

# Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes

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*Papillomaviruses cause certain common human cancers, most notably carcinoma of the cervix. The viral oncoproteins E6 and E7 are essential components in malignant conversion, although, in spite of being necessary, they are not sufficient for the development of the malignant phenotype. High risk HPV oncogenes fulfill dual functions in genome-harboring cells: their derived oncoproteins stimulate cell growth by pleiotropic effects. At the same time they act as progression factors by inducing mutations in host cell DNA and aneuploidy. The mechanism underlying the process towards malignant conversion, usually covering a long latency period between primary infection and cancer emergence, is presently not fully understood. It emerges, however, that cancer development depends on the interruption of at least two signalling cascades (labeled as C1F I and C1F II) that interfere with the function of viral oncoproteins (C1F I) and with the transcription of viral oncogenes (C1F II). Further modifications of host cell genes most likely mediate the escape from immune surveillance mechanisms of the host and the development of metastases.*

**Key words:** cervical cancer / E6 and E7 genes / oncogene regulation / immortalization / papillomavirus

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## Introduction

Specific types of human pathogenic papillomavirus (HPV) genotypes are the causative agents of anogenital cancers, most notably of cancer of the cervix, but probably also of approximately 20% of oral cancers.<sup>1</sup> The HPV types 16 and 18 have been most intensively characterized and shown to possess immortalizing

and transforming activity for a wide variety of human cell types.<sup>2</sup> HPV 16 is by far the most prevalent HPV types in HPV-linked human cancers.

The induction of continuous growth of human cells kept in tissue culture ('immortalization') is one of the consistent features of a subgroup of papillomaviruses that has been linked to human cancers. Initially discovered after transfection of whole HPV 16 or 18 genomes into human keratinocytes,<sup>3–5</sup> it has subsequently been demonstrated to depend entirely on two early viral genes E6 and E7<sup>6,7</sup> which appear to be uniformly expressed in HPV-positive human cancer cells.<sup>8</sup>

The molecular events underlying HPV-induced immortalization and subsequent malignant conversion have only recently been carefully analyzed and are understood only to a limited degree. This review tries to summarize our present understanding of those changes and to provide a provisional and still speculative scheme of molecular modifications accompanying those events.

## Specific viral oncogenes are necessary but not sufficient for cell immortalization, for malignant conversion and for the maintenance of the malignant state

Three viral oncogenes have been identified in high risk HPVs: the E5 gene which is obviously not necessary for immortalization of human cells and for the maintenance of the immortalized or malignant phenotype of HPV infected cells. E5 expression does not lead to immortalization of human cells, in addition, the gene is frequently deleted in cervical carcinoma lines.<sup>8</sup> E5 may be required for early events in natural HPV infections.

The E6 and E7 oncogenes, in contrast, mediate immortalization of a wide variety of human cells either individually or more effectively jointly.<sup>2</sup> Substantial evidence has been accumulated demonstrat-

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ing the requirement for viral oncogene expression in order to maintain the immortalized state in high risk HPV-DNA carrying cells.<sup>1</sup> Yet, it became clear in recent years that viral oncogene expression is not sufficient for cell immortalization: somatic cell hybridization of individual clones of immortalized human keratinocytes frequently results in senescence of the cell hybrids in spite of continued viral oncogene message expression,<sup>9</sup> similar to observations made in somatic cell hybrids with SV40-immortalized cells where senescent cells continue to express SV40 T antigen.<sup>10,11</sup> In both systems at least four complementation groups have been identified, pointing to the existence of several cellular genes, probably engaged within the same cellular signalling cascade, that regulate cellular senescence of normal human keratinocytes, whose interruption (in addition to viral oncoprotein expression) results in an immortalized phenotype.

Analogous observations have been made in HPV malignantly transformed cells or in cells derived from cervical carcinoma cell lines. The need for E6 and E7 oncogene expression for the maintenance of the malignant phenotype has been demonstrated in a number of different approaches: in a specific cervical carcinoma cell line (SW 756) addition of dexamethasone efficiently blocks HPV 18 E6/E7 gene expression.<sup>12</sup> Under these conditions the cells lose their malignant phenotype and cease to grow. Reintroduction of the same genes under the control of a dexamethasone-inducible promoter restores their carcinogenicity. E6/E7 anti-sense constructs are also able to selectively inhibit growth of carcinoma cells expressing these genes.<sup>13-16</sup> In addition, anti-sense oligonucleotides and specific ribozymes interfere with the growth of HPV-positive cervical cancer cells.<sup>17-19</sup>

Somatic cell hybridization with different cervical carcinoma cells or with *in vitro* malignantly transformed keratinocyte lines again reveal that viral oncogene expression is not sufficient for the maintenance of the malignant phenotype: such hybrids either succumb to senescence or complement each other to an immortalized phenotype or remain malignant when tested in immunocompromised animals.<sup>20</sup> This again indicates the recessive trait of the malignant phenotype, since in the case of complementation towards an immortalized state E6/E7 expression continues. Obviously a different signalling cascade, regulated by several genes, suppressing the tumorigenicity of immortalized cells has been interrupted in the course of malignant progression.<sup>21</sup>

Thus, although the E6 and E7 oncogenes are re-

quired for HPV-mediated cell immortalization and for the malignant phenotype of cervical carcinoma cells, their mere expression is clearly not sufficient to immortalize and transform cells. Host cell genes interfering with their function or expression have to be modified in the course of transition to a different growth pattern.<sup>21,22</sup>

### **E6 and E7 proteins are mutagenic and may act as progression factors**

The functions of E6 and E7 oncoproteins have been reviewed repeatedly<sup>23,24</sup> and will not be discussed here in detail. These genes are able to immortalize various human cell types independently, albeit at much lower efficiency in comparison to their joint function. They have been found to interact with a large number of host cell proteins,<sup>24</sup> of particular interest is the interaction of E6 with the multifunctional host cell protein p53. The interaction of E6 with p53 is mediated by the ubiquitinase E6-AP and results in substantially enhanced degradation of p53. One of the functions of p53, the checkpoint control of G1/S-phase transition and its blockade after DNA damage of the respective cell, is circumvented by the E6-mediated p53 degradation. This seems to be one reason for the accumulation of mutagenic events in E6 expressing host cells and possibly also for the subsequent development of an aneuploid karyotype. It seems, however, that E6 is not the sole factor in inducing chromosomal instability, since similar observations, although at lower efficiency, have also been made in E7 immortalized cells. The mechanism is here even less understood.

Mutagenic activity and the development of aneuploidy are common consequences of high risk HPV infection.<sup>25,26</sup> Expression of both viral oncogenes, jointly as well as individually, results also in increased uptake of foreign DNA.<sup>27</sup>

Binding to p53 and its functional inactivation are not required for immortalization of high risk HPV-infected cells. E6 protein binding to p53, however, is not a requirement for cell immortalization.<sup>28</sup> Yet, the need for modifications within cellular genes for effective immortalization and in addition for malignant conversion still points to an important role of p53 degradation as progression factor by eliminating an important control for DNA repair and apoptosis.

Similar to E6, E7 is able to induce mutations in host cell DNA,<sup>25,29</sup> enhance the mutagenicity of chemical carcinogens<sup>26</sup> and the integration of foreign

DNA.<sup>27</sup> The mechanism of these interactions is presently poorly understood. The induction of mutations and chromosomal rearrangements, however, should contribute to the modification of those cellular genes that control HPV expression in infected human keratinocytes.

The individual and joint mutagenicity of E6 and E7 oncoproteins points to their dual role in carcinogenesis: besides acting as oncogenic factors by stimulating host cell proliferation (see below), they are able to modify host cell genes, among others those which are engaged in their own control. Therefore, they can also be considered as progression factors.

### **E6 and E7 oncoproteins interact and inactivate cyclin-dependent kinase inhibitors as early events in immortalization**

In view of the continued transcription of viral oncogenes in immortalized cells succumbing to senescence after somatic cell hybridization,<sup>9</sup> interruption of one or more signalling cascades interfering with functions of viral oncoproteins emerges as the most likely reason for cell immortalization. This has been tentatively labeled as the *cellular interfering factor (CIF) cascade I*,<sup>1,21,22</sup> preventing in its active state, conversion of high risk HPV-infected cells to immortalization. Immortalization can be considered as a high risk state for malignant transition, since single hit events, like introduction of an activated ras gene,<sup>30-32</sup> the viral fos gene<sup>33</sup> or even overexpression of the cellular fos gene<sup>34</sup> result in malignant conversion of immortalized cells.

There exist some hints that cyclin-dependent kinase inhibitors may play a major role in restricting the functions of viral oncoproteins. A first set of studies indicated that uroepithelial cells immortalized by HPV16 E6<sup>35</sup> or HPV16-immortalized foreskin keratinocytes (Whitaker and zur Hausen, unpublished data) are commonly devoid of p16<sup>INK4</sup> expression. The respective gene was either switched off due to methylation, or became mutated or deleted. These data suggest that immortalization by E6 requires the inactivation of p16<sup>INK4</sup> which seems to represent an antagonist of E6 functions. Interestingly this presumptive inhibitory function is bypassed by E7, since E6 and E7 expressing cells or cells immortalized exclusively by E7 over-express p16<sup>INK4</sup>. The latter induces cell cycle arrest by inhibiting the phosphorylation of pRB by the cyclin-dependent kinases CDK4

and CDK6.<sup>36</sup> The interaction between E7 and pRB circumvents this blockade.

The functional regulation of E7 is even less clearly understood. E7 protein expression in keratinocytes can abrogate the p21<sup>CIP1</sup>-mediated inhibition of CDK2<sup>37,38</sup> and also block the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup>.<sup>39</sup> It is possible, though presently not proven, that this is a concentration-dependent effect and that a low level of E7 expression could lead to a functional impairment of this oncoprotein by high concentrations of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> proteins. In this respect it will be particularly interesting to analyze the level of E7 protein in somatic cell hybrids succumbing to senescence and to determine at the same time the functional activity of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> proteins.

### **E6 and E7 gene expression is controlled by an intercellular signalling cascade, triggered at least in part by TNF $\alpha$ and interrupted during malignant conversion**

During the past decade a number of cytokines have been identified capable of down-regulating high risk HPV oncogene transcription.<sup>24</sup> This suggested that besides functional impairment of viral oncoproteins, additional control mechanisms may regulate the oncogene activity. A striking observation resulted from the analysis of HPV transcription of immortalized cells after transplantation of these cells into nude mice.<sup>40,41</sup> Immortalized cells, in contrast to HPV-containing malignant cells, revealed a dramatic reduction in transcriptional activity, when compared to the same cells, kept in tissue culture. To some extent this seemed to correlate to the usually very low transcriptional activity of HPV16 E6/E7 genes in basal cell layers of low grade cervical squamous intraepithelial lesions (SIL) in marked contrast to high grade lesions containing the same virus type.<sup>42,43</sup> This suggested the existence of an intercellular regulatory pathway, initiated by surrounding cell compartments, blocking HPV transcription and thereby suppressing potentially invasive growth of HPV-immortalized cells.

The existence of a second *cellular interfering factor cascade (CIF II)* has been documented subsequently by *in vitro* studies: addition of human or murine macrophages to HPV-immortalized human keratinocytes effectively blocked HPV transcription selectively in immortalized but not in malignantly transformed cells. This effect is most likely mediated

by tumor necrosis factor (TNF)  $\alpha$  excretion, since TNF $\alpha$  addition results in the same effect.<sup>34,44</sup> These studies also revealed that TNF $\alpha$  treatment results in a modification of dimer composition of the transcription factor AP-1, one of the main regulatory factors in high risk HPV transcription. Whereas immortalized cells preferentially contain c-jun/c-jun homodimers, after TNF-treatment the AP-1 composition shifts selectively in immortalized cells to c-jun/Fra-1 heterodimers. This parallels the downregulation of HPV transcription, suggesting a relationship of both events. Introduction of c-fos overexpressing plasmids into immortalized cells modifies the AP-1 composition to c-jun/c-fos heterodimers and results in malignant conversion of these cells.<sup>34</sup>

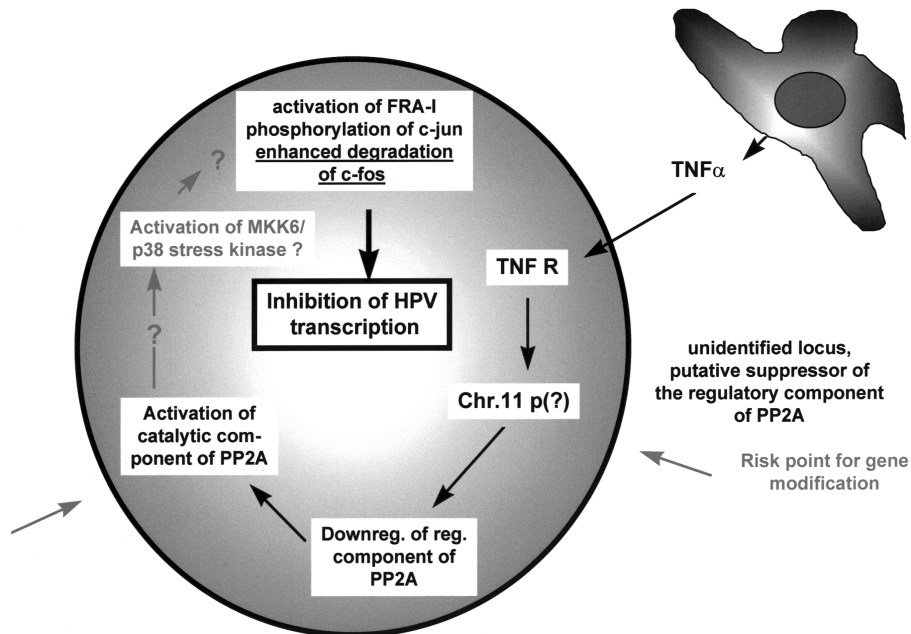
It is likely that protein phosphatase 2A (PP2A) is somehow engaged in this signalling cascade, since the upregulation of a regulatory component (PR55 $\beta$ ) of this complex enzyme or the blockade of its catalytic subunit result in the upregulation of persisting HPV oncogene transcription and increased transformability of the respective cells.<sup>45</sup> A certainly oversimplified scheme of the tentative CIF II cascade is shown in Figure 1.

Malignant conversion of HPV-immortalized cells,

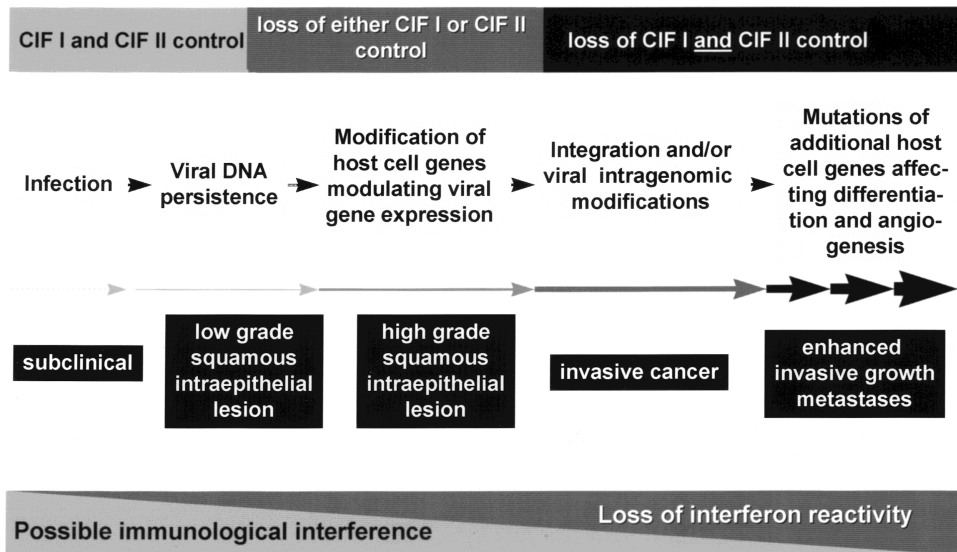
thus, seems to require the interruption of a second signalling pathway which intercellularly (most likely via macrophages and other cytokine excreting cells) interferes with HPV transcriptional events. In addition, however, other molecular events should contribute to the escape of these potential tumor cells from immune surveillance mechanisms and to the development of tumor metastases.

**Is TNF $\alpha$ -mediated interferon  $\beta$  responsiveness the key factor in preventing immortalized cells from malignant conversion?**

The assumption of a critical role of cellular regulatory events in HPV oncogene transcription in the prevention of malignant conversion has been repeatedly challenged: human keratinocytes immortalized by high risk HPV oncogenes under the control of foreign promoters, such as  $\beta$ -actin, should not be subject to this type of control and may thus convert directly to a malignant phenotype. This is, however, not the case.<sup>6</sup> Although mice transgenic for  $\beta$ -actin-regulated HPV 16 E6 and E7 genes develop anaplastic neuroepithelial tumors and chorion plexus carci-



**Figure 1.** Tentative scheme of a macrophage-mediated intercellular control of high risk human papillomavirus transcription. The gray arrows outside the circle indicate suggested risk points for cellular gene modifications in the course of transition from an immortalized towards a malignant phenotype. The gray arrows inside the circle mark as yet undefined speculative interactions within these signalling pathways.



**Figure 2.** Tentative scheme of papillomavirus-induced pathogenesis resulting in cervical cancer.

nomas,<sup>46</sup> keratinocytes immortalized by similar constructs fail to reveal malignant growth. TNF $\alpha$  treatment of HPV18 containing non-malignant HeLa fibroblast hybrids, containing also HPV16 DNA under the control of a  $\beta$ -actin promoter does not lead to a suppression of HPV16 oncogene expression (Hanke, Rösl and zur Hausen, unpublished data). Yet, these cells still remain non-malignant when inoculated into nude mice.

These observations raise the possibility that additional intercellular events, not directly affecting HPV transcription, may suppress malignant growth of these cells. One possible factor may be the specific induction of members of the interferon family. Ronco *et al*<sup>47</sup> demonstrated that HPV16 E6 binds and functionally inhibits the interferon regulatory factor 3 (IRF-3). IRF-3 is a potent transactivator of interferons and binds to the regulatory elements of the  $\beta$ -interferon promoter. The E7 protein abrogates signalling mediated by interferon  $\alpha$  and seems to prevent the translocation of p48, the DNA-binding component of the interferon-stimulated gene factor 3, to the nucleus upon interferon  $\alpha$  stimulation. Interferon- $\beta$  is able to induce apoptotic cell death in HPV16-immortalized keratinocytes.<sup>48</sup> Another interferon, interferon  $\tau$ , has been shown to efficiently suppress HPV E6/E7 oncoprotein expression.<sup>49</sup> These and other observations could suggest an important role of interferons in the suppression of invasive growth of HPV-immortalized keratinocytes.

In a recent study we could demonstrate that TNF $\alpha$  treatment of HPV-immortalized cells, but not of malignant cells, resulted in the effective induction of interferon  $\beta$  (Bachmann, Zawatzky, Rösl and zur Hausen, unpublished data). This occurs as well in cells containing HPV oncogenes under control of the  $\beta$ -actin promoter. It is, therefore, possible that the upregulation of  $\beta$  interferon, even in cells expressing viral oncoproteins controlled by foreign promoters, prevents a level of viral oncoprotein expression necessary for mediating invasive growth.

The emerging picture of high risk HPV-mediated pathogenesis is schematically outlined in Figure 2.

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