The E2F5 repressor is an activator of E6/E7 transcription and of the S-phase entry in HPV18-associated cells

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High-risk papillomavirus type 18 (HPV18) is one of the less represented HPV types in low-grade lesions of the anogenital tract, whereas it occupies the second place in cervical cancer, where it can be found in 16% of the cases worldwide, after HPV16 present in 54% of them. These epidemiological data indicate that HPV18 infection is more prone to carcinogenic progression. The main oncogenic proteins, E6 and E7 of HPV18, are functionally comparable to the homologous proteins of the other high-risk viruses, including HPV16. In this work, we investigated the possibility that the higher oncogenic potential of HPV18 might be due to transcriptional regulation of the E6/E7 oncogenes. By comparing the E6/E7 promoter and enhancer sequences of the mucosal HPV genomes, we identified E2F binding sites specific for HPV18. The E2F family of transcription factors contains activators (E2F1-3) and repressors (E2F4-8) that regulate the transcription of S-phase and mitotic genes and thereby have a crucial role in cell-cycle progression. Surprisingly, we identified E2F5 as a direct activator of HPV18 E6/E7 transcription by sequential silencing of E2F members in HeLa cells. In addition, we could show that E2F5 positively regulates S-phase entry in HeLa cells and that this activation of the cell cycle by a member of the E2F repressor family is specific for HPV18-expressing cells. Diverting the function of E2F5 from a cell-cycle repressor into an activator might contribute to the higher oncogenic potential of HPV18 when compared with other high-risk HPV types. Oncogene advance online publication, 19 July 2010; doi:10.1038/onc.2010.246

Keywords: papillomavirus; transcription; HPV; E2F; cell cycle

Introduction

High-risk human papillomaviruses (HPVs) are the causal agents of cervical cancer, and their genome is found in more than 99% of these cancers (Walboomers et al., 1999), where integration of the viral genome has a crucial role in carcinogenic progression. The pattern of the viral DNA integration implies conservation of the viral oncogenes, E6 and E7, which, respectively, induce p53 degradation through the ubiquitin-proteasome pathway (Scheffner et al., 1993) and pRb inactivation (Dyson et al., 1989) and the disruption of the E2 open reading frame encoding a potent repressor of E6/E7 transcription (Thierry and Yaniv, 1987; Bernard et al., 1989; Thierry and Howley, 1991). For high-risk HPV types HPV16 and 18 represent 70–80% of all cervical cancers worldwide (Castellsague et al., 2007). Prevalence of the two high-risk papillomavirus types, HPV16 and HPV18, in low-grade and high-grade lesions of the cervix, indicates that HPV16 is the most abundant genotype in all types of lesions (20.3% in low grade: first position and 54.4% in cervical cancer: first position), but the prevalence of HPV18 increases with the grade of the lesion (6.1% in low-grade lesions: 10th position and 15.9% in cervical cancer: second position) (Castellsague et al., 2007). This observation was supported by the finding that the transforming activity of HPV18 in vitro is better than that of HPV16 (Villa and Schlegel, 1991). As the known properties of the E6 and E7 oncogenes are well conserved among high-risk HPV types, a way to explain the differences in transforming efficiencies is to examine their transcriptional regulation. In a previous study, this difference correlated with the fact that HPV18 transcription of the oncogenes seemed to be approximately 10- to 50-fold higher than transcription of the HPV16 oncogenes in keratinocytes (Villa and Schlegel, 1991).

In HPV18, the E6 and E7 proteins are translated from an upstream tissue-specific enhancer, called enhanceosome, through efficient recruiting of coactivators such as CBP/P300, and can induce high transcriptional levels (Bouallaga et al., 2003). The E6 and E7 promoters are translated from a unique mRNA, the transcription of which is controlled by the P165 promoter and an upstream tissue-specific enhancer. The HPV18 enhancer has been shown to bind several transcription factors, such as AP1, HMG-I(Y) or the nucleolin (Bouallaga et al., 2000, 2003; Grinstein et al., 2002; reviewed in Thierry, 2009). It can form a three-dimensional structure, called enhanceosome, through efficient recruiting of coactivators such as CBP/P300, and can induce high transcriptional levels (Bouallaga et al., 2003).

In this study, we describe the role of a new cellular transcription factor in the HPV18 enhancer, which is a member of the E2F family. These transcription factors are involved in the G1/S transition of the cell cycle and, as discovered more recently, also in the G2/M transition (Ishida et al., 2001; Ren et al., 2002; Polager and Ginsberg, 2003; Thierry et al., 2004; Zhu et al., 2004).
There are eight E2F members; E2F1–3 activate the transcription of S-phase genes, whereas E2F4–8 repress their transcription. E2F5 (Sardet et al., 1995) had been shown to repress the cell cycle and to induce G0 in keratinocytes, depending on its sub-cellular localization and therefore probably favoring differentiation, although the mechanisms remain unclear (Apostolova et al., 2002; Reed et al., 2007).

In this work, we characterized an unexpected activating role of E2F5 in cell-cycle progression of HPV18-associated cells. This activation depends on the HPV18 transcription and involves the binding of E2F5 to the HPV18 enhancer. This observation suggests a new regulatory function of HPV18 in overcoming G0 arrest by changing the cell-cycle repressor E2F5 into an activator, through direct transcriptional activation of E7 and subsequent S-phase entry.

Results

E6/E7 transcription varies during the cell cycle together with the E2F transcription factors

To identify new potential transcription factors involved in cell-cycle-dependent HPV18 transcription, we analyzed the sequences of HPV18 regulatory region using the TRANSFAC software. Two potential E2F binding sites were found in the core of the enhancer as defined previously (Bouallaga et al., 2000) (Figure 1a). Sequences of the two sites were close to the consensus sequence for E2F binding, but the upstream site A marked the limit of the previously defined core enhancer region, indicating that this sequence was previously found to be essential for HPV transcription in sequential deletion experiments (Figure 1a). We reasoned that if the E2F family members were involved in the HPV18 E6/E7 transcription, their expression should vary concomitantly during the cell cycle. Analyses of the mRNA levels of six E2F family members by reverse transcriptase–PCR (RT–PCR) in synchronized HeLa cells indicated that mRNA levels of the E2F3, 4 and 6 were not modulated, whereas those of the E2F1 and 2 activators were decreased in G2 and, on the contrary, mRNA levels of the repressor, E2F5, were activated (Figure 1b).

We then investigated whether the endogenous E7 transcription in HeLa cells was also cell-cycle modulated. We used HeLa cells synchronized by thymidine block and released at different time points, as shown by flow cytometry analyses (Figure 1c). We then studied E6/E7 mRNA levels by RT–PCR and E7 protein expression by western blots in these cells at different phases of the cell cycle. E6/E7 mRNA levels were found to be higher in the late G1 and S phases compared with early G1 and G2/M (Figure 1d, upper panel). Modulation of the quantity of E7 protein was also detected, although with a clear delay compared with the transcription (Figure 1d, lower panel). The higher E6/E7 mRNA levels in the early S phase are therefore consistent with regulation of the HPV18 oncogene transcription by the E2F transcription factors (Mudryj et al., 1991; Johnson et al., 1993).

The HPV18 enhancer binds E2F2 and E2F5 in vitro

We then examined which of the three E2F factors is involved in HPV18 E6/E7 transcription by studying

Figure 1  Cell-cycle modulation of E2F and E7 transcription in HeLa cells. (a) Sequence of the core HPV18 enhancer containing two potential E2F binding sites A and B as indicated. (b) Detection of the levels of transcription of six members of the E2F family as shown by RT–PCR in synchronized HeLa cells as described in panel (c). (e) Fluorescence-activated cell sorting analyses of the phases of the cell cycle in HeLa cells after thymidine block at 0, 6 and 12 h release as indicated. (d) E6/E7 transcription varies during the cell cycle and is higher in late G1, decreasing progressively during S phase. These variations of mRNA levels are consistent with variation of the E7 protein levels observed by immunoblot (lower panel).
their binding to the two E2F putative binding sites A and B in gel-shift assays (Figure 2a). We compared the binding profiles of an E2F consensus binding site with two probes containing the HPV18 A and B sequences in gel-shift assays using HeLa nuclear extracts or purified E2F1 protein fused to GST. Only the upstream putative E2F binding site A of the HPV18 core enhancer bound efficiently to both the purified E2F1 and factors present in the HeLa nuclear extract. Supershift experiments, using specific antibodies against the E2F family members, indicated that from a HeLa cell extract (Figure 2b) both E2F2 and E2F5 were able to bind to the E2F binding site, whereas no supershift was found with the anti-E2F1 antibody, which was shown to efficiently shift the purified protein (data not shown). These experiments indicated that the HPV18 core enhancer sequence is able to bind to E2F and that this binding is presumably responsible for cell-cycle-dependent transcription of the HPV18 E6 and E7 genes in HeLa cells.

**E2F5 activates S-phase genes through activation of E7 transcription**

The putative involvement of the E2F5 repressor in the regulation of HPV18 transcription was intriguing and, to determine its role, we used an approach that relied on the silencing of the E2F transcription factors by pSUPER shRNA-expressing vectors. Silencing of the three E2F activators separately in HeLa cells did not reduce their respective mRNA levels, thus indicating that they could compensate each other (data not shown) as previously reported (Kong et al., 2007). We, therefore, transfected the three pSUPER vectors together and could obtain silencing of the transcription of E2F activators (Figure 3a). As for the E2F5 repressor, the silencing of the three activators did not modulate its mRNA levels, which were, however, efficiently silenced by the use of the specific E2F5 shRNA sequence (Figure 3a). Intriguingly, silencing of the E2F activators did not modulate E6/E7 transcription, whereas silencing of E2F5 strongly reduced the E6/E7 transcription by 70%, indicating a positive control of the endogenous HPV18 transcription by the E2F5 repressor in HeLa cells (Figure 3b). The effect of E2F5 silencing on E7 expression was also observed at protein level in western blot experiments (Figure 3b, lower panel).

We have previously shown that the HPV18 E7 expressed in HeLa cells is a strong activator of many E2F targets (Thierry et al., 2004; Teissier et al., 2007). If E2F5 is an activator of E7, its inactivation should reduce the transcription of S-phase target genes in HeLa. To verify this hypothesis, we transfected HeLa cells with the E2F shRNA expression plasmids and
found that, indeed, the transcription of the S-phase gene cyclin E1 was repressed both by the silencing of E2F activators and by silencing of E2F5 (Figure 3b). However, it is interesting that although silencing of the E2F activators repressed cyclin E1 mRNA levels, presumably by a direct effect, it had no effect on HPV18 transcription as already mentioned. Taken together, these data indicate that E2F5 might have an activating role for the E2F target genes in HeLa cells, although transcription of these genes remains under the control of the classical E2F activators (E2F1–3). When cells are transfected with pSUPER-expressing shRNA for E2F1, 2, 3 and 5 all together, there is no additive effect on cyclin E1 repression and the repression of E6/E7 transcription observed is comparable to that obtained using pSUPER-E2F5 alone (Figure 3b). From these experiments we could deduce that E2F5 is not a direct activator of cellular S-phase genes, as it is expected from previously published data, and that, for example, an S-phase gene promoter such as cyclin E1 remains controlled by the E2F activators. Activation of S-phase genes in HeLa cells would rather come from an indirect activation of the E7 transcription followed by activation of the E2F activators through modulation of the negative regulator pRb. An assumption from this hypothesis is that E2F5 can function as an activator of the cell cycle only when it is able to modulate E7 transcription.

We verified that the silencing of E2F5 did not affect the transcription of the cyclin E1 (S-phase gene) in HPV-negative C33-A cells (data not shown), thus implying that the regulation in HeLa cells was likely to be due to regulation of the E7 transcription.

To test whether E2F5 activation of the HPV18 E6/E7 transcription could be extended to other HPV18-associated cells, we transfected the C-41 cervical carcinoma cell line with pSUPER-E2F5 and could show that the endogenous E6/E7 transcription was repressed concomitantly with E2F5 silencing (Figure 3c).
detected repression of E6/E7 transcription of episomal DNA in nTERT (primary keratinocytes immortalized with telomerase hTERT) cells cotransfected with the HPV18 genome and the pSUPER E2F5 (Figure 3c), thus confirming that E2F5 is an activator of HPV18 E6/E7 transcription from both integrated and episomal HPV18 sequences.

We were intrigued by the fact that the transcriptional repressor E2F5 protein could modulate HPV18 transcription, and decided to check whether it could bind its regulatory region in vivo by chromatin immuno-precipitation (ChIP) experiments. We used a transfected expression vector expressing E2F5 fused to the green fluorescent protein (GFP) to perform ChIP experiments on the endogenous HPV sequences integrated in the cellular genome in HeLa cells. We also checked whether E2F5 will be recruited to the promoter of PolA, an S-phase gene used in this study as a typical E2F target. In both cases E2F5 was recruited, although it was not recruited by the negative control GAPDH, indicating a specific direct E2F5 binding to the HPV18 enhancer (Figure 3d).

To further confirm the direct involvement of E2F5 in transcriptional activation of the HPV18 early promoter, we transfected C33-A (HPV negative) cells with different constructs containing either the wild-type HPV18 LCR or mutants for E2F binding sites A and/or B (the same point mutations that impair E2F binding in Figure 2 were used), controlling luciferase transcription. Mutation of either site A or site B significantly decreased the LCR activity, with a stronger effect of site A mutation and no additive effect of the two mutations together (Figure 3e). Cotransfection of pSUPER-E2F5 induces a decrease of the luciferase activity, although not as strong and reproducible as the decrease of the E6/E7 mRNA observed in HeLa, C-41 and HPV18-transfected nTERT. Site A seems to be more sensitive to E2F5 silencing, which might reflect its greater affinity for E2F binding as shown earlier (Figure 2).

**E2F5 activates the cell cycle in HPV18-associated cells**

One important assumption of the modulation of S-phase genes by E2F5 is that silencing of E2F5 should modulate the cell cycle in HeLa cells. To test this hypothesis, we analyzed the effects of the silencing of E2F5 compared with the silencing of E2F activators and E7 in three cell lines: HeLa (HPV18), C33-A (HPV negative) and Caski (HPV16) cells by flow cytometry (Figures 4 and 5). In the presence of the siE7, HeLa cells did not release from G1/S block compared with control cells (Figure 4a). Inactivation of E2F5 in similar experiments also showed a block in G1, although less efficient than with E7 silencing, as only 54% of the cells were blocked in G1 after 6h of release, compared with 85% for shE7 and 32% for the control. This observation is consistent with the results obtained by RT–PCR, indicating that E2F5 inactivation has a weaker effect on the S-phase genes than E7 silencing itself (Figure 3b). Therefore, E2F5 silencing showed neither positive nor negative effect on the G1/S transition (Figure 4b), where E2F5 silencing did not change the cell-cycle release and did not start growing again. Cells transfected with either pSUPER-E2F1, 2 or 3 (all the activator members) or pSUPER E2F5 showed a decrease in their growth speed after 43 and 52h post-transfection, then eventually stop growing completely after 52h. In all cases, the growth arrest was sustained for more than 30h and none of these cells showed any relapse during this time frame, thus confirming a sustained anti-proliferative function of the E2F5 silencing in HeLa cells. To check whether the cell-cycle modulation is dependent on HPV18 E7 transcriptional modulation, we performed similar analyses in the HPV-negative C33-A cells. As expected, the shRNA against E7 did not affect their cell cycle, whereas E2F5 silencing showed neither positive nor negative effect on the G1/S transition (Figure 4b). These results confirmed that the positive role of E2F5 on the cell cycle depends on the presence of the HPV18 and expression of the E6/E7 oncoproteins.

**E2F5 is not a cell-cycle activator of HPV16-associated cells**

Using the TRANSFAC software we looked for E2F binding sites in the regulatory region of other HPV genomes, concentrating our efforts on mucosal HPV types. We could not find equivalent sequences in any of the HPV regulatory regions, even when looking at the HPV18 closest type, HPV45. We therefore decided to check whether the E2F5-dependent positive regulation of the cell cycle, which we observed in HPV18-positive cells, was also seen in the HPV16-positive Caski cells. We first studied the effect of the silencing of E7 and E2F5 on HPV16 E6/E7 transcription. Although E2F5 and E7 siRNAs very efficiently induced silencing of their respective targets, silencing of E2F5 did not change the level of endogenous E7 expression in Caski (Figure 5a). Cell cycle analyses confirmed that E2F5 did not function as a positive factor for S-phase entry in this cell line, as the silencing of E2F5 did not change the cell-cycle release (Figure 5b), contrary to that seen in HeLa cells (Figure 4a).

In this experiment, the behavior of HPV16-associated Caski cells was, therefore, comparable to that of HPV-negative C33 (compare Figures 5b and 4b). These data thus confirmed that E2F5 could function as an activator of the cell cycle and entry in S phase only in HPV18-associated cervical carcinoma and, therefore, correlate with the specifically aggressive behavior of this viral type in natural infection.
A model of regulation for S-phase genes by E2F5 is depicted in Figure 6, showing the HPV18-specific E2F5-mediated activation of S-phase genes through an amplification loop of E7 activation and re-routing of the E2F5 from a repressor to a transcriptional activator.

Discussion

E2F5 is one of the members of the E2F family and has been shown to have a crucial role in keratinocytes, as it is responsible for the cell-cycle arrest preceding differentiation (Apostolova et al., 2002). In HPV18-associated cells, we show that the E2F5 repressor does not function as a repressor of the cell cycle but rather as an activator, and furthermore, that this is specific for the cervical carcinoma associated with HPV18 and not with HPV16, for instance. Activation of S-phase genes by E2F5 in HeLa cells, such as cyclin E, was also clearly shown in silencing experiments. However, these experiments also indicated that cyclin E remained under the control of the E2F activators and that there were no additive effects on its repression when the E2F5 shRNA
was added to the shRNA activators. This observation indicated that probably the S-phase genes are not directly activated by E2F5, and this hypothesis is sustained by our finding that E2F5 is an activator of the HPV18 oncogenes. The effects of the silencing of E7 transcription by E2F5 were extended to C-41 cells and to nTERT transfected with the HPV18 episomal genome. We could deduce from our experiments that E2F5 activates the cell cycle in HPV18-positive cells by directly activating transcription of E6 and E7, which in turn activate E2F activity by degrading p53 and pRb. This point is reinforced by the cell proliferation assays indicating that silencing of E2F5 arrests cell growth with a delay of few hours compared with silencing of the E2F activators (E2F1–3).

The specificity of the transcriptional activation of E6/E7 by E2F5 in HPV18-positive cells is correlated with the presence of E2F binding sites only in the HPV18 LCR and their efficient binding to E2F in vitro and in vivo. In addition, mutation of these sites in a luciferase reporter assay clearly shows their functional importance in the regulation of HPV18 transcription. In contrast, no E2F binding sites could be found in the HPV16 regulatory region and consequently, E2F5 silencing in Caski cells associated with HPV16 affected neither the E6/E7 transcription nor the cell cycle. As E2F5 has been shown to have a key role in keratinocyte differentiation (Apostolova et al., 2002), we can speculate that in HPV18-infected keratinocytes, activation of E6/E7 transcription would be concomitant with induction of the differentiation through E2F5, therefore inducing a delay in cell differentiation.

The mechanism by which E2F5, classified as a repressor, leads to activation of the HPV18 transcription remains to be elucidated. Nevertheless, we can hypothesize that this property is carried by the enhancer of HPV18 itself. The localization of the E2F binding sites in an enhanceosome structure is unusual, as they are

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**Figure 5** E2F5 does not modulate the cell cycle in Caski cells. (a) Real-time RT–PCR in the HPV16-associated Caski cells indicate that the silencing of E2F5 has no effect on E6/E7 transcription. (b) Flow cytometry analyses were carried out as described in Figure 4.

**Figure 6** Model for the E2F-mediated control of the cell cycle in keratinocytes expressing HPV18. In normal keratinocytes, the S-phase genes are under the control of the E2F pathway and they are activated by the E2F activators, whereas they are repressed by E2F5. In HPV18-associated keratinocytes, the S-phase genes are controlled by E7 through negative regulation of pRB inducing the E2F activators and through direct activation of E7 transcription by E2F5, which, by both activating E7 and diverting the E2F repressor, leads to amplification of the S-phase activating signal.
more often found associated with promoter structures (Elkon et al., 2003; Linhart et al., 2005), and it might be a clue to explain the switch of a transcriptional repressor to an activator. The regulation of E2F5 is linked to the factors with which it interacts. Indeed, E2F5 contains a nuclear export signal but no nuclear localization signal and, therefore, needs to bind to another factor to enter the nucleus. One of the best known interactors of E2F5 is the pocket protein p130, which has been shown to be crucial for its function as a transcriptional repressor although, and despite the fact that p130 contains a nuclear localization signal, it does not seem responsible for E2F5 nuclear localization (Caracciolo et al., 2007).

In an attempt to study direct activation by E2F5, we overexpressed untagged or GFP–E2F5 in HeLa or in cotransfected C33-A cells with various luciferase reporters, but could not detect any functional activity. In addition, E2F5 overexpression did not modulate E2F target gene transcription, cell cycle or cell growth (data not shown). Cellular localization of GFP–E2F5 was mainly cytoplasmic, explaining, at least in part, its transcriptional defect (data not shown). To overcome this problem, we overexpressed an E2F5 containing an SV40 nuclear localization signal to increase its nuclear concentration, but that did not increase its functionality. We deduce from these data that to observe an increase in its functions, we probably should overexpress E2F5 together with its regulators, the difficulty being that they are not all known, including the one responsible for nuclear localization.

Another E2F repressor, E2F6, has recently been shown to interact with HPV16-E7 to induce a ‘stem-cell-like state’ of infected cells (McLaughlin-Drubin et al., 2008), although we could not show such direct interaction between HPV18-E7 and E2F5. In addition, we were unable to show any direct effect of E7 expression on the E2F5 transcriptional regulation, indicating that E7 itself does not induce the transcriptional switch. It has been shown that the high- and low-risk HPV-E7 onco genes can target p130 for degradation, thus leading to relief of the E2F5 transcriptional repression (Zhang et al., 2006). As this repression is required to arrest the cell cycle before keratinocyte differentiation, it seems that in both high- and low-risk HPV infection, this delay in cell differentiation is required for the viral cycle to proceed. We have found no obvious implication of either p130 or E7 itself in the HPV18 transcriptional regulation by E2F5 (not shown), which is consistent with the observation that this mechanism is specific for HPV18, thus not involving common regulatory mechanisms. This would imply that in HPV18-infected lesions, the regulation of E2F5 could occur by two different pathways, one common pathway involving degradation of p130 by E7 and one specific pathway involving the use of E2F5 as a transcriptional activator of the E7 transcription. We believe that this original mechanism of re-routing a transcription factor has a role in the high level of transcription of the viral onco genes in HPV18 and possibly in its higher transforming capabilities.

Materials and methods

Cell culture and transfections

HeLa, C33-A, C-41 and Caski cells were grown using standard procedures in a 37 °C humidified incubator with 5% CO2. All cell lines were grown in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Singapore) with 10% heat-inactivated fetal bovine serum. nTERT cells were cultivated in keratinocyte-SFM medium (Invitrogen, 13944) complemented with Bovine pituitary extract (25 μg/ml), epithelial growth factor (0.2 ng/ml) and CaCl2 (0.3 mM).

The cells were prepared the day before the transfection at 500 000 cells per 10-cm dish. For plasmid transfection, we used Lipofectamin LTX (Invitrogen) according to the manufacturer’s instructions. shRNA-expressing vectors were pSUPER plasmids (Oligogene, Seattle, WA, USA). In HeLa, C-41 and C33 cells, the control pSUPER plasmid used was pSUPER-E7-HPV16. The shRNA sequences cloned in pSUPER vectors are:

- pSUPER-E7-16: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E7-18: 5'-GATCCCCGGCATGGATGATATTTATTGCAGGAGGTGCGTACTTTCTTTTGGAAA-3';
- pSUPER-E2F1: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E2F3: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E2F5: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';

Caski cells were transfected with siRNA using Dharmafect (Thermo Scientific, Research Instruments, Singapore) according to the manufacturer’s instructions. The control used in these HPV16-positive cells was si-E7-HPV18.

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- pSUPER-E7-16: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E7-18: 5'-GATCCCCGGCATGGATGATATTTATTGCAGGAGGTGCGTACTTTCTTTTGGAAA-3';
- pSUPER-E2F1: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E2F3: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';
- pSUPER-E2F5: 5'-GATCCCCGAGCTGCAAACACAAUAUCUGTTGAAA-3';

Caski cells were transfected with siRNA using Dharmafect (Thermo Scientific, Research Instruments, Singapore) according to the manufacturer’s instructions. The control used in these HPV16-positive cells was si-E7-HPV18.

siE7-HPV18: 5'-GAUGGGAUUAUAUCAAAAACCTT-3';
- siE7-HPV16: 5'-GAGCGUCGAAAAACAAUCUAACdTdT-3';
- siE2F1: 5'-GCAAAAGGCCGGCGCAUCUAdTdT-3';
- siE2F2: 5'-GGGGCCUCAUGCAGUACCdTdT-3';
- siE2F3: 5'-GCCAAGUCGAGUACCdTdT-3';
- siE2F5: 5'-GCCACCUUCUGGGAACACTT-3';

The nTERT cells had been electroporated with NEON system (Invitrogen) as per the manufacturer’s instructions.

The RT-PCR experiments performed in HeLa with pSUPER-E2F5 were also carried out using an siE2F5 pool provided by ON-TARGETplus SMARTpool from Thermo Scientific (5'-GGUGUCUGCUGCUAUAUAUA-3'; 5'-UAAAGAAGAGACAGGUGGUAU-3'; 5'-GAACCCCAUUGCUACCdTdT-3'; 5'-UCAGCAGGAAUACCdTdT-3').

Luciferase assay was carried out by transfecting C33 cells with 5 μg of HPV18 LCR full-length cloned in pTL vector (Panomics, Fremont, CA, USA). The luciferase activity was normalized by the Renilla activity carried by 0.5 μg of control vector pRL-TK (Promega Pte Limited, Singapore). Point mutations are 5'-ATT GATGC-3' for site A and 5'-TTT GATGC-3' for site B.

Luciferase assay

Luciferase assay was carried out using a Promega kit (Cat no. E1960) according to the manufacturer’s instructions.

Gel-shift assay experiments

DNA probes were labeled with 50 μM alpha [³²P]-dATP for 30 min with Klenow enzyme (Roche, Singapore) in the provided buffer containing 50 μM of dCTP, dTTP and dGTP. Klenow enzyme was inactivated by heating for 10 min at 70 °C and the labeled probes were purified on columns (Amersham Bioscience, Uppsala,
Swedish). Sequences of the E2F binding sites in the probes are (underlined sequences are overhang for Klenow filling) as follows:

E2F consensus probe: 5'-CTAGATGTATTTAGCCCGCAGAA AACTTCTTAG-3'; HPV18 E2F site A: 5'-CTAGAAACAAATT GGGCGGCTTCTTAG-3'; HPV18 E2F site B: 5'-CTAGACC TCTTGGCAGATCTTAG-3'.

Nuclear extracts were prepared as previously described (Bouallaga et al., 2003) and were incubated for 30 min at room temperature in 12 mM Hepes, 0.5 mM EDTA, 4 mM MgCl2, 60 mM KCl, 0.1% NP40, 10% glycerol and 4 mM DTT, 3 μM spermidine, 0.5 μg/μl bovine serum albumin and 50 μg/μl Salmon Sperm DNA. The labeled probes were then incubated for 10 min before migration in a 5% native polyacrylamide 29:1 gel in 0.5% TBE. When antibodies were used, 1 μl of anti-E2F1, E2F2 or E2F5 rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (SC-193, SC-633 and SC-999) was added to the mixture. After migration, the gels were dried and revealed using a Phosphor-Imager. GST–E2F1 had been purified from bacteria as described previously (Heino et al., 2000).

Real-time RT–PCR
The RNA was extracted using RNeasy Mini Kit (Qiagen, Singapore) according to the manufacturer's instructions. A volume of 2.5 μg of total RNA measured with nanodrop ND-1000 was reverse transcribed with superscript II (Invitrogen) according to the manufacturer's instructions. Real-time PCR was carried out using the Stratagene MX3005p machine (Stratagene, Singapore) in 25 μl of mixture containing 25 ng of cDNA, 2.5 × 10−3 μM of each primer and 12.5 × 10−3 M of each dNTP (Bouallaga et al., 2003) and were incubated for 30 min at room temperature in 12 mM Hepes, 0.5 mM EDTA, 4 mM MgCl2, 60 mM KCl, 0.1% NP40, 10% glycerol and 4 mM DTT, 3 μM spermidine, 0.5 μg/μl bovine serum albumin and 50 μg/μl Salmon Sperm DNA. The labeled probes were then incubated for 10 min before migration in a 5% native polyacrylamide 29:1 gel in 0.5% TBE. When antibodies were used, 1 μl of anti-E2F1, E2F2 or E2F5 rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (SC-193, SC-633 and SC-999) was added to the mixture. After migration, the gels were dried and revealed using a Phosphor-Imager. GST–E2F1 had been purified from bacteria as described previously (Heino et al., 2000).

Flow cytometry
The cell cycle of HeLa, Caski and C33 cells was arrested 24 h after transfection with the various shRNAs by adding 2.5 mM thymidine to the medium for 24 h. T0 cells were then harvested by trypsinization at the release, whereas T2 cells were harvested 6 h after changing the medium to release the thymidine block. Cells were fixed in 70% ethanol and DNA was labeled with 20 μg/ml propidium iodide and 10 μg/ml RNase. Cell-cycle analyses were carried out using an LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell proliferation analyses
HeLa cells were reverse transfected and seeded on E-plates 96 (Roche). Cell growth was measured with the xCELLigence system (Roche); the cell density is given by the cell index, an arbitrary unit indicating the percentage of the well occupied by cells every hour during 88 h. The growth curves were normalized 27 h after the transfection.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgements
We thank Dr Felix Hoppe-Seyler and Professor Hanswalter Zentgraf, who provided us with the HPV18 E7 antibody. We thank Dr Sardet for providing us the E2F5-expressing vector.

References


E2F5 activates HPV18 E6/E7 transcription
S Teissier et al

Chromatin immunoprecipitation
Chromatin was purified and digested using the ChiP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. Pre-clearing was carried out for 1 h on a rotating wheel using protein A/G agarose (Santa Cruz Biotechnology). The chromatin was then incubated overnight at 4°C with the rabbit polyclonal anti-GFP antibody (Torrey Pines Biolabs, East Orange, NJ, USA, TP401). The protein A/G agarose mixture was added and incubated during 1 h at 4°C. Beads were centrifuged and washed three times with Mix1 and were resuspended in 200 μl of Mix2 and incubated overnight at 65°C. The mixture was then digested by 0.5 mg/ml proteinase K at 42°C for 2 h. Immunoprecipitated DNA was purified by phenol–chloroform (50–50%) extraction and ethanol precipitation.

Western blot
Immunoblotting experiments for the detection of HPV18 E7 were performed with an anti-HPV18 E7 antibody provided by Dr Zentgraf and Dr Hoppe-Seyler as described (Kuner et al., 2007); anti-actin rabbit antibody was from Sigma (St Louis, MO, USA) (A-2066).

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