with hTERT (HCK1T) resulted in the creation of highly potent tumor-initiating cells capable of forming tumors in nude mice when only 10 cells were transplanted subcutaneously (4). Since the cells having been cultivated in the differentiating medium containing high calcium and serum (DMEM + 10% fetal bovine serum; DMEM hereafter) were used for the assays, it is possible that such adaptation or selection was required for the tumor-initiating potential by adding further epigenetic or even genetic alteration(s). To exclude the possibility, we directly examined transformed phenotype of the cells soon after transduction of oncogenes cultivated in KGM and revealed that HRAS addition was sufficient for tumorigenic transformation of primary HCKs and HCKTs (HCKT where T is for hTERT) expressing HPV16 E6E7 (Table I, A). In the presence of HRAS, endogenous MYC protein levels were markedly elevated (Figure 1A). When 1 million cells were subcutaneously transplanted into nude mice, HCK4T-E (E is for E6E7) expressing HRAS formed large tumors within 2 weeks, irrespective of the presence of an exogenous MYC transgene although growth was marginally faster with the latter (Figure 1B; $P > 0.05$). A high proportion of tumor-initiating cells in populations expressing E6E7 and HRAS was confirmed by injecting only 200 cells of different batches of HCKTs expressing the same set of genes into nude mice, resulting in tumor formation within 2 months (Table I, B). Thus, we examined whether HPV16 E6E7 and HRAS with or without exogenous MYC could confer tumor formation properties on other human cell types, including HBEC and HFF immortalized with hTERT (Figure 1D and E). Although, exogenous MYC expression resulted in the faster tumor-forming ability of HBEC ($P < 0.0005$), E6E7 and HRAS was sufficient for tumorigenic potential of these human cells with increased endogenous MYC levels (Figure 1C).

Then, we examined the effect of induced expression of MYC^{T58A}, which is a form resistant to FBWX7-dependent proteasomal degradation (9), on the tumorigenic potential of HCK1T-E with HRAS cells. Although MYC^{T58A} accumulation was observed in doxycyclin-treated cells both *in vitro* and *in vivo*, it did not result in increased tumorigenic potential in this setting (Supplementary Figure 1 is available at *Carcinogenesis* Online), indicating the possibility that certain threshold levels of MYC stabilized by HRAS might be sufficient.

Table 1. Summary of xenograft transplantation of HCKs

(A) Tumor formation using E6E7 expressing HCKs with HRAS^{G12V}

Cells	No. of tumors per sites of injection 1 × 10^6 cells per site	Weight of tumors (mg) at the end of the experiment (weeks)
HCK1T	4/4	460 ± 137 (3 weeks)
HCK1Ta	4/4	640 ± 479 (2 weeks)
HCK1Tb	4/4	500 ± 338 (2 weeks)
HCK1Tc	4/4	140 ± 50 (2 weeks)
HCK4T	4/4	523 ± 148 (2 weeks)
HCK4	4/4	225 ± 50 (3 weeks)
HCK8	4/4	555 ± 140 (3 weeks)

(B) Tumor formation using E6E7 expressing HCKs with HRAS^{G12V}

	2 × 10^3 cells per site (weeks)	2 × 10^2 cells per site (weeks)
HCK1T	6/6 (4)	6/6 (4)
HCK4T	4/4 (4)	4/4 (6)
HCK8T	4/4 (4)	4/4 (6)

(C) Tumor formation using E6E7 expressing HCK1T cells with MYC or other downstream signals of HRAS^{G12V}

	No. of tumors per sites of injection (1 × 10^6 cells per site)				
HCK1T-E6E7-	vect (weeks)	MYC	MYC^{T58A}	Tet-inducible-MYC^{T58A}	
				Dox+ (weeks)	Dox− (weeks)
Vect	0/7	0/4	0/4	0/3	0/3
BCL2			1/4	1/3	0/3
AKT	0/4			3/3 (9)	3/3 (9)[a]
MEK1DD	0/4			4/4 (7)	3/3 (7)[a]
HRAS^{G12V}	12/12 (2)			10/10 (6)[b]	10/10 (6)[b]

(B) Latency was determined as the time taken before a palpable mass could be detected and indicated in parentheses (weeks). (C) Incidence of tumor formation within 20 weeks of observation period was scored otherwise observation was terminated at the time indicated in parentheses (weeks). No description indicates not determined.

[a]Only small tumors developed as in Figure 3B and C.

[b]10^3 ($n = 4$) or 10^2 ($n = 6$) cells were transplanted.

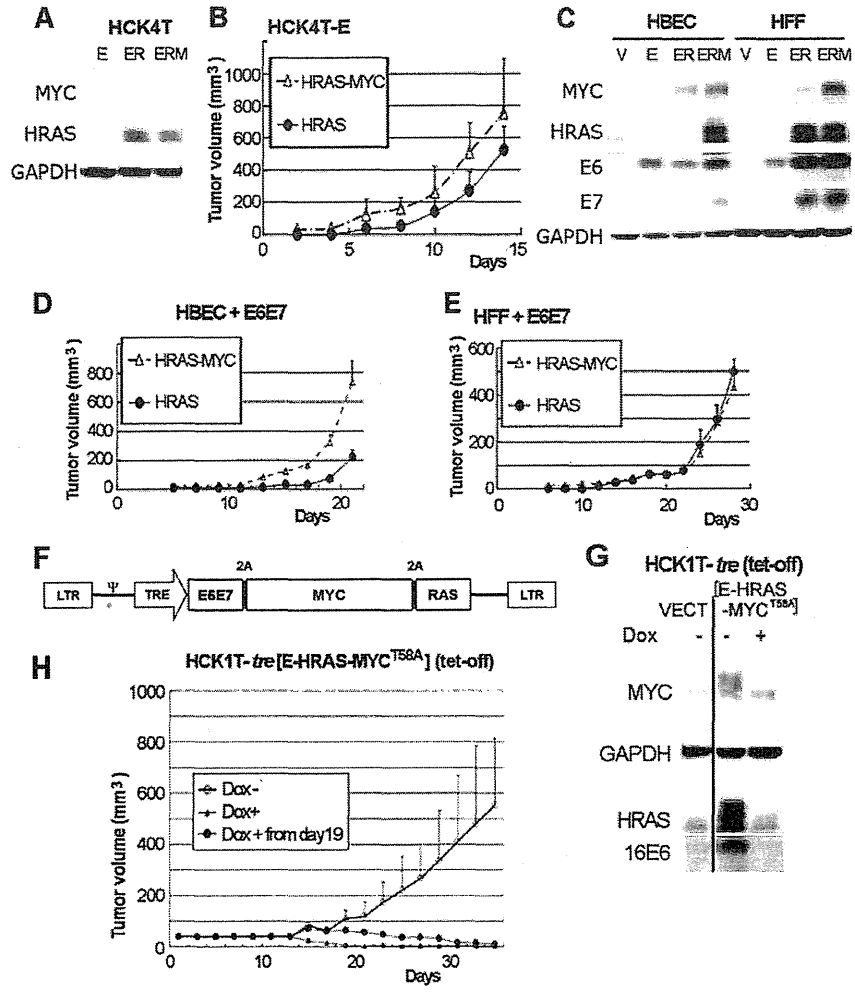

Fig. 1. HRAS with increased endogenous MYC expression is sufficient for full transformation of normal human cells expressing HPV16 E6E7. (**A**) Endogenous MYC levels in the presence of HRAS (ER) were compared with exogenous expression (ERM) by western blotting (ER for E6E7–HRAS and ERM for E6E7–HRAS–MYC). (**B**) *In vivo* tumor-forming ability of HCK4T-E cells with HRAS alone or HRAS–MYC. Cells were subcutaneously injected into nude mice (1×10^6 cells) and tumor size was measured every other day. The tumor volume (mm^3) was calculated as $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter. Each point is the mean of data for four to six samples ± SD. (**C**) Transgene products and MYC levels in human HBEC and HFF were determined by western blotting. (**D** and **E**) Tumorigenic ability of HBEC (D) and HFF (E) expressing HPV16 E6E7 and hTERT with HRAS alone or HRAS–MYC was determined as in (B). (**F**) Schematic of a single polycistronic virus in which expression of E6E7, MYC and HRAS are regulated by doxycyclin (Tet-off). These genes were separated by the sequences encoding the autonomous self-cleaving 2A peptides derived from foot-and-mouse disease virus (7). (**G**) The expression of the transgenes was determined by western blotting. (**H**) HCK1T cells transduced with this vector together with a Tet-off vector were transplanted into nude mice. When tumors had started to grow (the volume of the tumor exceeded 100 mm^3), the gene expression was terminated by adding doxycyclin in the drinking water.

To confirm that tumorigenicity is readily induced by expression of E6E7, HRAS and MYC (endogenous/exogenous) without further genetic changes and is reversible on cessation of such gene expression, E6E7, MYC^{T58A} and HRAS were cloned into a single lentiviral vector in which expression of transgenes was regulated by doxycyclin (Tet-off) (Figure 1F, G and H). HCK1T cells transduced with this vector together with a Tet-off (tTA) vector were transplanted into nude mice. When tumors had started to grow (the volume of the tumor exceeded 100 mm^3), the gene expression was terminated by adding doxycyclin to the drinking water and this resulted in halted tumor growth followed by complete regression (Figure 1H). These data support the idea that E6E7, HRAS and MYC are sufficient for tumor-forming ability of human cells without additional genetic alterations.

MYC stabilization by HRAS

We have reported that endogenous as well as exogenous MYC protein stability is increased in the presence of HRAS in exponentially growing HCK1T cells adapted to calcium and serum (grown in DMEM) (4). Because most of the data in this report were prepared with cells kept in KGM, which does not contain serum and high calcium, endogenous MYC protein stability was determined using HCK1T-E cells with a vector, AKT (myr-AKT1), MEK1DD [constitutively active form of MEK1which activates the extracellular signal-regulated kinase (ERK) pathway (10,11)] or HRAS in KGM. Endogenous MYC protein levels were increased in the order of the genes listed above (vector, AKT, MEK1DD and HRAS) (Figure 2A). In parallel with the MYC levels, Survivin (12), phosphorylated 4EBP1 (13) and phosphorylated p70S6K levels were increased and TSC2 levels were decreased (14). Increased ERK phosphorylation was observed in MEK1DD- and HRAS-expressing cells. Although similar trends were observed in subconfluent culture (data not shown), they were more evident under post-confluent culture conditions (Figure 2A).

Furthermore, increased MYC protein stability was found in the presence of HRAS (Figure 2C, also with post-confluent cells; data not shown) without significant increase in MYC messenger RNA

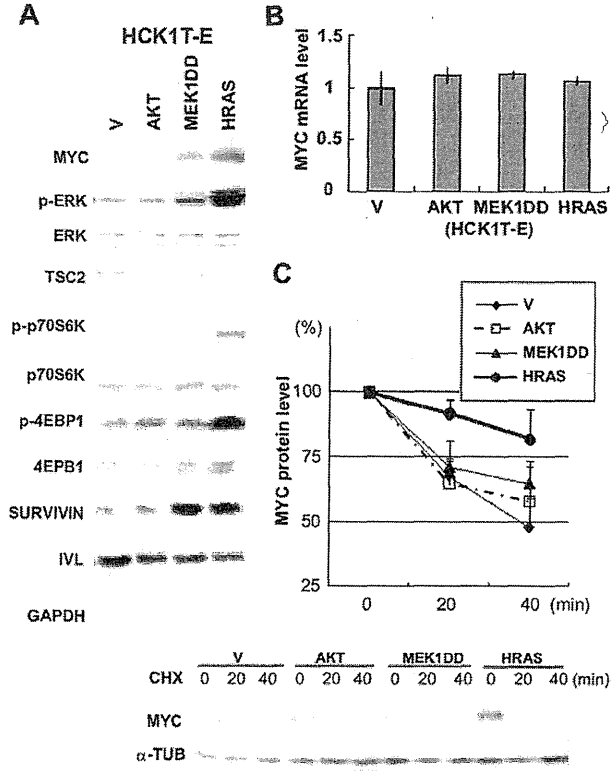

Fig. 2. Increased MYC protein stability in HRAS-positive cells. (**A**) Endogenous MYC protein levels in HCK1T-E with vector, AKT (myr-AKT1), MEK1DD (constitutively active form of MEK1) and HRAS from post-confluent (3d) cells were determined by western blotting. (**B**) Quantitative reverse transcription–PCR of MYC transcript levels from exponentially growing cells. Experiments were performed in triplicate and results were normalized to beta-2-microglobulin and presented as mean ± SD. (**C**) Exponentially growing cells were treated with 25 μg/ml cycloheximide (CHX) for the indicated time periods and the MYC degradation rate was assessed by western blotting. Results of quantitation in three experiments are shown with ± SD (**D**).

levels (Figure 2B). The levels of phosphorylated MYC, which has been reported to correlate with its function (15,16), were increased in the presence of HRAS (Supplementary Figure 2 is available at *Carcinogenesis* Online). From these observations, it is suggested that in HRAS-expressing cells, endogenous MYC expression is maintained at a higher level than in other cells.

Dissection of HRAS signaling pathways in tumor formation

In order to evaluate the importance of stabilized MYC for tumorigenic potential of HCK1T-E, we examined biological effects with MYC^{T58A}. Although we speculated that increased MYC expression is crucial for HRAS-induced tumorigenic potential, HCK1T-E with induction of MYC^{T58A} alone did not give rise to tumors. Though MYC^{T58A} mutant is reported to have reduced activity to induce apoptosis than MYC (16,17), it increased cleavage of caspase 3 in HCK1T-E cells (Figure 3A). To reduce the negative effect of MYC^{T58A}, BCL2 was introduced to HCK1T-E cells (Figure 3A). HCK1T-E with BCL2 cells showed weak tumor-forming ability upon MYC^{T58A} induction (Table I, C), but 4–5 months were necessary for palpable tumor masses, indicating a possible requirement for additional genetic and/or epigenetic alterations. MYC^{T58A} induction together with expression of either active AKT or MEK1DD conferred tumorigenic potential on HCK1T-E cells (Figure 3B and C). Although without induction of MYC^{T58A} they eventually started to form small tumors at the end of observation period (7–9 weeks), this might have been due to leakage of MYC^{T58A}

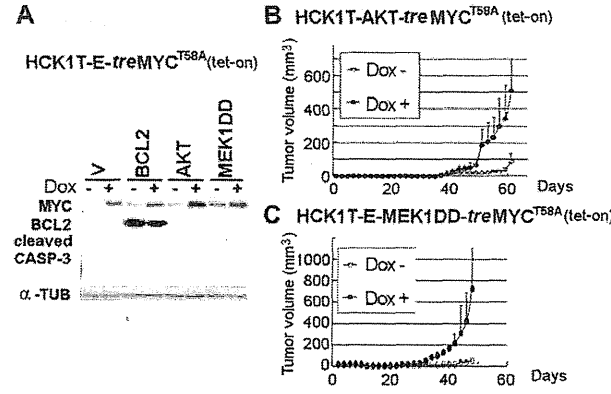

Fig. 3. Stabilized MYC expression is required for the tumorigenic potential of HCK1T-E cells with activated AKT or ERK. (**A**) MYC^{T58A} expression with the Tet-regulated expression system was determined by western blotting. Dox +; cells were treated with 1 μg/ml doxycycline (Dox) for 5 days. *In vivo* tumor-forming ability of AKT (**B**) and ERK (**C**) activated HCK1T-E cells with induction of MYC^{T58A} were determined as for Figure 1B. Mice were treated with doxycycline (1 μg/ml in their drinking water) or the vehicle (ethanol).

from the Tet-regulated expression system because HCK1T-E with AKT or MEK1DD alone did not form tumors within 20 weeks (Table I, C). Since tumorigenic potential was less than with HCK1T-E-RAS cells, it is evident that multiple RAS signaling pathways other than simply MYC stabilization are cooperatively involved in tumorigenic transformation of HCK1T-E cells.

MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells

Then, the biological effects of MYC on HCK were examined. Upon induction of MYC^{T58A} in HCK1T cells with BCL2, the expression levels of carbamoyl phosphate synthase/aspartate transcarbamoylase/dihydroorotase, a bona fide MYC target gene (18) and survivin (12) were increased, whereas the levels of a differentiation marker, involucrin, and a key inducer of keratinocyte differentiation, NOTCH1 (19), were decreased (Figure 4A). Furthermore, repression of TSC2 accompanied by activation of the mammalian target of rapamycin (mTOR) pathway was observed upon MYC^{T58A} induction. Activation of NOTCH1 and accumulation of involucrin induced by exposure to calcium and serum were largely canceled by MYC^{T58A} expression (Figure 4A). Similar effects of MYC^{T58A} induction were also observed in HCK1T-E cells with AKT or MEK1DD, although they were less marked, probably because E6E7 and AKT or MEK1DD influenced MYC regulation (17,20) (Figure 4B; data not shown). Induction of MYC^{T58A} significantly supported the growth of these cells in differentiating medium containing serum and high calcium, whereas no significant effects were observed in KGM (Figure 4C and D). Thus, MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells.

Inhibition of tumorigenic potentials of HCK1T-E–HRAS cells by inhibition of MYC or mTOR

Finally, we examined the role of endogenous MYC with HRAS in tumorigenic potential of HCK cells and cervical cancer cell lines (CaSki, SiHa, HeLa and C33A; Supplementary Figure 3B and C is available at *Carcinogenesis* Online; data not shown) by introducing the MYC inhibitor, OmoMYC (6), with Tet-regulated expression system, for which potential tumor-suppressive effects were recently reported in a mouse lung cancer model featuring KRAS mutation (21). OmoMYC induction levels were determined with an anti-MYC monoclonal antibody that recognizes both endogenous MYC and OmoMYC (Figure 5A). In contrast to the observations with MYC^{T58A} induction (Figure 4A and B), OmoMYC induction resulted

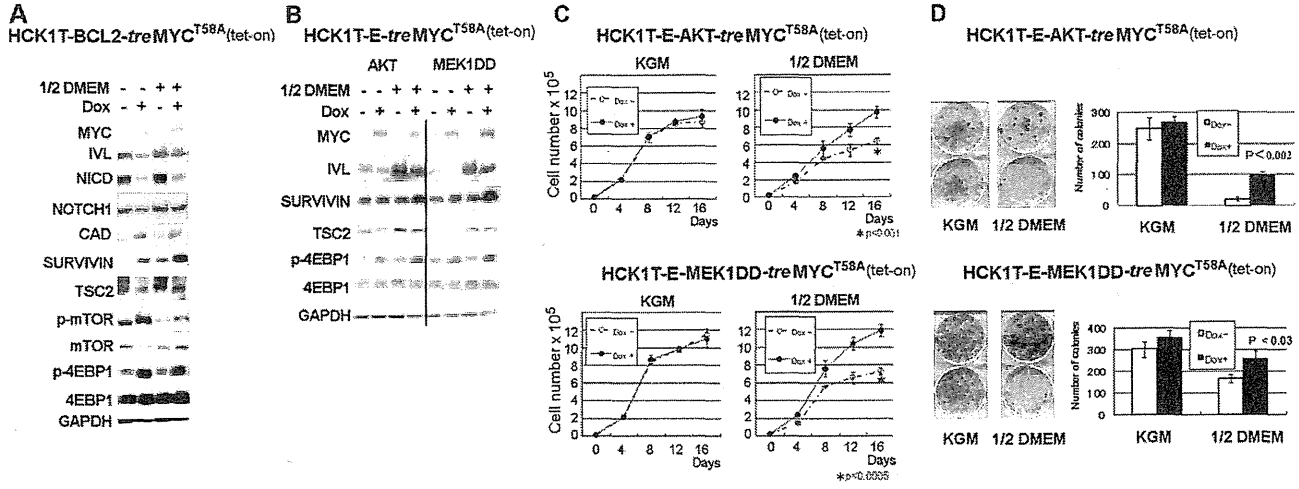

Fig. 4. Resistance to differentiation and mTOR activation by MYC. (**A**) HCK1T with BCL2 cells were treated with 1 μg/ml Dox for 3 days to induce MYC^{T58A} expression. Some cells were exposed to calcium and serum (KGM and DMEM were mixed at one to one ratio; 1/2 DMEM hereafter) for the last 1 day to induce calcium- and serum-induced terminal differentiation. Differentiation markers and MYC regulated genes were determined by western blotting. (**B**) HCK1T-E with AKT or MEK1DD with inducible MYC^{T58A} cells were treated as in (A). (**C**) Growth of cells in KGM (C left) and 1/2 DMEM (right, medium was replaced on day 1) were determined. A total of 2×10^4 cells were seeded on 22 mm dishes (BD Bioscience 3043) with the addition of Dox 1 μg/ml after 6 h and then counted on the indicated days. (**D**) The cells were seeded at a cell number of 2000 in wells of 35 mm dishes of six-well plates and 1 μg/ml Dox were added to the medium of three wells (dark bar) after confirmation of cell attachment (5 h). In half of the experiments, medium was replaced with 1/2 DMEM on day 2. After cultivation for 10 days, the cells were stained with Giemsa's dye, and the numbers of colonies were counted. Note that HCK1T-E with MEK1DD cells formed huge dense colonies with induction of MYC^{T58A} in clonogenic assay.

in increased involucrin and TSC2 (Figure 5A), further supporting regulation of these molecules through MYC in HCK cells. With induction of OmoMYC not exceeding endogenous MYC levels, HCK1T-E and HCK8T-E with HRAS cells did not result in significant reduction of growth (Figure 5B). Anchorage-independent growth ability of these cells was dramatically reduced with OmoMYC induction (Figure 5C) and tumorigenic potential was also profoundly reduced (Figure 5D). We obtained essentially the same result by moderate silencing of endogenous MYC in HCK1T-E with HRAS (Supplementary Figure 3A is available at *Carcinogenesis* Online). The induction of OmoMYC in cervical cancer cell lines also resulted in the suppression of their transforming abilities (Supplementary Figure 3B and C is available at *Carcinogenesis* Online). Although overexpression of MYC was not obvious in these cell lines, even in HeLa cells with a low level of MYC amplification (22), MYC might also play a critical role in these cells.

Because we found activation of the mTOR pathway in HRAS-transduced HCK cells, effects of an mTOR inhibitor, Rapamycin, on their transformation were tested. The clonogenicity of HCK1T-E with HRAS cells was reduced with Rapamycin in a dose-dependent manner (Figure 5E). Although either 10 nM Rapamycin or OmoMYC induction alone did not result in complete repression of clonogenic potential, simultaneous use of them blocked clonogenicity completely (Figure 5E), while strongly suppressing tumorigenic potential in nude mice (Figure 5F). These data indicate that the mTOR pathway is a major downstream effector activated by HRAS through MYC.

Discussion

MYC and RAS oncogenes can cooperatively induce full transformation of mouse cells but cause apoptosis and senescence, respectively, when expressed individually. Unlike the mouse cell case, transduction of MYC and RAS oncogenes into human cells does not suffice for full transformation, possibly because of more sophisticated tumor-suppressive failsafe mechanisms. However, we recently demonstrated that MYC and RAS can cooperatively transform human cells (HCKs) with the help of HPV16 E6 and E7 (4). In the development of cervical cancer, deregulated expression of E6 and E7 precedes disease progression, and E6 and E7 can immortalize HCKs and alleviate both MYC-induced apoptosis and RAS-induced senescence, mainly through inactivation of p53 and pRB. Here, we showed that oncogenic RAS on a background of E6E7 expression can induce full transformation of HCKs, and that stabilization of MYC by RAS is critical for tumorigenic transformation. Many mechanisms have been reported to be involved in MYC stabilization. A major ubiquitin ligase of MYC, FBXW7, preferentially recognizes and induces degradation of MYC with phosphorylated Thr58 and unphosphorylated Ser62, and thus the MYC^{T58A} mutant is very stable (23). Phosphorylation of Ser62 by ERK1/2 and inhibition of Thr58 phosphorylation through inactivation of GSK3β by AKT/PI3K are reported to be involved in RAS-induced MYC stabilization (17). Recently, CDK2 and downstream target(s) of PDK1 were also documented to phosphorylate Ser62 (24,25). Activities of these kinases can be regulated by multiple RAS signaling pathways as well.

If we could identify core gene sets, which promote reprogramming of normal human cells into cancer-generating cells, it would be of great advantage to understanding the complicated molecular mechanisms of carcinogenesis. In this study, we clarified that transduction of only three factors, namely oncogenic HRAS, E6 and E7, is sufficient for tumorigenic transformation of HCKs, though early studies have already suggested cooperation between E6E7 and oncogenic RAS (26–29). Thus, E6, E7 and HRAS might constitute one such core gene set. It also proved sufficient to induce full transformation of other normal human cell types, including human tongue keratinocytes, HBECs and HFFs though the HBECs and HFFs had been transduced with hTERT. Our recent study indicates that the role of E6E7 could be largely but not completely replaced by the blockade of the pRB and p53 pathways in human tongue keratinocytes (30). We previously found that ovarian surface epithelial cells could not be fully transformed by transduction of oncogenic KRAS and MYC with blockade of the pRB and p53 pathways by CDK4/CYCLIN D1 and a dominant-negative form of p53 (5). Other than inactivation of p53 and pRB, E6 and E7 proteins have many functions and it is very conceivable that these could be involved in full transformation. Indeed, in HCKs, the PDZ-binding motif of E6 is critical for full transformation of HCKs through degradation of several PDZ-containing proteins (our unpublished results).

Increased tumorigenic potential by exogenous MYC was observed with variation (Figure 1), indicating certain threshold levels of MYC are required for tumorigenicity depending on the cell type. However,

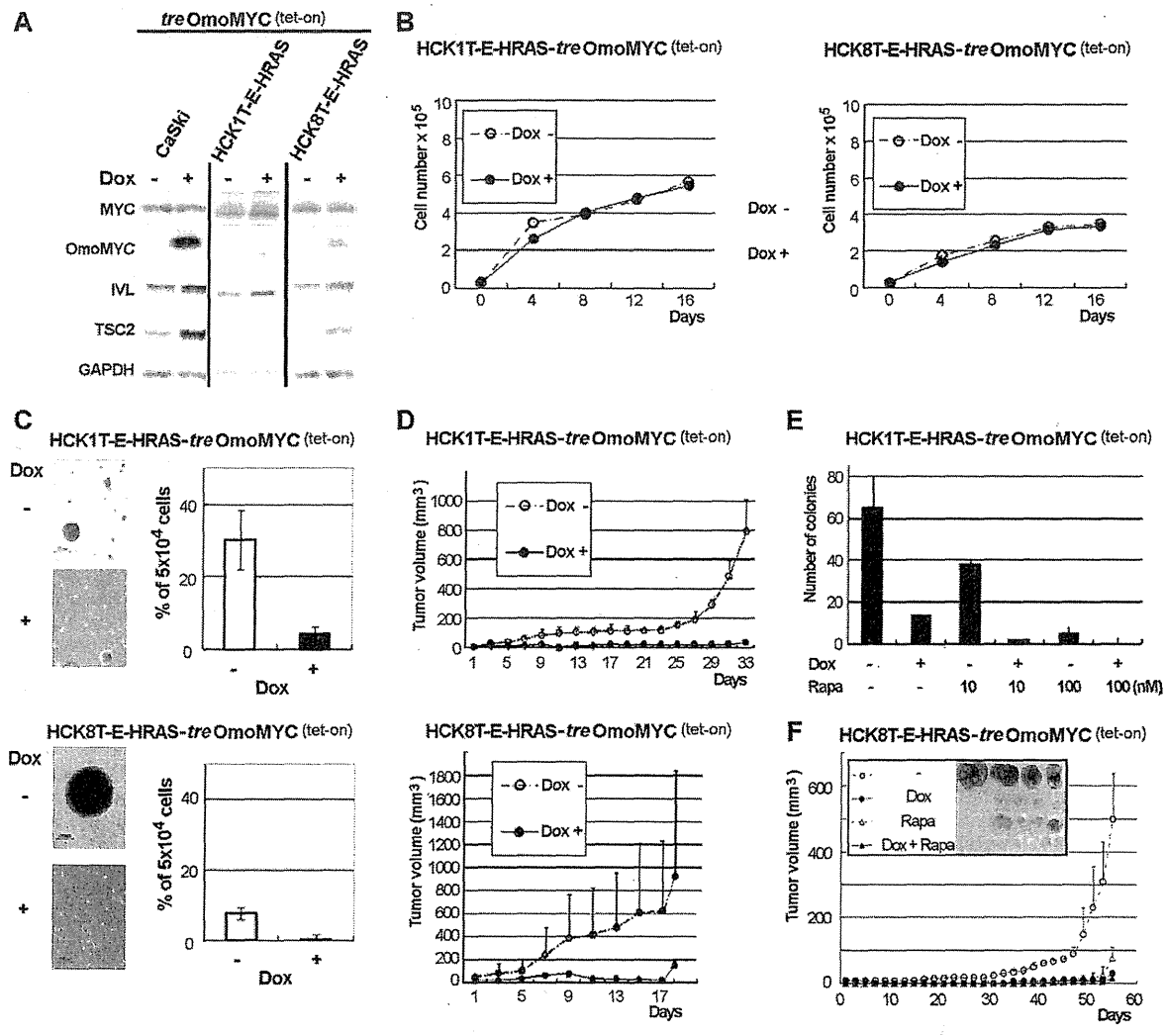

Fig. 5. Inhibition of MYC and/or mTOR pathway repressed tumorigenic potential of HCK cells with E6E7 and HRAS. (**A**) Induction of OmoMYC, an MYC inhibitor (Dox 1 μg/ml 5 days) and alteration of involucrin and TSC2 in HCK and CaSki cells were determined by western blotting. (**B**) Effects of OmoMYC induction on growth of HCK cells with E6E7 and HRAS were determined as for Figure 4C. (**C**) For assessment of anchorage independent growth of HCK cells with OmoMYC induction, aliquots (5×10^4 cells) were seeded in 35 mm dishes. After 3 weeks, the numbers of colonies (≥50 μm in diameter) were counted. (**D**) Effects of OmoMYC on tumor-forming ability of HCK cells were determined as for Figure 3B. (**E**) Clonogenic potential of HCK1T cells with OmoMYC or Rapamycin was determined as for Figure 4D. Indicated concentration of Rapamycin was added to the cells at day 1. (**F**) The effect of OmoMYC with Rapamycin on tumor-forming ability of HCK8T-E with HRAS cells was determined as in D. Mice were treated with 5 mg/kg Rapamycin administered by intraperitoneal injection twice a week.

transduction of four factors, E6, E7, RAS and MYC, proved sufficient for tumorigenic transformation of normal human cells tested here and broader cell types, including colon epithelial cells and pancreatic duct epithelial cells (data not shown), though hTERT might be additionally required for cells, such as HFFs, in which E6 cannot activate telomerase. In our previous study, HCK1T-E–HRAS–MYC cells adapted to DMEM showed much higher MYC expression than those kept in KGM (Supplementary Figure 4 is available at *Carcinogenesis* Online) with higher tumorigenicity, i.e. 10 DMEM-adapted cells formed huge tumors in ~50 days in contrast to the same cell number kept in KGM forming tiny tumors after ~100 days [(4) and data not shown]. DMEM-adapted cells might have gained the capacity to permit high levels of MYC and might give us a clue to understand further malignant conversion. Our preliminary data indicate that the DMEM-adapted cells exhibit epithelial–mesenchymal transition like changes, as determined by immunoblotting and microarray analysis (Supplementary Figure 4 and Supplementary Table 1 are available at *Carcinogenesis* Online).

We tried to dissect the RAS signaling pathways in order to define the critical factors for the promotion of cancer and found that activation of AKT or ERK pathway alone on the background of E6 and E7 expression was insufficient for full transformation. However, with additional induction of MYC^{T58A}, the cells acquired tumorigenicity in nude mice (Figure 3 and Table I, C). These results allow us to hypothesize that one critical player to promote cancer 'stemness' downstream of HRAS signaling is elevated function of MYC. In normal HCK1T, induced expression of MYC^{T58A} inhibited terminal differentiation and increased expression of Survivin, which is implicated as a cancer stem cell marker (31) (Figure 4A). Furthermore, we found that TSC2 expression was repressed with induction of MYC^{T58A}, as reported recently for another cell type (14), accompanied by activation of the mTOR pathway.

It was recently reported that MYC sustains pluripotency of induced pluipotent stem and embryonic stem (ES) cells through repression of the primitive endoderm differentiation regulator, GATA6 (32), and our results indicate that MYC confers resistance to calcium- and

serum-induced terminal differentiation (Figure 4A–D) and the tumorigenic potential on non-tumorigenic HCKs (Figure 3). Although MYC-^{T58A} has reduced activity to induce apoptosis compared with wild-type MYC in the mammary gland (23), it does occur in a dose-dependent manner, even in the presence of E6 and E7 (Supplementary Figure 5 is available at *Carcinogenesis* Online) and remaining cells form tumors (Figure 3). We did not observe significant differences in the tumorigenic potential with the induction of MYC^{T58A} in HCK1T-E with HRAS cells (Supplementary Figure 1 is available at *Carcinogenesis* Online). It is also reported that low levels of deregulated MYC are competent to drive ectopic proliferation of somatic cells and oncogenesis, but overexpression of MYC wakes up the apoptotic and ARF/p53 intrinsic tumor surveillance pathways (33). These results clearly indicate that a certain threshold level of MYC is sufficient for tumor development, which is not affected by further overexpression, though such surplus expression of MYC might affect other pathological features such as metastasis.

MYC has been identified as one of four genes, which can reprogram fibroblasts into ES cells (34). Analysis of the ES cell-specific gene expression signature revealed that core pluripotency factors such as OCT4 and SOX2 are active in ES and induced pluipotent stem cells but not in cancer stem cells (35), but MYC regulatory networks are activated in both ES and cancer stem cells. Thus, MYC seems to play a role in normal ES cell biology and also cancer stem cells. MYC expression is deregulated in a wide range of human cancers and the rate of overexpression is generally more than the level of amplification (36). Cancers without amplification of MYC but with alterations in other oncogenes, such as RAS and growth factor receptors, which activate the function of MYC, could also be considered as MYC deregulated. Here, inhibition of endogenous MYC functions with Omo-MYC resulted in significant reduction of tumor formation and when the mTOR pathway activated by MYC was suppressed with Rapamycin, the tumorigenic potential of HCK cells was suppressed profoundly (Figure 5). To our knowledge, this is the simplest *in vitro* carcinogenesis model for human cancer and the first report indicating that endogenous MYC is a critical regulator of HRAS-induced tumor formation by human cells. The contribution of MYC to the cancer stemness might be broader than generally considered, and attempts to inhibit MYC functions with small molecules (37) as cancer therapy might be applicable to a wide range of malignancies.

Supplementary material

Supplementary Table 1 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/.

Funding

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare (10103828) and National Cancer Center Research and Development Fund (23-B1) to T.K., and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (21590452, 23300345) to M.N.-S. and T.K.

Acknowledgements

We thank Dr Kanai and Dr Ojima, Division of Molecular Pathology and the members of the Department of Gynecology for normal HCKs and would like to express our appreciation to Ms. Ishiyama for her expert technical assistance. We are grateful to Hiroyuki Miyoshi (RIKEN, BioResource Center) for CSII-EF-MCS and the related constructs.

Conflict of Interest Statement: None declared.

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Received July 14, 2011; revised January 19, 2012; accepted February 12, 2012

Virology 422 (2012) 99–104

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Molecular cloning and characterization of a novel human papillomavirus, HPV 126, isolated from a flat wart-like lesion with intracytoplasmic inclusion bodies and a peculiar distribution of Ki-67 and p53

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ARTICLE INFO

Article history:
Received 9 August 2011
Accepted 10 October 2011
Available online 5 November 2011

Keywords:
Human papillomavirus type 126
Gamma papillomavirus
Flat warts
Adult T-cell leukemia
Ki-67
p53
Homogeneous intracytoplasmic inclusion body (Hg-ICB)

ABSTRACT

Infection with certain human papillomavirus types induces warts with specific macroscopic and microscopic features. We observed multiple flat wart-like lesions on the chest, neck and extremities of an adult T-cell leukemia patient. Histologically, atypical intracytoplasmic inclusion bodies currently known to be pathognomonic for genus gamma or mu papillomaviruses were disclosed in some cells of the epidermis showing histological features compatible with flat warts. In the present study, a novel human papillomavirus was identified and its whole genome, 7326 bp in length, was cloned and characterized. Phylogenetic analysis showed the virus designated as HPV126 to be a novel type of genus gamma papillomavirus. Strikingly, Ki-67 and p53 expression was found to be increased in all layers of the epidermis except for horny layer, contrasting to expression restricted to the basal and lower spinous layers in ordinary flat warts.

Introduction

So far, more than one hundred twenty human papillomaviruses (HPVs) have been characterized based on nucleotide sequence diversity (Bernard et al., 2010). Infections of distinct types of HPVs are characterized by type-specific cytopathic/cytopathogenic effects (CPEs), i.e., macro- and microscopic features, pathological properties, and tissue tropisms. Hence, unusual CPEs which had not previously been described may suggest that lesions could be induced by a novel type of HPV (Egawa, 2005). We recently observed intracytoplasmic inclusion bodies (ICBs) resembling the HPV 4/60/65-associated homogenous ICB (Hg-ICB) (Egawa, 1994, 2005; Egawa et al., 1993) in flat wart-like lesions of a patient with adult T-cell leukemia (ATL). However, the clinical features of the lesions proved quite different from those of HPV 4/60/65-associated skin lesions, i.e., pigmented warts (Egawa, 1988; Egawa et al., 1993) or ridged warts (Honda et al., 1994), suggesting the presence of a previously unidentified papillomavirus. While the HPV type-specific CPEs are important in understanding the biological nature of the viruses, many of the novel HPV genotypes recently isolated lacked specific cell biological aspects.

The present report describes not only isolation and molecular biological characterization of a novel HPV genotype, HPV126, but also a clinical, histopathological and immunohistochemical characterization of HPV 126-associated skin lesions, revealing this novel human genus gamma papillomavirus to induce flat wart-like lesions with Hg-ICBs. Strikingly, Ki-67 and p53, well-known cell cycle proteins, were established to be expressed in all layers of the epidermis except for horny layer in the lesions, quite different from the expression pattern restricted to basal and lower spinous layers seen in ordinary flat warts.

Results

Histopathological features of wart lesions

Disseminated hypopigmented macules clinically resembling flat warts or epidermodysplasia verruciformis-related tinea versicolor-like lesions (Jablonska and Orth, 1985) were seen on the chest, neck, and extremities of a 56-year-old Japanese patient (Fig. 1A) (Kawai et al., 2009). A biopsy was taken from the disseminated fused lesion and adjacent normal-looking skin. Microscopically, at least two independent wart-

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0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.10.011