

**Fig. 9** Comparison of immune interactions at the human maternal-fetal interface during normal pregnancy with those proposed during anti- $\beta$ 2GP1 mAb-related pregnancy loss. (Upper) In normal pregnancy, interaction of CD1d on the extravillous trophoblast cells (EVT) and maternal iNKT cells induces low level secretion of IL12 from trophoblast cells. IL12, in turn, appropriately stimulates IFN $\gamma$ -producing NK, NKT, T helper and cytotoxic T cell activity, thereby initiating a potent but tightly controlled local inflammatory cascade. This enables invasion of fetally derived EVT into the maternal decidua for appropriate placentation. (Lower) Maternal anti- $\beta$ 2GP1 antibodies are present in the decidua and in the maternal blood bathing placental villi. Direct interaction between anti- $\beta$ 2GP1 antibodies and the PS- $\beta$ 2GP1 complex presented by CD1 molecules ligates CD1d and induces strong IL12 production. Unchecked induction of the inflammatory cascade at the maternal interface might then result in pregnancy loss in the absence of placental coagulation.

direct access to CD1d-bearing trophoblast cells during human pregnancy. Through such interactions, maternal aPL may deter the otherwise well-localized and tightly controlled inflammatory process at the site of implantation.

In this study, we have shown that interactions between anti- $\beta$ 2GP1 antibodies and trophoblast CD1d molecules promote IL12 release from trophoblast cells, and IFN $\gamma$  release from decidual lymphocytes via CD1d ligation. These interactions are made possible by the surface presentation of a PS- $\beta$ 2GP1 complex by CD1d. Although it has been previously reported that PS and  $\beta$ 2GP1 form a complex at the cell surface,<sup>26,27</sup> ours is the first to demonstrate that CD1d is able to present both PS and  $\beta$ 2GP1. We also demonstrate that CD1d ligation and downstream signaling can be initiated upon exposure to anti- $\beta$ 2GP1 antibodies alone without the requirement for the secondary anti-IgG antibodies necessary in standard *in vitro* CD1d cross-linking

methods. As it is known that anti- $\beta$ 2GP1 antibodies can bind to two molecules,<sup>31</sup> these antibodies have the capacity to crosslink CD1d bearing  $\beta$ 2GP1 *in vivo*. Such *in vivo* cross-linking may, in fact, be very efficient. In our *in vitro* models, ligation of the CD1d/PS/ $\beta$ 2GP1 complex by anti- $\beta$ 2GP1 mAbs resulted in a much stronger transient induction of IL12 transcription than ligation of CD1d using anti-CD1d antibodies. Our co-culture data further demonstrate that the presence of decidual lymphocytes alone can stimulate downstream signaling thru trophoblast-expressed CD1d and that anti- $\beta$ 2GP1 mAbs can ligate the CD1d/PS/ $\beta$ 2GP1 complex regardless of the presence of decidual lymphocytes. In fact, our data suggests that antibody-mediated ligation of CD1d in the presence of decidual lymphocytes can initiate a local inflammatory cascade via transient IFN $\gamma$  release from decidual lymphocytes which are, in turn, activated by trophoblast-derived IL12. Transient cytokine release after *in vitro* CD1d ligation is known to be rapid as shown previously.<sup>29</sup> This might be the reason why the peak of cytokine release was observed at 18 hrs but not at 24 hrs. IL4, type 2 cytokine from iNKT cells, release was not observed in this co-culture system (data not shown).

We propose the following mechanism for anti- $\beta$ 2GP1 antibody-related pregnancy loss (Fig. 9; lower panel). In a mother positive for anti- $\beta$ 2GP1 antibodies, CD1d-bearing EVT will be exposed to these antibodies at the maternal-fetal interface during early gestation. Direct interaction between anti- $\beta$ 2GP1 antibodies and the PS- $\beta$ 2GP1 complex presented by CD1d molecules ligates CD1d and induces potent downstream IL12 production. IL12 activates maternal IFN $\gamma$ -producing NK, NKT, and T cells. IFN $\gamma$  derived from maternal lymphocytes upregulates CD1d expression on the surface of the EVT.<sup>16</sup> This overexpression of CD1d enhances anti- $\beta$ 2GP1 antibody-mediated cross-linking in a feed-forward fashion. Unchecked induction of the inflammatory cascade at the maternal interface could then result in pregnancy loss in the absence of placental coagulation. Further study using additional clinical materials is needed to verify this novel mechanism for aPL-related pregnancy loss.

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# Productive lifecycle of human papillomaviruses that depends upon squamous epithelial differentiation

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Human papillomaviruses (HPVs) target the stratified epidermis, and can cause diseases ranging from benign condylomas to malignant tumors. Infections of HPVs in the genital tract are among the most common sexually transmitted diseases, and a major risk factor for cervical cancer. The virus targets epithelial cells in the basal layer of the epithelium, while progeny virions egress from terminally differentiated cells in the cornified layer, the surface layer of the epithelium. In infected basal cells, the virus maintains its genomic DNA at low-copy numbers, at which the viral productive lifecycle cannot proceed. Progression of the productive lifecycle requires differentiation of the host cell, indicating that there is tight crosstalk between viral replication and host differentiation programs. In this review, we discuss the regulation of the HPV lifecycle controlled by the differentiation program of the host cells.

**Keywords:** HPV, differentiation, epithelial cell, keratinocyte

## INTRODUCTION

Human papillomavirus (HPV) infections of the anogenital organs are a very common “sexually transmitted disease (STD).” Although the incidence of cancer progression is low, a HPV infection is frequently detected in anogenital cancers. As for cervical cancer, HPV DNA is detected in more than 90% of cases. Approx. 5.5 million new cases of HPV infection are reported and there are c.a. 450,000 diagnoses of cervical cancer per year worldwide, leading to approximately 200,000 deaths each year, which ranks second among cancers in women (Parkin and Bray, 2006). HPV infections have also been associated with the head and neck squamous cell carcinomas (HNSCCs).

Human papillomavirus is categorized as a small virus containing DNA. More than 120 types of HPV have been identified and one-third of them target mucosal membranes, the remainder target the cutaneous membranes. Mucosa-tropic HPVs can be classified into two types based on their association with malignant carcinomas: a high-risk type (such as HPV type 16, 18, 31) and a low-risk type (such as HPV6 and 11; Howley, 1996). Prophylactic vaccines for HPV16 and 18, Cervarix (GlaxoSmithKline), and for HPV6, 11, 16, and 18, Gardasil (Merck & Co.), have been developed recently and effectively prevent primary infections. They, however, cannot be used as therapeutic vaccines, indicating the importance of a Pap smear and the development of effective treatment strategies (Carter et al., 2011). In order to inhibit HPV-induced cancer, an understanding of the molecular basis of the infection and the characteristics of the infected lesions is important.

## GENOME ORGANIZATION OF HPV AND FUNCTIONS OF VIRAL PROTEINS

Human papillomaviruses have a common gene organization (Figure 1): an early region encoding non-structural genes, the late

region for structural genes, and a regulatory region (long control region: LCR).

The functions of each viral protein are summarized in Table 1. E1 and E2 are cooperatively involved in the initiation of viral DNA replication. E2 also functions as a transcriptional transactivator. E6 and E7 modulate the cell cycle control and contribute to viral genome maintenance (Frattoni et al., 1996; Stubenrauch et al., 1998; Thomas et al., 1999). They also contribute to cancer development (Münger et al., 2004). Though E4 and E5 are speculated to modulate the productive phase of the HPV lifecycle, their biological roles remain unclear (Fehrmann et al., 2003; Genther et al., 2003; Nakahara et al., 2005; Wilson et al., 2005, 2007; Fang et al., 2006). Both L1 and L2 are capsid proteins.

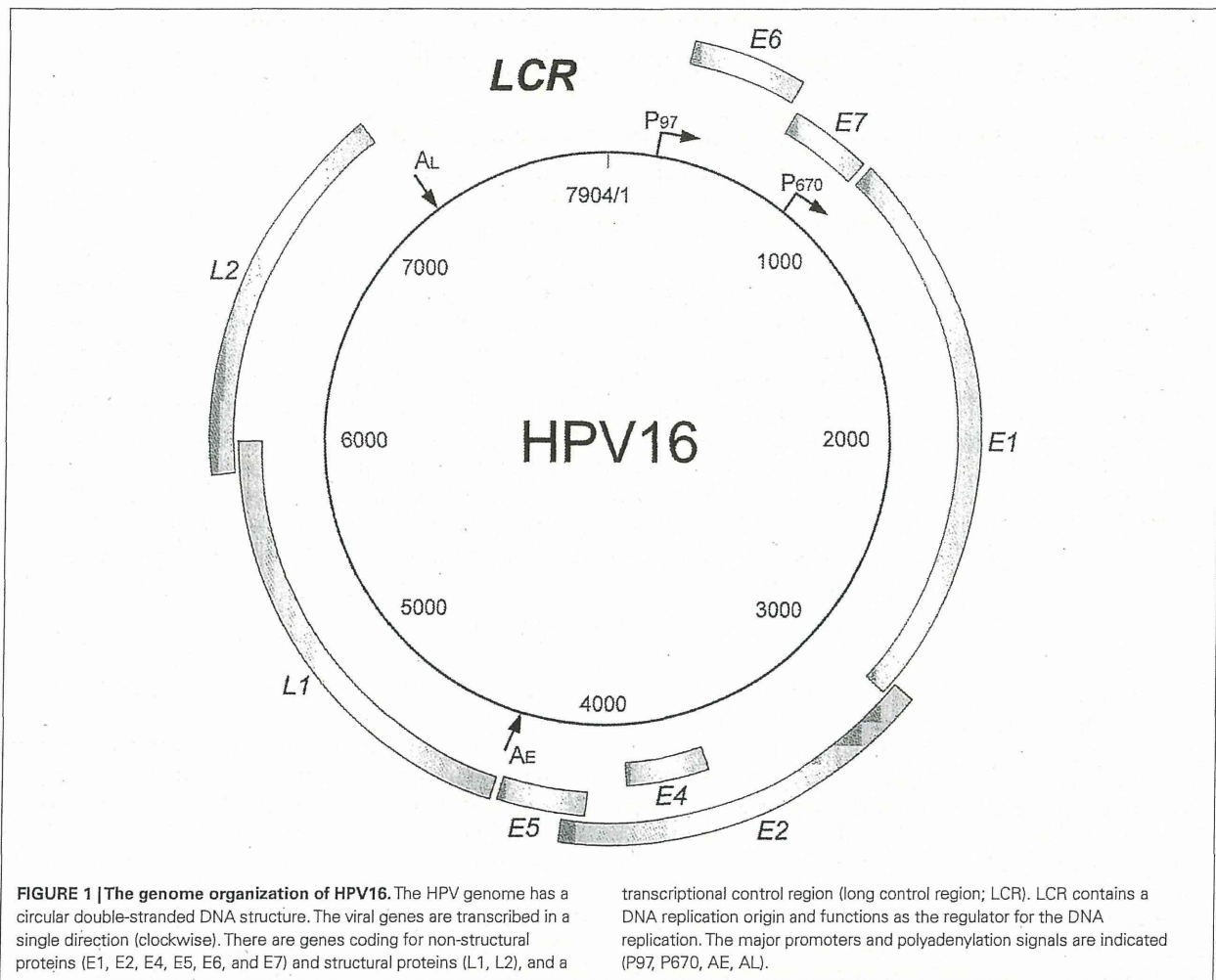
## HPV LIFECYCLE

The target of a HPV infection is the stratified epithelium. In the normal stratified epithelium, the cell attached to the basal membrane (basal cell) is the only cell that has the potential to proliferate. The basal cell divides into a new basal cell and a daughter cell that is detached from the basal membrane, and the daughter cell launches its differentiation process. The daughter cells exit from the cell cycle and change their gene expression pattern, proceeding to terminal differentiation, then peel off from the epithelium (Jones et al., 2007). The lifecycle of HPV is tightly regulated by the differentiation program of the host cells (Figure 2). In this section, the differentiation-dependent lifecycle of HPV is briefly summarized.

## ENTRY OF HPV INTO THE BASAL CELLS OF STRATIFIED EPIDERMIS

Human papillomavirus virions invade through damaged areas of the epithelium and infect the basal cells. Although the receptor for the HPV infection has not been fully characterized, the following





model has been postulated; virions initially attach to the heparan sulfate proteoglycan (HSPG) on the basal membrane, and transfer to the receptor expressed on the keratinocytes moving on the basal membrane in the wound-healing process, then enter the cells (Kines et al., 2009).

#### LOW-LEVEL EXPRESSION OF VIRAL GENES AND GENOME MAINTENANCE IN THE BASAL LAYER

Following viral entry and uncoating, HPV genomic DNA is transported into the nucleus and maintained at a low-copy number in the basal cells (50 ~ 100 copies per cell; in the basal layer, Figure 2; Moody and Laimins, 2010). Genome maintenance as episomal status is essential for the establishment of the early phase of the viral lifecycle (McBride et al., 2006).

#### PRODUCTIVE REPLICATION OF HPV IN THE DIFFERENTIATED CELLS

After leaving the basal membrane, the infected cells initiate the differentiation program. Because HPV does not encode DNA polymerase activity for viral genome replication, the host DNA replication machinery is required. However, the DNA replication activity is suppressed in the differentiated cells that exit from the

cell division cycle. To ensure that the viral genome is replicated, HPV needs to reactivate cell division among the differentiation-initiated cells. E6 and E7 inactivate p53 and retinoblastoma protein (pRb), respectively, which enables the cells to maintain their DNA replication potential (Münger et al., 2004).

In the upper layers of the stratified epithelium (in the spinous layer, Figure 2), the expression of viral genes that are required for viral genome replication is markedly accelerated (Hummel et al., 1992; Ozbun and Meyers, 1997), inducing viral genome amplification to thousands of copies per cell (Bedell et al., 1991). Following the genome amplification, in the terminally differentiated cells, the synthesis of capsid proteins is triggered. The capsid proteins assemble into virions that encapsidate viral genomic DNA. The progenitor virions are released externally with peeled keratinocytes.

#### DIFFERENTIATION-DEPENDENT CONTROL OF HPV LIFECYCLE

The differentiation-dependent lifecycle of HPV is controlled of multiple levels, such as transcription, post-transcriptional processing, translation, and DNA replication. In the following sections, each regulatory mechanism is summarized.

Table 1 |

Function in viral lifecycle	Activities	Target factor
<b>E1</b> Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
<b>E2</b> Transcription of viral genes Replication of viral genome Maintenance of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChR1
<b>E6</b> Reactivation of cellular replication mechanisms Proliferation, immortalization, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	p53, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
<b>E7</b> Reactivation of cellular replication mechanisms Proliferation, genomic instability, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	RB, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
<b>E4</b> ?	Destruction of keratin network, induction of G <sub>2</sub> M arrest of cell cycle	Cytokeratin 8/18
<b>E5</b> ?	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
<b>L1</b> Major capsid protein		
<b>L2</b> Minor capsid protein		

### TRANSCRIPTIONAL REGULATION OF VIRAL GENES

Human papillomavirus has two major promoters, the early promoter and the late promoter. In HPV16, P97, and P670 have been identified as the early and late promoters, respectively (Figure 1). Transcriptional activity is mainly controlled by the LCR. A transcriptional enhancer is located within the LCR, with which various cellular transcription factors can associate (Figure 3).

The binding sites for the viral transcriptional regulator, E2, are found in HPV16 LCR. Viral gene expression is regulated by the occupancy status of the E2-binding sites (E2BSs; Figure 3), which is partly defined by the E2 expression level controlled by cellular differentiation status (Steger and Corbach, 1997; Hadaschik et al., 2003).

E2 functions in viral genome segregation by tethering the viral DNA to the mitotic chromatin, in which a cellular protein, bromodomain-containing protein 4 (Brd4), has been reported to be involved (McPhillips et al., 2006). Interaction between E2 and Brd4 is also required for the E2-mediated transcriptional activation and repression (McPhillips et al., 2006; Wu et al., 2012).

A ubiquitous transcription factor, Sp1 is a well-known regulator for HPV gene expression. The Sp1-binding site partially overlaps with one of the E2BSs (E2BS#2), and a TATA box element is located close to the promoter-proximal E2BS (E2BS#1; Figure 3). The binding of E2 to those E2BSs, therefore, interferes

with the assembly of the transcriptional initiation complex, resulting in a suppression of E6/E7 expression that is governed by the early promoter activity (Tan et al., 1992). It was also reported that Sp1 altered the chromatin structure of HPV16 LCR, offering the accessibility of transcription factors (Stümel and Bernard, 1999).

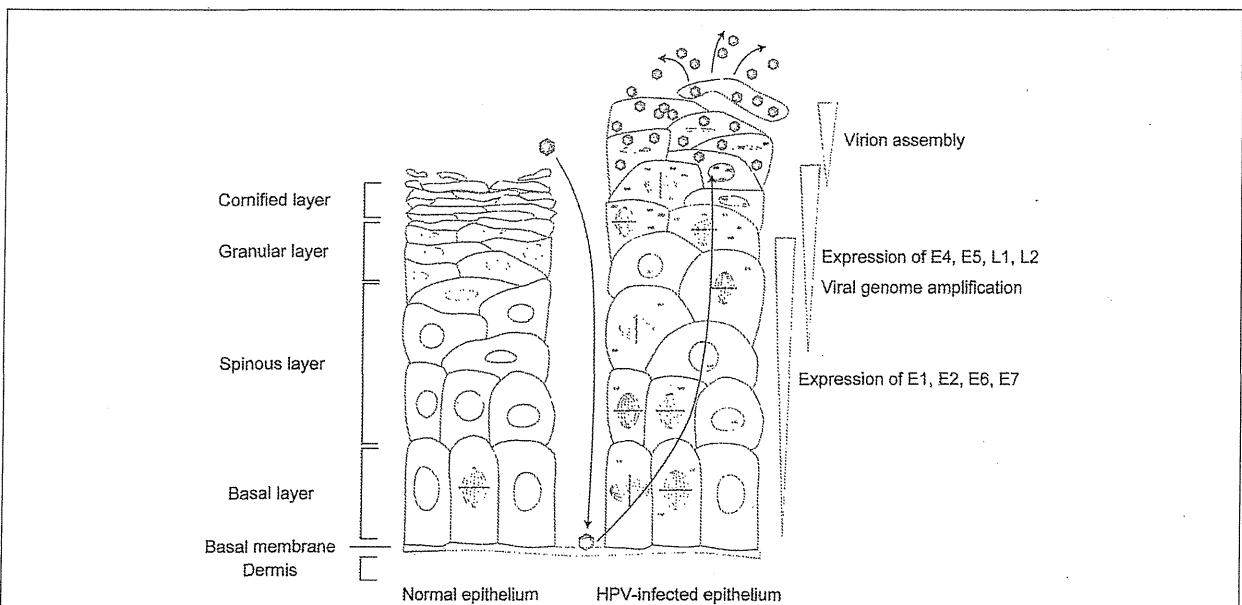
### TRANSCRIPTIONAL CONTROL IN THE UNDIFFERENTIATED CELLS

Transcripts of viral early genes are expressed in the infected basal cells, which is essential for the viral DNA replication (Dürst et al., 1992). It was reported that a unique promoter, P14, was utilized for E1 expression and the E2BSs were considered as necessary for the P14 activity (Lace et al., 2008). The transcript initiated from P14 is a poly-cistronic mRNA containing E6, E7, and E1, in which the shunting in ribosomal scanning process enables the translation of E1 (Reimm et al., 1999). The regulatory mechanism for E2 expression has not been clarified. The early promoter is used for E6 and E7 expression, in which several transcription factors, including AP-1, glucocorticoid receptor, NF1, Oct-1, Sp1, YY-1, and CDP, are involved (Figure 3; Desaintes and Demeret, 1996).

### TRANSCRIPTION IN THE DIFFERENTIATED CELLS

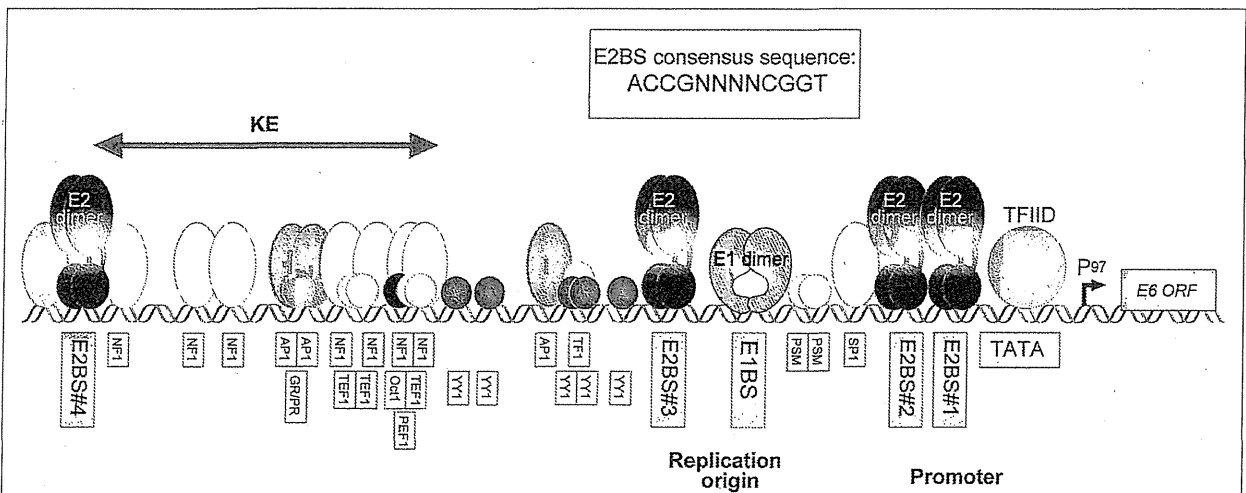
The early promoter is activated in association with the differentiation process, increasing the E1/E2 expression (Hummel et al., 1992; Ozburn and Meyers, 1997). Although levels of E6 and E7 also





**FIGURE 2 |** The lifecycle of human papillomaviruses (HPVs). HPVs infect specifically the cells in the basal layer of the stratified epithelium through lesions. Viral genomes are maintained as episomal DNA in the nuclei of infected cells. The viral lifecycle is

strictly controlled by host cell differentiation, and the late lifecycle (productive lifecycle) occurs in upper layers of the epithelia that are terminally differentiated, and the progenitor virions are released from the cornified keratinocytes.



**FIGURE 3 |** The structure of HPV16 LCR (region of the control of early promoter P97). The early promoter P97 and replication origin are located in LCR, which are regulated by various cellular factors. Activity of P97 is regulated by AP-1, NF1, SP1, TFIID, TF1, Oct-1, PSM, and the viral transcription factor E2. Four E2-binding sites (E2BS) have been identified

in HPV16 LCR and the consensus sequence for E2BS is shown in an inset. A glucocorticoid receptor and progesterone receptor (GR/PR) recognition element was also identified in the LCR. The existence of a keratinocyte-specific enhancer (KE) has been proposed (Desaintes and Demeret, 1996).

increase with the early promoter's activation, the E2 overexpressed in the upper layer is thought to suppress their transcription via the mechanism mentioned above. E6 and E7 are important in maintaining infected cells in an undifferentiated state, but terminal differentiation is required for the productive replication of HPV.

The inhibition of E6/E7 expression by E2 might promote cellular differentiation, and the cells undergo terminal differentiation, which is suitable for the viral productive lifecycle.

AP-1, a heterodimer composed of Fos and Jun, is considered to be involved in the differentiation-dependent transcriptional

control in keratinocytes; there are reports that the expression profiles of Fos and Jun family members were modified, and that the interaction between AP-1 and KRF-1, a keratinocyte-specific transcription factor (Mack and Laimins, 1991), was strengthened in the differentiation process (Desaintes and Demeret, 1996; Thierry, 2009). Several transcriptional factors were reported to be involved in the differentiation-dependent control of LCR function; EPOC-1/Skn-1a, C/EBP- $\alpha$ , - $\beta$ , c-Myb, NF1, NFATx, Pax5, and WT1 (Desaintes and Demeret, 1996; Thierry, 2009).

The late promoter is specifically activated in the differentiated layers of epithelium. The late promoter activity is suppressed by CDP (CCAAT displacement protein) and YY-1, whose binding potential was reported to be decreased in differentiated keratinocytes (Ai et al., 1999, 2000). There was also a report that the expression ratio of a transcription factor, Sp1 and its antagonist, Sp3, was altered through the differentiation, which activated the late promoter activity (Apt et al., 1996). The binding of hSkn-1a and C/EBP $\alpha$  to the proximal region of the late promoter contributes to the control of the late promoter activity (Kukimoto and Kanda, 2001; Woodriddle and Laimins, 2008). The involvement of E7 in the regulation of the late promoter activity was also described (Bodily and Laimins, 2011; Bodily et al., 2011). It still remains necessary to clarify the regulatory mechanism for the late promoter in the differentiation of epithelial cells.

**METHYLATION OF THE HPV GENOME DURING THE CELL DIFFERENTIATION PROCESS**

HPV gene expression is controlled by the methylation of HPV genomic DNA. As E2BSs contain CpG dinucleotides (see inset in Figure 3), they can be modified by DNA methylation in the host

cell. E2BSs are reported to be highly methylated in undifferentiated cells, inhibiting E2-binding, and demethylation at the E2BSs occurs in association with the cell differentiation (Kim et al., 2003; Vinokurova and von Knebel Doeberitz, 2011).

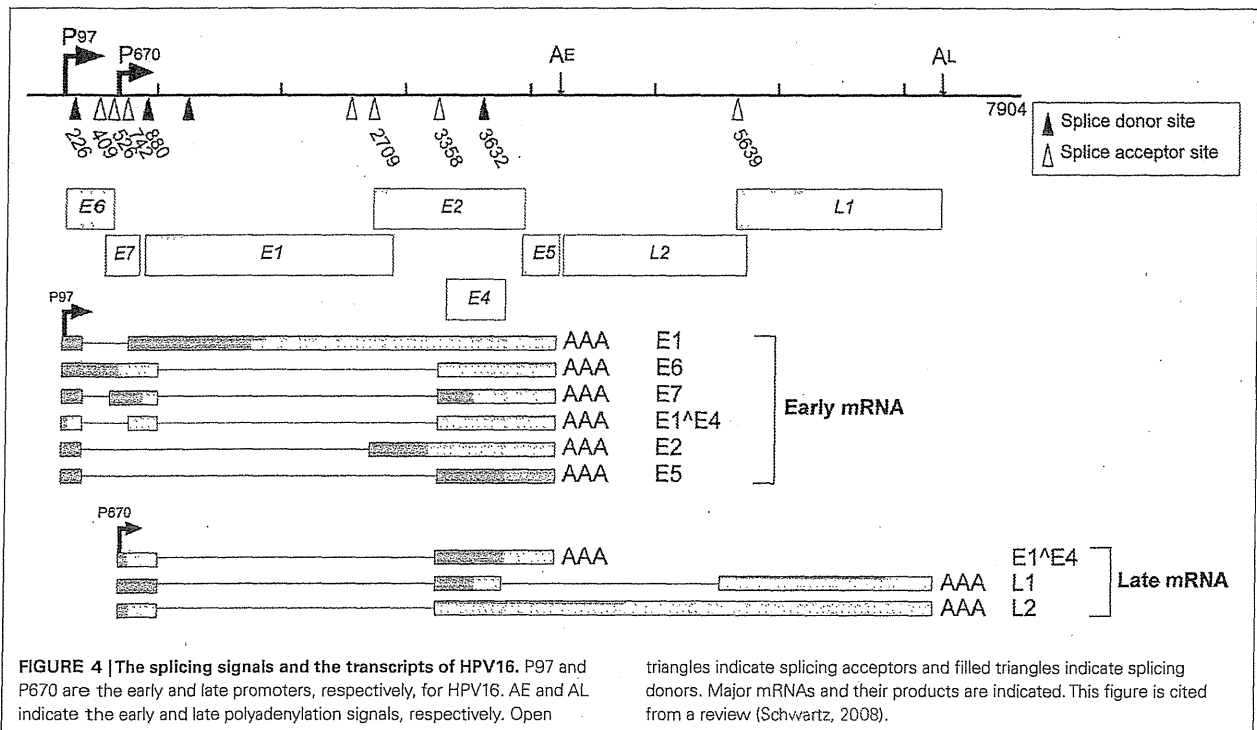
**RNA PROCESSING**

For conversion of the gene expression profile from the early to late phase of viral replication, RNA processing is considered critical. The primary transcript of HPV encodes multiple viral genes, and precise RNA processing is essential to produce the mRNA for each viral gene at an appropriate stage of cell differentiation (Schwartz, 2008).

In the early phase of the viral lifecycle, the primary transcription initiated by the early promoter is terminated at the early poly(A) signal, AE (Figure 1), and the transcript is processed by using the early splicing signals, which produces the mRNAs encoding the viral early genes. In the differentiated cells, the transcripts for the late genes are expressed from the late promoter and utilize a late poly(A) signal, AL (Figure 1), and late splicing signals. The early and late splicing signals compete for the splicing factors, so their usages are generally exclusive.

Multiple splicing signals are found in the HPV genome, which are utilized for the expressions of various viral genes (Figure 4). These splicing signals can be categorized into three groups; early phase-specific signals (DS226, SA409, SA526, SA742 in HPV16), late phase-specific signals (SD 3632 and SA5639), and non-specific signals (SD880, SA2709, SA3358; Schwartz, 2008).

Early splicing events have three major roles; regulation of the expression ratio of early genes, production of splicing variants of viral genes, and suppression of late gene expressions. The early



**FIGURE 4 |** The splicing signals and the transcripts of HPV16. P97 and P670 are the early and late promoters, respectively, for HPV16. AE and AL indicate the early and late polyadenylation signals, respectively. Open

triangles indicate splicing acceptors and filled triangles indicate splicing donors. Major mRNAs and their products are indicated. This figure is cited from a review (Schwartz, 2008).



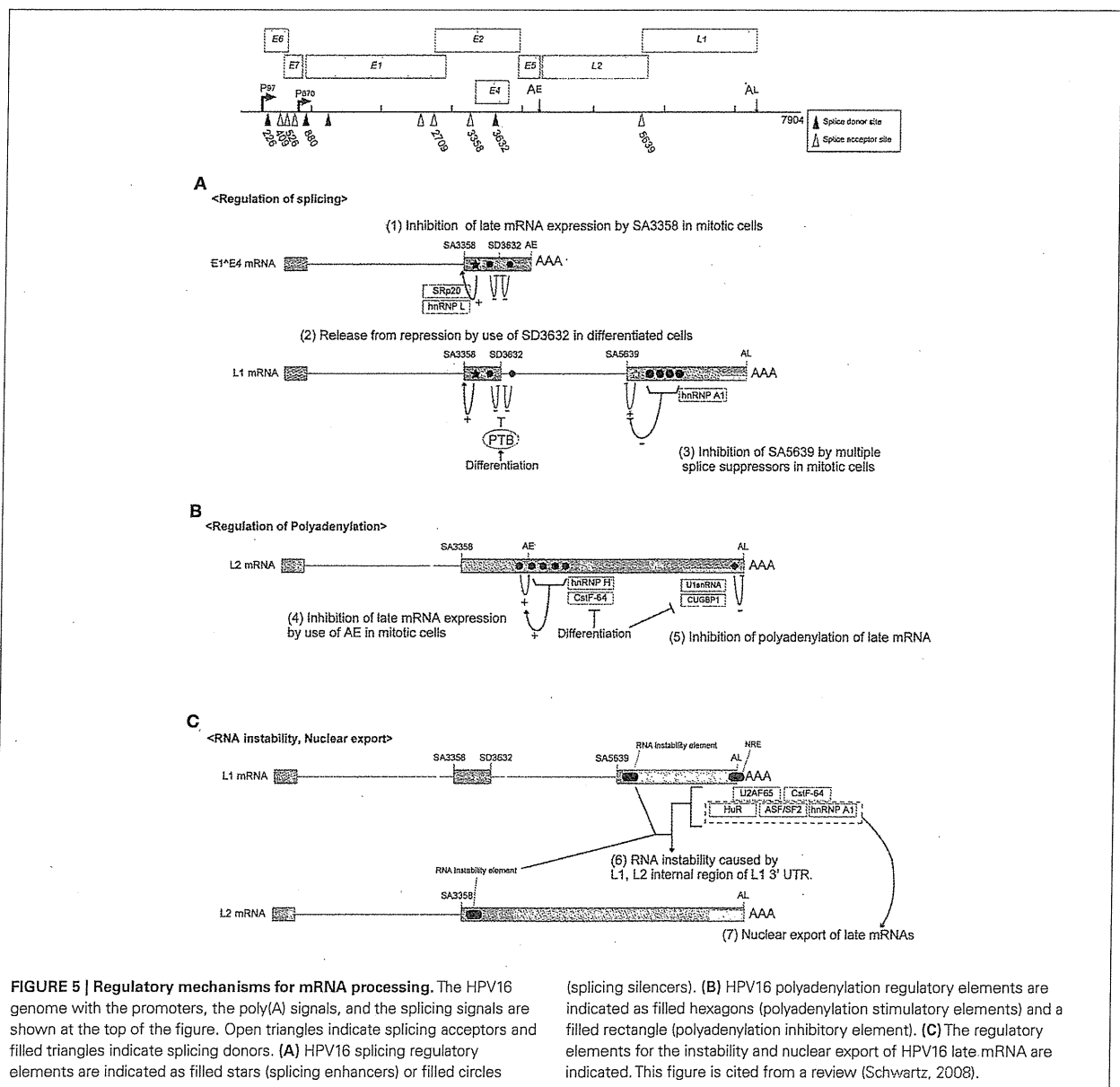
splicing sites of HPV16, SD226, SA409, and SA526, are located in the E6 ORF, which enhances the translation efficiency of E7. Because the initiation codon for E6 is leaky and there are several splicing signals in the E6 ORF, various variants of E6 can be expressed. Those variants were reported to counteract the full-length E6, which might be important for the fine-tuning of E6 activities.

SA3358 is utilized in both the early and late phases of viral replication to produce HPV16 E1<sup>+</sup>E4 mRNA. A strong splicing enhancer was identified downstream of SA3358, and it accelerated the polyadenylation at AE and suppressed the late gene expression in the early phase (Figure 5A). In undifferentiated cells, SA3358 competed with the late splicing signals for the splicing factors

(SRp20, hnRNPL etc.), which might contribute to the suppression of late gene expression (Rush et al., 2005; Jia et al., 2009).

The late mRNAs are transcribed from the late promoter, and a major splicing event occurs between SD880 and SA3358 in HPV16. For the production of L1 mRNA, additional splicing between SD3632 and SA5639 is required. The mRNAs for L1 and L2 are poly-cistronic, and the mechanism for bypassing the upstream ATG remains to be explained.

SD3632 in HPV16 is used exclusively for late mRNA production. SD3632 is located close to SA3358 and AE, and the usage of SD3632 was suppressed by a splicing suppressor in dividing cells. It was indicated that the polypyrimidine tract-binding protein (PTB) interfered with the splicing suppressor in differentiated



cells, which potentiated late gene expression (Figure 5A; Somberg et al., 2008).

A late phase-specific SA5639 in HPV16 was reported to be regulated by the cis-acting elements found in the L1 coding region (Zhao et al., 2004). A splicing enhancer was identified in the 3' region of SA5639. Although the enhancer was activated in dividing cells, its function was hindered by multiple splicing suppressors located in the L1 coding region. It was reported that hnRNP A1 is associated with those multiple splicing suppressors (Figure 5A; Zhao et al., 2007).

To produce the late mRNAs, it is essential to bypass the polyadenylation at AE. Enhanced utilization of AE could, therefore, inhibit the late gene expression. The 5' 400 nt of the HPV16 L2 ORF was reported to enhance the polyadenylation at AE, where multiple-G-motifs are located (Oberget et al., 2005). The hnRNP H interacted with the G-motifs and accelerated the polyadenylation at AE, and the expression of hnRNP H decreased as the cell differentiation progressed. CstF-64 was also reported to bind the L2 coding region of HPV31 and enhance the polyadenylation at AE, and the expression of CstF-64 diminished during the cell differentiation process (Figure 5B; Terhune et al., 2001).

Within the 3'UTR of the late mRNA (late UTR), a motif highly homologous to the U1snRNA was identified. It was reported that U1-70K, a component of U1snRNP, bound to the U1snRNA motif of BPV1 and interfered with the polyadenylation (Furth et al., 1994). Although U1-70K binding was not detected with HPV16, CUG binding protein 1 (CUGBP1) was reported to interact with the late UTR element of HPV16 and inhibit the polyadenylation process (Figure 5B; Goraczniak and Gunderson, 2008).

#### CONTROL OF LATE mRNA STABILITY

There are RNA instability elements within the L1 and L2 coding mRNAs of HPV16, which function in undifferentiated cells (Mori et al., 2006), although the mechanism for RNA destabilization remains to be clarified (Sokolowski et al., 1998; Collier et al., 2002). There is a GU-rich negative regulatory element (NRE) in the late UTR of HPV16, which is a RNA instability element (Kennedy et al., 1991). Various factors, ASF/SF2, CstF64, U2AF65, hnRNPA1, and HuR, are reported to regulate the differentiation-dependent events of HPV replication through binding to the NRE (Figure 5C; Dietrich-Goetz et al., 1997; Koffa et al., 2000; Cheunim et al., 2008).

A highly U-rich region was located in the early UTR of HPV16 and reduced mRNA stability; a U-rich region is a signature feature of unstable mRNA (Jeon and Lambert, 1995; Barreau et al., 2005).

#### NUCLEAR EXPORT OF LATE mRNAs

The L1 mRNA of HPV16 was retained in the nucleus in undifferentiated W12 epithelial cells (Koffa et al., 2000), suggesting that the nuclear export of late mRNAs was inhibited in the dividing cells. Although the factor(s) that mediates the nuclear export of late mRNAs has not been identified, candidates include HuR, hnRNA, and ASF/SF2, which are proteins shuttling between the nucleus and cytoplasm (Figure 5C; Carlsson and Schwartz, 2000; Koffa et al., 2000; McPhillips et al., 2004; Zhao et al., 2004).

#### TRANSLATIONAL CONTROL OF LATE GENE EXPRESSION

As the inhibitory mechanism for late gene expression, the involvement of translational inhibition was also reported. Translation efficiency was suppressed with *in vitro* translated RNA containing the late UTR of HPV1, suggesting the late UTR had the potential to inhibit the translation. For the inhibition, poly(A)-binding protein (PABP) was considered to be responsible (Wiklund et al., 2002). It was also reported that poly(C) binding protein 1 and 2 (PCBP-1 and -2) and hnRNP K bound to the L2 coding region of HPV16 mRNA and inhibited the *in vitro* translation (Collier et al., 1998). The rare codon usages found in L1 and L2 might also contribute to the inhibition of late gene translation (Gu et al., 2004). In terminally differentiated cells, the altered expression ratios of tRNA species could compensate for the inhibitory effect of the rare codon usages (Fang et al., 2007).

#### REGULATION OF VIRAL DNA REPLICATION

E1 and E2 have essential roles in the HPV genome's replication (Kadaja et al., 2009). E2 is a DNA-binding protein that recognizes E2-binding sites (E2BSs) located in the LCR (Figure 3). E2 has transcriptional transactivator activity, as well as the capacity to bind to the viral DNA replication factor E1. E1 has DNA helicase and ATPase activities and weak DNA-binding capacity. Through its interaction with E2, E1 is recruited to the replication origin (ori), which is essential for the initiation of viral DNA replication (Chiang et al., 1992a,b). E2 also contributes to the segregation of viral DNA in the cell division process by tethering the viral DNA to the host chromosome through interaction with Brd4 and/or ChlR1, both of which can bind to chromatin (McBride et al., 2006). Accurate segregation of the viral genome is essential to maintain the HPV infection in the basal cells, in which the copy number of the viral genome is very low.

The replication potential of E1 is regulated by its interaction with cellular proteins. p56, one of the interferon stimulated genes (ISGs), directly interacts with E1 and translocates it to the cytoplasm, which might contribute to the interferon-mediated inhibition of HPV replication (Terenzi et al., 2008). The interaction of WD repeat protein p80 (WDR80) with E1 is reported to be required for the efficient maintenance of the viral genome in undifferentiated keratinocytes (Côté-Martin et al., 2008).

As mentioned, the expression levels of E1 and E2 increase in association with the differentiation of the epithelial cells, which could be responsible for the vegetative genome amplification.

Recently, it was reported that E6 and E7 activated the ATM DNA damage pathway in differentiation-dependent manner, by which Chk2 was activated. Chk2 potentiated caspase-3 and -7, and the caspases in turn cleaved the E1 protein, which might be required for viral DNA amplification in the differentiated cells (Moody et al., 2007; Moody and Laimins, 2009).

Nuclear accumulation of E1 blocks cell cycle progression in early S-phase and triggers the activation of a DNA damage response (DDR) and of the ATM pathway (Fradet-Toucotte et al., 2011), and the activation of DDR might facilitate HPV DNA replication (Sakakibara et al., 2011). The nuclear accumulation of E1 is regulated by phosphorylation of the nuclear export signal (NES) found in E1 via Cyclin E/A-Cdk2 (Fradet-Toucotte et al., 2010).



DNA replication of HPV utilizes the replication mode with a “ $\theta$ -structure” in undifferentiated cells, and the mode changes to “rolling circle replication” in differentiated cells, which enables the rapid synthesis of multiple copies of viral DNA. The molecular mechanism supporting the DNA replication in the differentiated cells, however, has not been fully explained (Flores and Lambert, 1997).

#### INVOLVEMENT OF CELLULAR TRANSCRIPTION FACTORS IN VIRAL DNA REPLICATION

It was reported that the binding of hSkn-1a to its recognition site proximal to the ori region was required for the viral genome replication of HPV16 (Kukimoto et al., 2008). In other HPV types, Sp1 and TATA box binding protein (TBP) inhibit viral genome replication, in which competition between E2 and Sp1 or TBP may be involved (Demeret et al., 1995; Hartley and Alexander, 2002). These transcription factors might alter the chromatin structure, which could inhibit the access of E1 to the origin (Demeret et al., 1995). The inhibition of STAT-1 expression by E6 and E7 was also reported to be important for viral genome amplification (Hong et al., 2011).

#### VIRION PRODUCTION

A report described that HSP70 was activated in response to the keratinocyte differentiation and co-localized with HPV31 L1 in the differentiated layer of epithelium (Song et al., 2010). It was reported that the disulfide bond among the HPV16 L1 pentamer was formed in a differentiation-dependent manner and had an important role in virion stability (Conway et al., 2011), indicating that virion production was regulated not only by the quantity of the late gene products but also by a differentiation-dependent mechanism.

#### THE MODULATION OF CELL PROLIFERATION/DIFFERENTIATION BY HPV GENE PRODUCTS

As described above, HPV replication is strictly regulated by the differentiation program of the host cells. Conversely the HPV infections modulate the proliferation/differentiation status of the host cells, indicating tight communication between the virus and the host cell, which is required for completion of the viral replication.

#### ACCELERATION OF CELL PROLIFERATION AND INHIBITION OF CELL DIFFERENTIATION

The acceleration of cell proliferation and inhibition of differentiation are induced by the expression of E6 and E7 (Longworth and Laimins, 2004; Moody and Laimins, 2010). E7 inhibits the functions of the pocket protein family, activates the E2F-dependent promoter, and induces S-phase-specific gene expression (Moody and Laimins, 2010). E7 maintains Cyclin E or Cyclin A-CDK2 activity in differentiated cells by targeting p21 and p27, important regulators for growth arrest in the differentiation process. E6 mediates ubiquitination of p53 in association with E6AP, causing the proteasomal degradation of p53 (Moody and Laimins, 2010), and disturbs p53-mediated growth arrest. The association between E6 and histone acetyltransferases (HATs) might be also involved in the inhibition of p53 function (Moody and Laimins, 2010). E6 was reported to target cellular PDZ proteins, which might contribute to the immortalization of the infected cells (Thomas et al.,

2008). E6 was reported to activate telomerase activity by inducing the overexpression of c-Myc and by modulating the expression of NFX1-91, which also contributed to the immortalization process (Gewin et al., 2004).

The functions of E6 and E7 in the activation of the DNA replication machinery of the host cell are required to ensure the viral genome's replication in the differentiated cells (Thomas et al., 1999), resulting in the aberrant proliferation and the retarded differentiation of the host cells. With a transgenic mouse model expressing HPV16 E6 and/or E7 under the K14 promoter, dysplasia was observed at the stratified epidermis (Griep et al., 1993).

Although the normal differentiation of keratinocytes is not fully understood, reports describe the involvement of Notch, MAPK, NF- $\kappa$ B, p63, the AP2 family, C/EBP, IRF6, GRHL3, and KLF4 (Blanpain and Fuchs, 2009). There are also papers describing the contribution of c-Myc to the differentiation of epithelial stem cells, and differentiation-dependent demethylation at histone H3K27 (Blanpain and Fuchs, 2009). Recently, some of these factors were found to be associated with HPV gene products (Lathion et al., 2003; Chakrabarti et al., 2004; An et al., 2008; Melar-New and Laimins, 2010), and it is reasonable that these associations modify the cell differentiation program of the infected cells.

#### INHIBITION OF THE APOPTOTIC INDUCTION

The aberrant proliferation and/or DNA replication in the HPV-infected cells induce p53-dependent apoptotic cell death. The inactivation of pRb by E7 also potentiates the p53 activity. The p53-dependent apoptosis is counteracted by E6 activity (Garnett et al., 2006; Moody and Laimins, 2010). E6 induces the proteasomal degradation of p53. It has also been reported that E6 directly binds to p53 and inhibits its DNA-binding potential (Lechner and Laimins, 1994), and that E6 interacts with HDAC p300, ADA3, and/or CREB-binding protein (CBP), which destabilizes p53 (Patel et al., 1999; Zimmermann et al., 1999; Kumar et al., 2002). The HPV-infected cells also escaped from anoikis by the E6-mediated expression of FAK and the phosphorylation of paxillin, which activates FAK (McCormack et al., 1997). It was reported that interaction between E7 and p600 was involved in the inhibition of anoikis (Huh et al., 2005).

Several membrane-spanning death receptors have been identified, such as TNF receptor type 1 (TNFR1), FAS receptor, and TRAIL receptor. The high-risk type E6 was reported to interact with TNFR1 and suppress TNF $\alpha$ -induced apoptosis (Filippova et al., 2002). E6 is also known to interact with FADD and caspase8, which might inhibit the apoptosis mediated by FAS and TRAIL (Filippova et al., 2004; Garnett et al., 2006). In addition, E6 was reported to be associated with pro-apoptotic Bcl2 members, BAK and BAX, and to interfere with intrinsic apoptosis (Garnett et al., 2006). It was reported that E5 could inhibit FAS- and TRAIL-mediated apoptosis (Garnett et al., 2006). In addition, there was a paper that described the inhibitory effect of E7 on apoptosis, in which the upregulation of cellular inhibitor of apoptosis protein (c-IAP) by E7 was involved (Garnett et al., 2006).

#### THE MODULATION OF miRNA EXPRESSION THROUGH CELL DIFFERENTIATION

Recently, it was reported that HPV E6 and E7 induced the aberrant expression of tumor suppressive miRNAs (Zheng and Wang,

2011). E6 and E7 are known to target c-Myc, p53, and E2F, and these transcription factors are reported to be involved in the regulation of miRNA expression, so E6 and E7 could cause the uncoordinated expression of those miRNAs. E6 and E7 target p53 and pRb, respectively, and cause the unregulated expression of the miR-15/16 cluster, the miR-17-92 family, miR-21, miR-23b, miR-34a, and the miR-106b/93/25 cluster. Such aberrant expression was suspected to be involved in the development of cervical cancer. It was also reported that E6, E7, and E5 suppress the expression of miR-203 which participates in the differentiation of epithelial cells (McKenna et al., 2010; Greco et al., 2011). Through the suppression of miR-203, the expression level of p63 is upregulated in the differentiated cells, which might contribute to the genome amplification and the late gene expression in the upper layers of epithelium (Melar-New and Laimins, 2010).

**EPIGENETIC ALTERATION INDUCED BY THE HPV INFECTION**

There was a report describing the epigenetic alteration of cells mediated by HPV gene expression (Hsu et al., 2011; Hyland et al., 2011; McLaughlin-Drubin et al., 2011; Zheng and Wang,

2011). The epigenetic alteration induced by the HPV infection was considered to modify the miRNA expression pattern, which might change the cell differentiation program. Although there is a possibility that some epigenetic alteration occurs also in the normal cell differentiation process, there has been no report related to it.

**CONCLUSION**

Human papillomavirus suppresses its replication to a “maintenance level” or “latent infection mode” in the basal cells, and maintains the DNA synthesis potential of the infected cells detached from the basal membrane to maintain viral genome replication. In terminally differentiated cells, a tremendous level of genome amplification and late gene expression takes place. After completion of virion assembly, the virions are released externally with the cornified cells (the regulation of the differentiation-dependent lifecycle of HPV is summarized in Figure 6). One of the reasons for this unique lifecycle of HPV is escape from the immune-surveillance system (Bodily and Laimins, 2011). Because both L1 and L2 could have immunogenicity, their expressions should be suppressed until the infected cells move to the upper layer of the

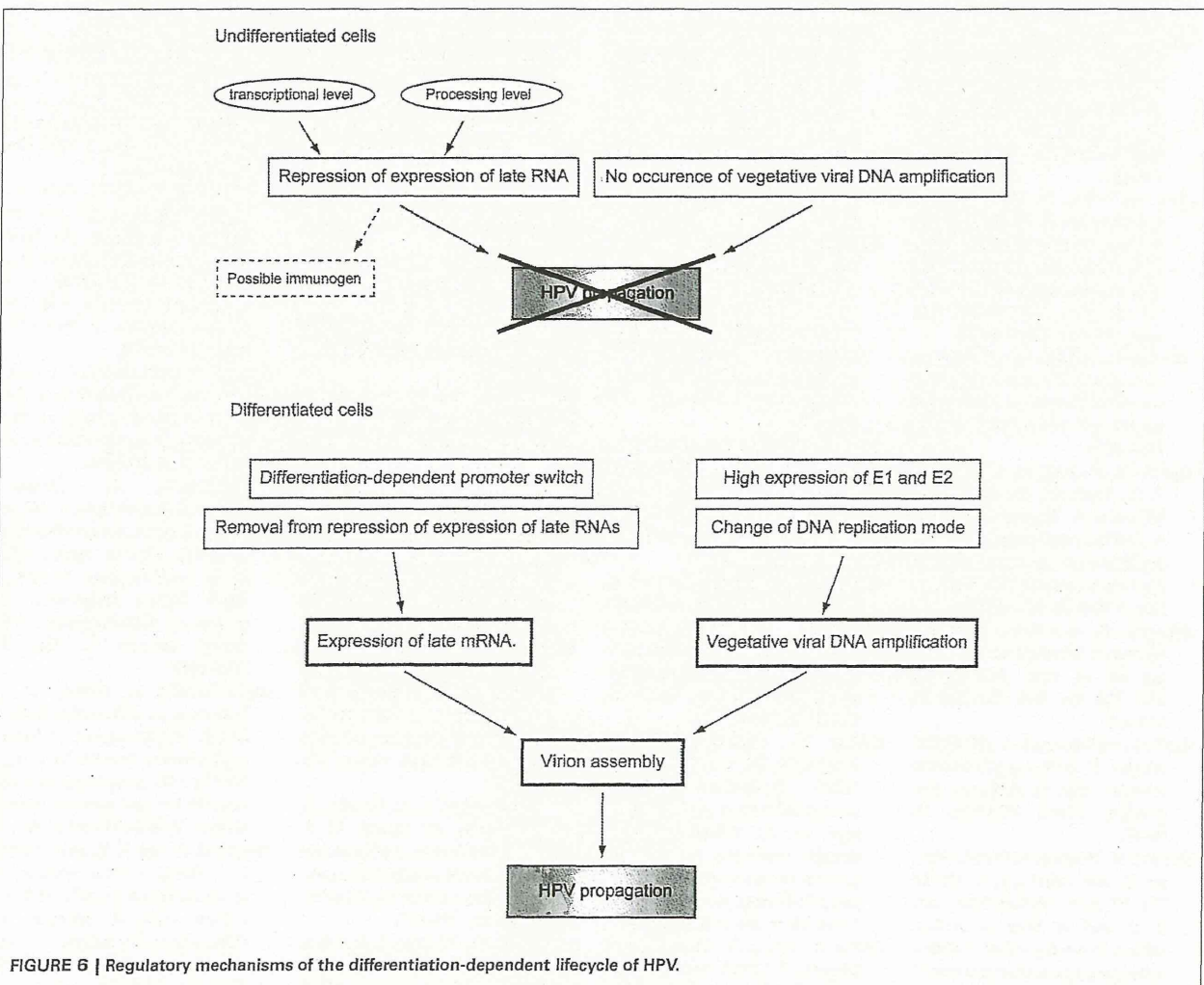


FIGURE 6 | Regulatory mechanisms of the differentiation-dependent lifecycle of HPV.



epithelium, where the host immune-surveillance system does not well function. The hyperproliferation induced by E6 and E7 is required for viral genome amplification and contributes to the augmentation of progeny virion production by expanding the pool of the infected cells.

Tight communication between the virus and the host cell is a unique character of HPV biology, and raises the possibility of using HPV as a probe to investigate the development of the stratified epithelium. In this review, we did not describe the details of the cancer progression induced by HPV infections.

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# Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers

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Genotyping human papillomavirus (HPV) in clinical specimens is important because each HPV type has different oncogenic potential. Amplification of HPV DNA by PCR with the consensus primers that are derived from the consensus sequences of the *L1* gene has been used widely for the genotyping. As recent studies have shown that the cervical specimens often contain HPV of multiple types, it is necessary to confirm whether the PCR with the consensus primers amplifies multiple types of HPV DNA without bias. We amplified HPV DNA in the test samples by PCR with three commonly used consensus primer pairs (L1C1/L1C2+C2M, MY09/11, and GP5+/6+), and the resultant amplicons were identified by hybridization with type-specific probes on a nylon membrane. L1C1/L1C2+C2M showed a higher sensitivity than the other primers, as defined by the ability to detect HPV DNA, on test samples containing serially diluted one of HPV16, 18, 51, 52, and 58 plasmids. L1C1/L1C2+C2M failed to amplify HPV16 in the mixed test samples containing HPV16, and either 18 or 51. The three consensus primers frequently caused incorrect genotyping in the selected clinical specimens containing HPV16 and one or two of HPV18, 31, 51, 52, and 58. The data indicate that PCR with consensus primers is not suitable for genotyping HPV in specimens containing multiple HPV types, and suggest that the genotyping data obtained by such a method should be carefully interpreted. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01922.x, 2011)

Human papillomavirus (HPV), composed of an icosahedral capsid and a circular double-stranded DNA genome, is classified into more than 100 genotypes based on the nucleotide sequence homology of the *L1* gene encoding the major capsid protein.<sup>(1)</sup> The HPV types found in lesions of the skin and genital mucosa are grouped as cutaneous and genital HPVs, respectively. Of genital HPVs, 15 types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) that have been found in cervical cancer are called high-risk HPVs<sup>(2)</sup> and the types, such as HPV6 and HPV11, that have been found in benign genital warts are called low-risk HPVs.<sup>(3)</sup>

For detection and genotyping of HPV DNA in the clinical specimens, such as cervical swabs and Pap smears, a part of the *L1* gene is amplified by PCR then grouped based on the susceptibility to various restriction enzymes, the binding capacity to type-specific probes, or the nucleotide sequences of the amplicons.<sup>(4)</sup> Several consensus primer pairs have been developed and used as standard primers for PCR-based genotyping of HPV in the clinical specimens. L1C1/L1C2+C2M was developed in 1991,<sup>(5)</sup> and has been used in more than 10 articles describing HPV prevalence in the Japanese population.<sup>(5-16)</sup> MY09/11<sup>(17)</sup> and GP5+/6+<sup>(18)</sup> were developed in 1989 and 1995, respectively, and have been used in numerous studies worldwide.<sup>(19)</sup> These primers are derived from the consensus sequences of the *L1* gene and the amplicons contain type-specific sequences. Recently new primers, PGMY09/11<sup>(20)</sup> and modified GP5+/6+

(MGP),<sup>(21)</sup> which are composed of several type-specific primers, were developed to improve the accuracy of HPV genotyping. The World Health Organization HPV Laboratory Network, which was founded to improve the quality of laboratory services for effective surveillance and monitoring of HPV vaccination impact, recommends PCR with PGMY09/11 followed by reverse blotting hybridization with type-specific probes, as a standard procedure for HPV genotyping.<sup>(22)</sup>

Recent studies showed that many HPV-positive women are infected with multiple genotypes.<sup>(23,24)</sup> Therefore, the methods capable of detecting and genotyping HPV DNA of multiple types in a single clinical specimen are necessary to know the precise prevalence of HPV types and the impact of HPV vaccines. As PCR does not always amplify different DNA fragments with equal efficiency, we examined whether PCR with consensus primers can amplify HPV DNA of multiple genotypes in a single sample without bias. We found that PCR with consensus primers sometimes results in mistyping.

## Materials and Methods

**Plasmids.** The pUC plasmid containing the complete genome of HPV16, 18, 31, 51, or 52, or the *L1* gene of HPV58 was used. Purified plasmids were quantified with the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The copy numbers of HPV genomes were calculated from the concentration of plasmid expressed as molarities and Avogadro's number.

**Clinical specimens.** Cervical exfoliated cells were collected from outpatients who visited the NTT Medical Center Tokyo, with their informed consent. The study design and sample collection were approved by the institutional review board. One case of normal cytology, two cases of cervical intraepithelial neoplasia (CIN) grade 1, two cases of CIN2, two cases of CIN3, and one case of unknown cytology were selected for this study. DNA was purified using the QIAamp DNA blood kit (Qiagen, Hilden, Germany).

**Polymerase chain reaction.** Table 1 shows the sequences of primers used in this study: three consensus primer pairs, L1C1/L1C2+C2M,<sup>(5)</sup> MY09/11,<sup>(17)</sup> and GP5+/6+;<sup>(18)</sup> and two mixtures of type-specific primers, PGMY09/11<sup>(20)</sup> and modified GP5+/6+ (MGP).<sup>(21)</sup> Polymerase chain reaction amplification was done in a 50  $\mu$ L reaction mixture containing 1 $\times$  PCR buffer II (Life Technologies, Carlsbad, CA, USA), 1.25 units AmpliTaq Gold DNA polymerase (Life Technologies), and 50 ng cellular DNA extracted from human HaCaT cells. The 5'-end of either the forward or reverse primer was biotinylated. The concentrations of MgCl<sub>2</sub>, dNTPs, and primers, and the reaction temperature were adjusted to those used in the original article

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Table 1. Nucleotide sequences of primers used in this study

	Primer set	Forward (5'-3')	Reverse (5'-3')
Consensus primers	L1C1/L1C2+C2M	CGTAAACGTTTTCCCTATTTTTT	TACCCTAAATACTCTGTATTG TACCCTAAATACCCTATATTG CGTCCMARRGGAWACTGATC
	MY09/11 GP5+/6+	GCMCAGGGWCATAAYAATGG TTTGTTACTGTGGTAGATACTAC	GAAAAATAAACTGTAATCATATTC CGTCCCAAAGGAAACTGATC
Multiple primers	PGMY09/11	GCACAGGGACATAACAATGG	CGACCTAAAGGAAACTGATC
		GCGCAGGGCCACAATAATGG	CGTCCAAAAGGAAACTGATC
		GCACAGGGACATAATAATGG	GCCAAGGGGAAACTGATC
		GCCAGGGCCACAACAATGG	CGTCCCAAAGGATACTGATC
		GCTCAGGGTTTAAACAATGG	CGTCCAAGGGGATACTGATC
			CGACCTAAAGGGAATTGATC
			CGACCTAGTGGAAATTGATC
			CGACCAAGGGGATATTGATC
			GCCCAACGGAAACTGATC
			CGACCAAGGGGAAACTGGTC
	CGTCCCTAAAGGAAACTGGTC		
	GCGACCCAATGCAAAATGGT		
MGP		ACGTTGGATGTTTGTACTGTGGTGACTACTAC	ACGTTGGATGGAAAAATAAACTGTAATCATATTC
		ACGTTGGATGTTTGTACCCTGTGGTAGACTACTAC	ACGTTGGATGGAAAAATAAACTGTAATCATACTC
		ACGTTGGATGTTTGTACTAAGGTAGATACCACTC	ACGTTGGATGGAAATATAAACTGTAATCAAATTC
		ACGTTGGATGTTTGTACTGTTGGATACAAC	ACGTTGGATGGAAAAATAAACTGTAATCATATTC
		ACGTTGGATGTTTGTACTATGGTAGATACCAAC	ACGTTGGATGGAAAAATAAACTGCAATCATATTC

MGP, modified GP5+/6+.



Fig. 1. Amplicons produced from the human papillomavirus (HPV)16L1 gene by PCR with primers tested in this study. Both ends of amplicons are indicated by the nucleotide (nt) numbers of the HPV16L1 gene. MGP, modified GP5+/6+.

describing the method.<sup>(5,17,18,20,21)</sup> Figure 1 shows the location of primers on the HPV16L1 ORF and the size of amplicons.

**Reverse blotting hybridization.** Reverse blotting hybridization was done as described previously.<sup>(22)</sup> Briefly, 15  $\mu$ L denatured amplicons, of which the 5'-ends were labeled with biotin, were allowed to hybridize with the type-specific probes immobilized on a nylon membrane using the Miniblotter MN45 (Immunic, Cambridge, MA, USA). The hybridized amplicon was detected using streptavidin-HRP (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and ECL detection reagents (GE Healthcare Bio-Sciences). The chemiluminescence was detected with the Light-Capture AE-6972 (ATTO, Tokyo, Japan). The intensities of dots were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The specific density was calculated by the subtraction of the background from the integrated density. Samples showing the specific densities of more than 1000 intensity units were defined as positives.

The nucleotide sequences of type-specific probes for MY09/11, GP5+/6+, PGMY09/11, and MGP primers were described previously.<sup>(25,26)</sup> Type-specific probes for L1C1/L1C2+C2M primers were newly designed in this study. The nucleotide sequences were as follows: HPV16, 5'-GTTATTGTTAGGTTTTTTAA; HPV18, 5'-CCACCACCTGCAGGA-

ACCT; HPV31, 5'-AGGATTGTCAGATTTAGGTA; HPV51, 5'-TAGCAGCACGCGTTGAGGTT; HPV52, 5'-ACCATTACCACTACTGGTGT; and HPV58, 5'-TATTGTTATTGGGACTTTTG.

**Real-time PCR.** Copy numbers of HPV DNA in a clinical sample were determined by real-time PCR using type-specific primers and SYBR-green dye. A reaction mixture (20  $\mu$ L) containing 2  $\mu$ L sample, 10  $\mu$ L Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan), 0.4  $\mu$ L ROX reference dye, and 0.9  $\mu$ M each primer was subjected to PCR with the Applied Biosystems 7900HT (Life Technologies). The reaction was done in triplicate. The copy number was calculated with the standard curve obtained by using serially diluted HPV plasmids. The nucleotide sequences of type-specific primers are as follows: HPV16 forward, 5'-CAGAACCATATGGCGACAGC and reverse, 5'-GTACATTTTCACCAACAGCA; HPV18 forward, 5'-GATATTTACAAATGTCTGCA and reverse, 5'-GCACAGTGTACCCCATAGTA; HPV31 forward, 5'-GATTATCTTA-AAATGGTTGCT and reverse, 5'-GGACCGATTACCAACC-GTG; HPV51 forward, 5'-AGCTATGGATTTGCTGCC and reverse, 5'-AGCAAAGATTTGCTCCCTGC; HPV52 forward, 5'-GATTATTTGCAAATGGCTAGC and reverse, 5'-GGCACAGGGTCACCTAAGGTA; HPV58 forward, 5'-AGTGAACCTTATGGGGATAG and reverse, 5'-AAAGGTCATCCGGGA-

CAGCC. Polymerase chain reaction with these primer sets amplified target HPV DNA without non-specific reaction. For example, PCR with the primers for HPV16 and a test sample containing HPV16 produced a single DNA fragment that formed a single peak in the dissociation curve.

## Results

**Amplification of HPV DNA in a test sample containing a single HPV genotype.** Figure 2 shows the results of reverse blotting hybridization of the amplicons obtained by PCR from test samples (50  $\mu$ L) containing 6, 60, 600, or 6000 copies of the plasmid having genomic DNA of HPV16, 18, 51, 52, and 58, all prevalent types among Japanese women, and 50 ng sheared human DNA. Three consensus primer pairs (L1C1/L1C2+C2M, MY09/11, and GP5+/6+) and two sets of mixed multiple primers (PGMY09/11 and MGP) were used for PCR. The biotinylated amplicons were allowed to hybridize with the type-specific probes immobilized on a nylon membrane and the biotin on the membrane was detected by streptavidin labeled with peroxidase.

Polymerase chain reaction with L1C1/L1C2+C2M produced detectable amplicons of HPV16, 18, 51, 52, and 58 from the samples containing 60, 6, 6, 60, and 60 copies of HPV DNA, respectively. Polymerase chain reaction with MY09/11 produced detectable amplicons of HPV16, 18, and 58 from the samples containing 600 copies of HPV DNA but did not produce detectable amplicons from the samples containing 6000 copies of HPV51 and 52. Polymerase chain reaction with GP5+/6+ produced detectable amplicons of HPV16, 18, 52, and 58 from the samples containing 60, 60, 6000, and 6000 copies, respectively, but failed to produce detectable amplicons from the sample containing 6000 copies of HPV51. Thus, PCR with L1C1/L1C2+C2M amplified HPV in the test samples more efficiently than the other PCR with the consensus primers.

Polymerase chain reaction with PGMY09/11 produced detectable amplicons of HPV16, 18, 51, 52, and 58 from the samples containing 6, 6, 60, 60, and 60 copies of HPV DNA, respectively. The PCR with MGP produced detectable amplicons of HPV16, 18, 51, 52, and 58 from samples containing 6, 60, 60, 6, and 600 copies of HPV DNA, respectively.

**Amplification of HPV DNA in a mixed test sample containing HPV16, and either HPV18, 51, 52, or 58.** Figure 3 shows the results of reverse blotting hybridization of the amplicons obtained by PCR from test samples containing 6000 copies of HPV16 and 6, 60, 600, and 6000 copies of either HPV18, 51, 52, or 58 and 50 ng sheared human DNA.

Polymerase chain reaction with L1C1/L1C2+C2M failed to amplify HPV16 DNA from the samples containing 6000 copies of HPV18 or 51 and the level of HPV16 amplicons was greatly reduced in the presence of 600 copies of HPV18 or 51. However, the amplification of HPV18 and 51 DNA was not influenced by the presence of 6000 copies of HPV16 DNA (Figs 2,3). The PCR with L1C1/L1C2+C2M amplified HPV16 DNA in the presence of HPV52 or 58 DNA. The data clearly indicate that amplification of HPV16 DNA by PCR with L1C1/L1C2+C2M was significantly interfered with by the presence of HPV18 or 51 DNA.

Polymerase chain reaction with MY09/11, GP5+/6+, PGMY09/11, or MGP amplified HPV16 DNA in the presence of HPV18, 51, 52, or 58 DNA but the level of HPV16 amplicons was reduced by co-existence of 6000 copies of HPV18 DNA. The PCR with GP5+/6+, PGMY09/11, or MGP produced reduced levels of HPV18, 52, or 58 amplicons in the co-existence of 6000 copies of HPV16 DNA (Figs 2,3).

**Amplification of HPV DNA in clinical specimens containing two or three HPV genotypes.** Table 2 shows the detection and genotyping of HPVs in clinical specimens using PCR with consensus primers. Eight clinical samples in which two or three HPV types had been detected by PCR with PGMY09/11 or MGP were selected, and the copy numbers of the detected HPV DNA in the samples were measured by real-time PCR using type-specific primers. Then the HPV in the samples was examined by PCR with L1C1/L1C2+C2M, MY09/11, or GP5+/6+. The HPV types detected with consensus primers are listed in decreasing order of amplicon levels.

In no. 165 containing HPV16 (16 000 copies), 18 (5200), and 31 (3100), HPV18 was detected by PCR with L1C1/L1C2+C2M but neither HPV16, which was three times more abundant than HPV18, nor 31 were detected. All three HPVs were detected by PCR with MY09/11. HPV16 and 18 were

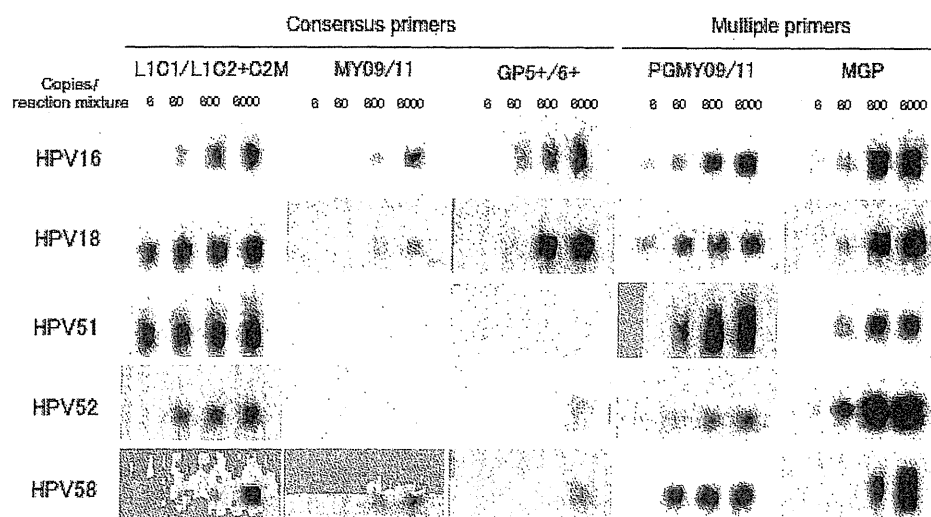


Fig. 2. Amplification of human papillomavirus (HPV) DNA in test samples containing HPV DNA of single genotype. The HPV DNA in the test sample was amplified by PCR with primers indicated. The test sample contained 6, 60, 600, or 6000 copies of plasmid DNA having HPV genomic DNA of the indicated type. The biotin-labeled amplified DNA fragments were hybridized with type-specific probes fixed on a membrane and reacted with streptavidin-HRP. MGP, modified GP5+/6+.

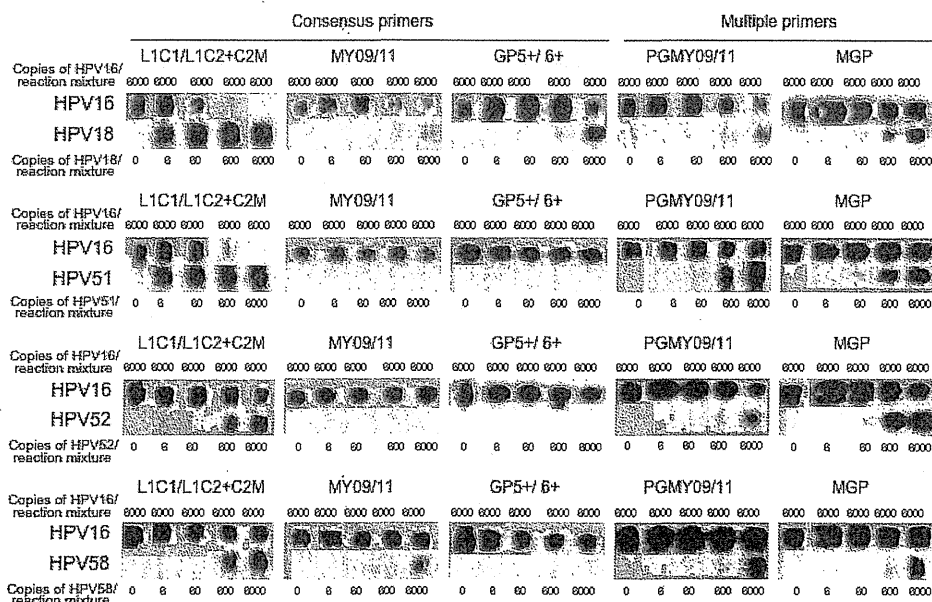


Fig. 3. Amplification of human papillomavirus (HPV) DNA in test samples containing HPV16 DNA and either HPV18, 51, 52, or 58 DNA. HPV DNA in the test sample was amplified by PCR with primers indicated. The test sample contained 6000 copies of HPV16 plasmid together with 6, 60, 600, or 6000 copies of HPV18, 51, 52, or 58 plasmid. The biotin-labeled amplified DNA fragments were hybridized with type-specific probes fixed on a membrane and reacted with streptavidin-horseradish peroxidase. MGP, modified GP5+/6+.

Table 2. Genotyping of clinical samples containing multiple types of human papillomavirus (HPV)

Sample no. (cytology)	Genotypes: copy number <sup>†</sup>	Consensus primers		
		L1C1/L1C2+C2M	MY09/11	GP5+/6+
165 (CIN2)	16:16 000	18	18 <sup>‡</sup>	16
	18:5200		16	18
	31:3100		31	
258 (CIN3)	52:1700	52	16	16
	16:1300	16	58	
	58:180	58		
352 (normal)	16:200		16	16
	52:25			
402 (CIN2)	52:7300	51	52	16
	51:120	52	16	
993 (CIN1)	16:110			
	16:5600	16	16	16
	52:3300	52		
996 (CIN1)	16:47 000	16	16	16
	52:25 000	52		
1061 (unknown)	18:4300	18	18	18
	16:290		16	16
1245 (CIN3)	16:78 000	58	58	16
	58:78 000	16	16	

<sup>†</sup>Genotypes detected using PGMY09/11 and modified GP5+/6+ primers. Copy numbers were determined by real-time PCR and expressed as copy numbers subjected to PCR for genotyping. <sup>‡</sup>The HPV types detected with consensus primers are listed in decreasing order of amplicon levels.

detected by PCR with GP5+/6+ but HPV31, the least component, was not detected by PCR with GP5+/6+.

In no. 258 containing HPV52 (1700), 16 (1300), and 58 (180), all three HPVs were detected by PCR with L1C1/L1C2+C2M. HPV16 and 58 were detected by PCR with

MY09/11 but HPV52, the most abundant type, was not detected. Only HPV16 was detected by PCR with GP5+/6+.

In no. 352 containing HPV16 (200) and 52 (25), neither HPVs were detected by PCR with L1C1/L1C2+C2M. Only HPV16 was detected by PCR with MY09/11 and PCR with GP5+/6+.

In no. 402 containing HPV52 (7300), 51 (120), and 16 (110), HPV16 was not detected by PCR with L1C1/L1C2+C2M. HPV16 and 52, but not HPV51, were detected by PCR with MY09/11. Only HPV16 was detected by PCR with GP5+/6+.

In no. 933 containing HPV16 (5600) and 52 (3300), and in no. 996 containing HPV16 (47 000) and 52 (25 000), HPV52 was not detected by PCR with MY09/11 and GP5+/6+.

In no. 1061 containing HPV18 (4300) and 16 (290), HPV16 was not detected by PCR with L1C1/L2C2+C2M.

In no. 1245 containing HPV16 (78 000) and 58 (78 000), HPV58 was not detected by PCR with GP5+/6+. Thus, amplification of HPV DNA in the clinical specimens containing multiple HPV genotypes by PCR with consensus primers was biased and sometimes caused misjudgment in typing.

## Discussion

We evaluated HPV consensus primers for PCR amplification of HPV DNA in specimens containing multiple HPV types and concluded that PCR with consensus primers is not suitable for simultaneous amplification of multiple types of HPV DNA. The low sensitivity of HPV51 and 52 detection by PCR with MY09/11 and GP5+/6+ is consistent with previous reports.<sup>(20,21,27)</sup> Polymerase chain reaction with L1C1/L1C2+C2M, which amplified HPV DNA tested more efficiently than PCR with MY09/11 or GP5+/6+ (Fig. 2), failed to amplify HPV16 DNA when HPV18 or 51 DNA coexisted in the samples (Fig. 3, Table 2).

Polymerase chain reaction with L1C1/L1C2+C2M amplifies HPV18 and 51 DNA very efficiently, as shown in Figure 2. The resultant abundant HPV18 and 51 amplicons might inhibit reactions amplifying the other HPV types in the samples. Similarly, it is reported that interference in PCR was observed even



between two closely related plant virus isolates that have identical binding sites for consensus primers, although the mechanism of the interference is not fully explained.<sup>(28)</sup>

As PCR with L1C1/L1C2+C2M has been used widely,<sup>(5-16)</sup> the biased amplification may have caused mistyping in the previous numerous studies of genotyping of clinical specimens. Previous studies using L1C1/L1C2+C2M showed that the rate of multiple infections among HPV-positive Japanese women was 10-20%.<sup>(15,16)</sup> In other countries, studies using PGMY09/11 showed that 30-45% of HPV-positive women were infected with multiple genotypes.<sup>(20,29-31)</sup> It is possible that reevaluation of HPV prevalence in Japanese women by using mixtures of type-specific primers, such as PGMY09/11 and MGP, increases the rate of multiple infections. The data of this

study suggest that the genotyping data obtained by PCR with consensus primers should be carefully interpreted.

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## Disclosure Statement

The authors have no conflict of interest to declare.

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