Effects of human papillomavirus type 16 E5 deletion mutants on epithelial morphology: functional characterization of each transmembrane domain

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Human papillomavirus type 16 (HPV-16) is the cause of cervical cancer. The HPV genome encodes three transforming proteins, E5, E6 and E7. E6 and E7 are the main transforming proteins of HPV, while the role of E5 is still poorly understood. Using three dimensional organotypic raft cultures we show that HaCaT human keratinocytes expressing HPV-16 E5 form a very perturbed epithelium, with simultaneous hyperkeratinization of some cells and defective differentiation of other cells. The basal layer is disturbed and many cells invade the collagen matrix. Many cells among the differentiated layers show characteristics of basal cells: progression through the cell cycle, expression of cytokeratin 14, lack of cytokeratin 1 and production of matrix metalloproteases (MMP). Using deletion mutants which encompass the three hydrophobic domains of E5, we have assigned the ability to promote invasion of the matrix to the first hydrophobic domain, and the capacity to induce MMP9 to the C-terminal four amino acids. We also show that invasion and production of MMP9 can be dissociated, as mutants that are still capable of invasion do not produce MMP9 and vice versa.

INTRODUCTION

Human papillomaviruses (HPVs) infect mucosal and cutaneous epithelia and induce lesions that can persist and progress to cancer; this is particularly so for HPV-16, the major cause of cervical cancer. The HPV genome encodes three transforming proteins, E5, E6 and E7. E6 and E7 are the main transforming proteins of HPV, while the role of E5 is still poorly understood. While E6 and E7 are expressed throughout the course of the disease and are necessary for the maintenance of a transformed phenotype, E5 is expressed during the early stages of infection and its expression is often, but not always, extinguished as the lesion progresses towards malignancy (Cavuslu et al., 1996; Chang et al., 2001; Kell et al., 1994).

E5 of HPV-16 is a hydrophobic membrane-associated protein 83 aa long, with three well-defined hydrophobic regions, believed to be transmembrane domains, and short regions at the N and C termini that may extend beyond the lipid bilayer. E5 is localized in the endomembranes of the infected cells, the Golgi apparatus and the endoplasmic reticulum (Suprynowicz et al., 2006). The functions of E5 appear to be multiple. E5 binds to the 16K subunit c (a component of the proton pump vacuolar ATPase) (Conrad et al., 1994) and reduces acidification of the endomembrane compartments (Schapiro et al., 2000; Straight et al., 1995), resulting in the sustained activation of the receptor for epidermal growth factor (EGF-R) (Straight et al., 1993). Likely through the activation of EGF-R, E5 is also responsible for the activation of other kinases, such as MAP kinase and PI3 kinase (Crusius et al., 1997; Kim et al., 2006). The impeded acidification of the Golgi apparatus and other organelles is likely to be
responsible also for E5-mediated inhibition of FAS-mediated apoptosis (Kabsch et al., 2004), and for downregulation of surface MHC class I and class II (Ashrafi et al., 2005; Zhang et al., 2003). The downregulation of surface MHC class I is additionally due to a direct binding between E5 and the heavy chain of the complex (Ashrafi et al., 2005, 2006). In transgenic mice, E5 alters the growth and differentiation of stratified epithelia and induces epithelial tumours at a high frequency in an EGF-R-dependent fashion (Genther Williams et al., 2005), contributing to both promotion and progression of skin carcinogenesis (Maufort et al., 2007). E5 has also been implicated in subtly regulating the viral genome copy number during viral DNA replication (Fehrmann et al., 2003; Genther et al., 2003).

The first transmembrane domain of E5 is responsible for the downregulation of MHC class I (Ashrafi et al., 2006), and for anchorage-independent growth in monolayer (Lewis et al., 2008), while the second and third transmembrane domains co-operate in binding the 16K subunit c (Adam et al., 2000; Rodriguez et al., 2000). Although the impact of expression of E5 on some cell functions, such as the activation of the EGF-R, the downregulation of connexin 43 and the inhibition of FAS- and TRAIL-mediated apoptosis, has been studied in organotypic cultures of differentiating cells (Tomakidi et al., 2000a, b), the specific contribution of each of the three transmembrane domains to cell differentiation is not known.

In this communication we report the effects that HPV-16 E5 and its deletion mutants (Fig. 1) have on the differentiation and behaviour of human keratinocytes in organotypic three-dimensional raft cultures.

RESULTS AND DISCUSSION

HPV-16 E5 is not capable of immortalizing primary human keratinocytes and has to be studied in an immortal cellular background. The established HaCaT human keratinocyte cell line has been extensively used both by us and by others for the analysis of the functions of E5 (Ashrafi et al., 2005, 2006; Oelze et al., 1995; Crusius et al., 1998; Tomakidi et al., 2000a, b; Kabsch et al., 2004; Lewis et al., 2008; Kim et al., 2009; Hu et al., 2009). HaCaT keratinocytes (Boukamp et al., 1988), although aneuploid and immortal due to heterozygous mutations in the p53 gene (Lehman et al., 1993), differentiate similarly to normal keratinocytes both in xenografts (Boukamp et al., 1988) and in organotypic raft cultures (Supplementary Fig. S2, available in JGV Online) thus justifying their use in HPV studies. Additionally, for their studies of HPV-16 E5, other authors have used either non-human cells (Straight et al., 1993; Tsao et al., 1996), or cells previously immortalized by other agents such as HPV-16 E6 and E7 oncogenes or SV40 large T oncogene (Stoppler et al., 1996; Tsao et al., 1996; Krawczyk et al., 2008; Regan & Laimins, 2008).

Organotypic three-dimensional raft cultures have been particularly useful in unravelling the impact of HPV genes on the fate of keratinocytes (Chow & Broker, 1997). Here, we have studied the effects of E5 on cell proliferation and differentiation in raft cultures of HaCaT keratinocytes. Efforts have been made to present the same areas of each raft culture for ease of comparison. However, occasionally areas from other parts of the rafts are presented to exemplify a particular point more clearly.

In our HaCaT cells, E5 is expressed constitutively. Expression of E5 was detected throughout the thickness of the HaCaT epithelium (Supplementary Fig. S3, available in JGV Online), similar to that reported by Chang et al. (2001) in high grade cervical intraepithelial neoplasia (CIN) and cervical cancer. However, it is not possible to directly compare levels of expression of E5 in HaCaT cells and in in vivo lesions as there are no available and reliable antibodies against E5. Given these limitations, HaCaT-16E5 cells represent the best model currently available to investigate E5 functions.

HPV-16 E5 alters the differentiation programme of HaCaT keratinocytes

**Morphology.** HaCaT cells, harbouring empty pCI-neo plasmid, full-length wild-type E5 or the deletion mutants, all expressed from the constitutive immediate-early (IE) promoter of hCMV (Fig. 1), were grown on collagen rafts and allowed to differentiate. Haematoxylin and eosin (H&E) staining showed that HaCaT-pCI-neo cells differentiated into a fairly ordered epithelium with a regular, unbroken basal layer and spinous, granular and squamous differentiating layers (Fig. 2a), resembling normal foreskin keratinocytes (Supplementary Fig. S2). In contrast, HaCaT-16E5 formed an epithelium with dysplastic/neoplastic characteristics: a very disturbed basal layer with many vacuolated cells and numerous invading pockets (Fig. 3a). Furthermore, the upper epithelium

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**Fig. 1.** Schematic diagram of wild-type HPV-16 E5 and deletion mutants. The rectangles indicate the hydrophobic domains and the lines indicate the non-hydrophobic portions of the proteins. The numbers refer to amino acids. The four di-leucine motifs are indicated in full-length E5. The protein is tagged with the HA-epitope but the amino acids of the epitope have not been taken into account in the numbering of E5 residues. Expression of the E5 gene is controlled by the constitutive hCMV IE promoter.
showed areas of hyperkeratinization and also nucleated cells (Fig. 3b).

The vacuolization of the basal cells in E5-expressing cells has already been observed both in bovine cells expressing E5 of BPV-1 and BPV-4 (Ashrafi et al., 2000) and in HPV-16 E5-expressing human foreskin keratinocytes immortalized by E6 and E7 (Krawczyk et al., 2008).

This morphological pattern was repeated in two clones of HaCaT-16E5 and one clone of HaCaT-pL2-16E5 in which 16E5 is expressed from the epithelial cell-specific promoter of Epstein–Barr virus (EBV) LMP2 gene (data not shown), thus excluding clonal differences.

**Cell cycling.** To see if E5 induces unscheduled cell cycling, the sections were analysed for the expression of mcm5 and/or Ki67; both are markers of cell cycle progression (Alazawi et al., 2002; Gerdes et al., 1984). Very similar results were obtained for mcm5 and Ki67 (Fig. 3c–f). In HaCaT-pCI-neo, only the occasional cell of the basal layer showed nuclear Ki67 staining (Fig. 2b, inset). On the contrary in HaCaT-16E5, mcm5/Ki67 staining was detected in the basal and suprabasal layers (Fig. 3d, f), including cells among the differentiated keratinocytes and at the very surface of the epithelium (Fig. 3d, f), showing that some cells were capable of cell cycle progression even in the uppermost layers. The invading cells were also positive for these markers (Fig. 3c, d).

**Differentiation.** The differentiation status of the cells was assessed by looking at the expression of cytokeratin 14 (CK14), specific for basal layer cells, and CK1, specific for differentiating keratinocytes. In HaCaT-pCI-neo cells, CK14 was expressed in the basal layer and CK1 was present throughout the section except in the basal layer (Fig. 2c, d). In HaCaT-16E5 cells, CK14 was expressed in the basal layers and in the outer cells of the invasion pockets, and also, more faintly, in patches of the upper layers (Fig. 3g, h), the latter suggesting faulty differentiation. CK1 was present throughout the thickness of the raft and in the inner mass of the invasion pockets, showing that in these invading masses differentiation proceeded inwards (Fig. 3i). CK1 was not present in the basal layers and in the upper layer patches where CK14 was present (Fig. 3i, j), confirming that some cells have not differentiated properly.

The presence of cycling cells and CK14 in the upper layers of the HaCaT-16E5 epithelium is somewhat reminiscent of the epithelium observed in mice transgenic for HPV-16 E5 (Genther Williams et al., 2005), where BrdU-positive nuclei were found in the upper layers and CK14 expression was observed throughout the thickness of the epithelium.

**Invasion.** Matrix metalloproteases (MMPs) are produced by invading cells; they digest the extracellular matrix thus allowing the cells to move into the surrounding tissues (Björklund & Koivunen, 2005; Deryugina & Quigley, 2006). To see if the invading HaCaT-16E5 cells were producing MMPs, the sections were analysed for the presence of MMP9 (gelatinase B) and MMP1 (collagenase-1) by the use of the appropriate antibodies. HaCaT-pCI-neo
cells did not produce any MMP9 (Fig. 2e), but MMP1 was produced by the basal layer (Fig. 2f). The production of MMP1 by these control cells is likely to be due to the fact that the ‘dermal’ portion of the raft cultures was made of type I collagen and it has been shown that type I collagen stimulates production of MMP1 by keratinocytes in vitro and that MMP1 is necessary and sufficient for keratinocyte migration over type I collagen (Pilcher et al., 1999). HaCaT-16E5 cells were positive for both MMP9 and MMP1 both in the basal layer and in the invading pockets (Fig. 3k, l). Additionally, MMP1 was present in much greater amounts than in HaCaT-pCI-neo cells and also in suprabasal layers (Fig. 3l), indicating that the abundant production of MMP1 by the HaCaT-16E5 cells was not only due to the interaction with type I collagen but also predominantly to the effect of E5.

E5-induced invasion might substantially contribute to tumour progression in persistently infected cervical epithelial cells, although this remains to be confirmed.

The gene for MMP1 is overexpressed in HPV-associated cervical cancer and in BPV-1-associated equine sarcomas (Narayan et al., 2007; Yuan et al., 2008), but the papillomavirus proteins involved in the transcriptional activation of the MMP1 gene in these tumours have not yet been identified. MMP9 too has been reported to be activated by papillomavirus (Akgül et al., 2006; Behren et al., 2005). The MMP promoters are responsive to the transcription factor AP-1 (Clark et al., 2008). It is known that E5 promotes the upregulation of c-fos (Chen et al., 1996) and that c-jun is activated (phosphorylated) in equine sarcomas expressing BPV-1 E5 (Borzacchiello et al.,

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**Fig. 3.** Organotypic cultures of HaCaT-16E5 cells. (a, b) H&E, note the vacuolated basal cells and the numerous invasion pockets in (a) and the areas of hyperkeratinization in (b) (arrowheads); (c, d) mcm5; (e, f) Ki67, note the areas of cycling cells in the upper and superficial layers in (d) and (f) (arrowheads); (g, h) CK14 in the basal layer and in the outer layers of the invading pockets, note the upper layers staining (arrowheads), particularly in (h); (i, j) CK1 in the upper layers, note the lack of staining in areas of superficial layers (arrowheads), roughly where there was staining for CK14, also note in (i) the keratinization in the central part of the invasion pocket (arrow); (k) MMP9 in the basal layer and in the outer portion of the invading pockets; (l) MMP1 in the basal and suprabasal layers and in the pockets of invasion. (b), (d), (f), (h) and (j) at ×10 magnification, all other panels at ×5 magnification.
2009). Both c-jun and c-fos are components of AP-1. It is therefore plausible that the observed production of MMP1 and MMP9 in the HaCaT-16E5 epithelium is due to the effect of E5 on c-fos/c-jun with consequent activation of MMP genes. This would not be the first example of a viral oncoprotein activating MMP production through AP-1 as the membrane-associated LMP1 oncoprotein of EBV induces expression of MMP9 through AP-1 (Wakisaka & Pajano, 2003; Yoshizaki et al., 1998). It is to be noted, however, that in warts induced by the cottontail rabbit papillomavirus the activation of MMP9 has been ascribed to E2 (Behren et al., 2005).

It is clear that the expression of E5 alters the differentiation programme of HaCaT keratinocytes, leading to both hyperkeratinization and defective differentiation in the upper layers, disruption of the basal layer and invasion.

**HPV-16E5 deletion mutants allow the assignment of functions to the domains of E5**

We have shown that HPV-16 E5 downregulates MHC class I (Ashrafi et al., 2005) and studies of deletion mutants of E5 lacking one or more of the hydrophobic domains have assigned the ability to downregulate and physically bind MHC class I to the first hydrophobic domain (Ashrafi et al., 2006). The availability of these mutants prompted us to identify the E5 domains responsible for cell cycling, differentiation and invasion. E5R79 lacks the C-terminal four amino acids, E5A54 additionally lacks the third transmembrane domain, E5R30 contains only the N terminus and the first transmembrane domain, and Del-1 lacks only the first transmembrane domain (Fig. 1). These E5 mutants have been described previously by Rodriguez et al. (2000) and Ashrafi et al. (2006). The cell lines express the E5 deletion mutants to levels similar to those of HaCaT-16E5 (Ashrafi et al., 2006; Supplementary Fig. S1, available in JGV Online). Three clones each of HaCaT-E5A54, HaCaT-E5R30 and HaCaT-E5Del1, and one clone of HaCaT-16E5R79 were grown in organotypic raft cultures and each cell clone was analysed at least two times as described for HaCaT-16E5 cells, with reproducible results.

HaCaT-E5R79, HaCaT-E5A54 and HaCaT-E5R30 cultures exhibited very similar phenotypic characteristics, suggesting that the third and second transmembrane domains and the intervening regions did not markedly influence the morphology of the epithelium. Therefore, only HaCaT-E5R30 will be described and discussed in detail as emblematic of that formed by the other two mutants. Some aspects of HaCaT-E5R79 rafts are shown for comparison.

**Morphology.** In all clones analysed, the epithelium formed by HaCaT-E5R30 was generally of a more homogeneous thickness than that formed by HaCaT-16E5 (Fig. 4a). The basal layer was not vacuolated and the lack of vacuolization in these epithelia is in accordance with the lack of koilocytosis in keratinocytes expressing a 20 C-terminal amino acid deletion mutant of E5 (Krawczyk et al., 2008). In the HaCaT-E5R30 epithelium, there were numerous pockets of invasion, as observed for E5 (Fig. 4a). The morphology of a raft culture of HaCaT-E5R79 is shown for comparison (Fig. 4g).

**Cell cycling.** The only cells expressing mcm5 were those of the basal layer and the invasion pockets (Fig. 4b). Little or no mcm5 staining was observed in the upper layers.

**Differentiation.** Hyperkeratinization was not as extensive as in the rafts formed by HaCaT-16E5. CK14 was expressed in the basal layers and in the outer cells of the invasion pockets (Fig. 4c). CK1 was expressed in the upper layers and in the centre of the invasion pockets (Fig. 4d). Little or no CK14 was seen in the upper layers.

**Invasion.** There were numerous pockets of invasion as in the HaCaT-16E5 epithelium, but the major and most noticeable difference was that invasion was not accompanied by the production of MMP9. MMP9 was expressed only in very few cells and the great majority of the epithelium was negative for this marker (Fig. 4e). A similar MMP9 expression pattern was observed for HaCaT-E5R79 (Fig. 4h). On the contrary, MMP1 was present in the basal layer and in the invasion pockets (Fig. 4f), but its levels were not dissimilar from those observed in HaCaT-pCI-neo cells.

These patterns were observed with three clones each of HaCaT-E5A54 and HaCaT-E5R30 and one of HaCaT-E5R79. The reproducibility of these results and the fact that three different mutants induced the same phenotype rules out clonal variations.

The behaviour of HaCaT cells expressing E5 mutants, including HaCaT-E5R79 in which E5 has lost only the C-terminal four amino acids, suggests that the most C-terminal four amino acids are responsible for ectopic cell cycling and production of MMP9. Whether the C-terminal amino acids signal to the nucleus to activate transcription of the MMP9 gene and whether AP-1 is involved in this process remains to be established. It is also possible to conclude that invasion does not depend on the production of MMP9, although it is possible that MMP1 contributes to the invasion of these cells.

**Removal of the first hydrophobic domain abrogates invasion**

**Morphology.** The epithelium formed by HaCaT-E5Del1 cells had characteristics similar to those of HaCaT-16E5 cells: the basal layer had an increased thickness and many vacuolated cells. The main difference between the two epithelia was the absence of invading pockets (Fig. 5a).

**Cell cycling.** Ki67 staining revealed that most of the cells of the basal layers and some cells in the upper layers were...
progressing through the cell cycle (Fig. 5b, c), although not as many or as superficial as in HaCaT-16E5 cells.

Differentiation. CK14 and CK1 expression patterns were again almost mirror images, with CK14 in the lower layers and in patches of upper layers and CK1 in the upper layers but less in those areas where CK14 was expressed (Fig. 5d, e).

Invasion. Although there was no invasion, both MMP9 and MMP1 were expressed in the basal layer (Fig. 5f, g). Again, the levels of MMP1 were similar to those of HaCaT-pCI-neo cells. This confirms that invasion can indeed be independent of MMP9 (and MMP1) production and that MMP9 production is due to the C terminus of E5 (Fig. 4h).

The di-leucine motifs in the first hydrophobic domain do not appear to contribute to raft culture phenotype. From the above it is clear that the removal of the first hydrophobic domain impairs the ability of the cells to invade collagen. This domain contains four di-leucine (LL) motifs at positions 11/12, 16/17, 22/23 and 27/28. It has been reported that mutation of LL22/23 causes the localization of a small amount of E5 away from the Golgi apparatus and into lamellipodia (Auvinen et al., 2004). To see if the LL motifs were at all responsible for the ability of the first hydrophobic domain to promote invasion, we used two HaCaT cell lines expressing E5 with LL11/12 mutated to valine (HaCaT-E5LL11/12VV) or LL22/23, 27/28 mutated to VV (HaCaT-E5LL22/23, 27/28VV) (Cortese et al., 2009). Both these cell lines were capable of invasion and the epithelium they formed was not dissimilar from that formed by E5 (Fig. 5g, h). Additionally, both cell lines showed cycling cells in the upper layers and MMP9 and MMP1 production was similar to E5 (data not shown). These results show that the LL motifs investigated here do not contribute noticeably to the tested functions of E5.

The first hydrophobic domain of E5 appears critical for the function of the protein: it is responsible for downregulation of MHC class I and binding to the heavy chain of the complex (Ashrafi et al., 2006), for growth in an anchorage-independent fashion (Lewis et al., 2008) and now, as shown here, for invasion. However, the di-leucine LL motifs present in this domain do not appear to contribute in an obvious way to the ability of the first hydrophobic domain to induce invasion. A biochemical analysis of E5R30 (first
domain, invasion promoting but MMP9 negative) and E5Del1 (first domain missing, non-invading but MMP9 positive) mutants will shed light on invasion and MMP9 production. Although HaCaT keratinocytes provide a convenient means of dissecting the effect of the domains of E5 on tissue morphology, differentiation and invasion, it would be useful in the future to back up our observations in another system. W12 cells are an epithelial cell line from a low grade CIN lesion, which contain episomal HPV-16 genomes and express E5 (Stanley et al., 1989). W12 cells can differentiate in raft culture to give tissue with many characteristics of CIN (Aasen et al., 2003). The contributions of E5 to the morphology, differentiation and other characteristics of W12 cells could be dissected from the effects of E6 and E7 by ablating E5 expression using siRNA. Alternatively, normal human keratinocytes could be transfected with HPV-16 genomes containing deletions or point mutations in the E5 gene. Raft tissues could then be generated and analysed as we have described.

**METHODS**

**Antibodies.** The following antibodies were used: anti-mcm5 [mouse monoclonal antibody (mAb) SPM197, 1:500; Cambridge Bioscience], anti-Ki67 (mouse mAb MiB, 1:200; Dako), anti-MMP1 (rabbit Ab 38929, 1:320; Abcam), anti-MMP9 (goat Ab AF911, 1:800; R&D Systems), anti-cytokeratin (CK) 1 (mouse mAb NCL-CK1, 1:100; Novocastra) and anti-CK14 (mouse mAb RCK107, 1:700; Abcam). Very similar results were obtained with mcm5 and Ki67 antibodies.

**Plasmids.** pCI-neo-16E5 contains the full-length HPV-16 E5 open reading frame (ORF), tagged with the HA epitope at the N-terminus, inserted into the commercial cloning vector pCI-neo (Promega) (further cloning details are as in Ashrafi et al., 2005). The expression of the E5 ORF is controlled by the constitutive IE promoter/enhancer of human cytomegalovirus (hCMV). The deletion mutants of E5 were made as described in Rodriguez et al. (2000) and Ashrafi et al. (2006). These were R79, lacking the hydrophilic C terminus; A54, lacking the third domain; R30, lacking the second and third domains, and Del1, lacking the first domain (Fig. 1). Additionally, two point mutants of the first hydrophobic domain of E5 were used, available as part of another study (Cortese et al., 2009). One point mutant had the leucine pair at residues 11/12 changed to valine (E5LL11/12VV) and the other had the leucine pairs at 22/23 and 27/28 likewise mutated to

![Fig. 5. Organotypic cultures of HaCaT-E5Del1, HaCaT-E5LL11/12VV and HaCaT-E5LL22/23, 27/28VV cells. (a–g) HaCaT-E5Del1; (a) H&E, note the vacuolated cells in the basal layers, areas of hyperkeratinization in the upper layers and the absence of invasion pockets; (b, c) Ki67 in the basal layers and occasionally in the upper layers (arrowhead in c); (d) CK14 in the basal layer and in the areas of the more superficial layers (arrowhead); (e) CK1 in the upper layers, note the lack of staining in areas of superficial layers (arrowheads), roughly where there was staining for CK14; (f) MMP9; (g) MMP1, note the presence of MMP9 and MMP1 in the basal layer despite the absence of invasion. (h, i) H&E of HaCaT-E5LL11/12VV and HaCaT-E5LL22/23, 27/28VV cells, respectively. Note the similarity of these epithelia to that of HaCaT-16E5 cells. (c) ×10 magnification, all other panels at ×5 magnification.](http://vir.sgmjournals.org)
valine (E5LL22/23, 27/28VV). The point mutants were constructed using site-directed mutagenesis on the original pCI-neo-16E5 plasmid.

Additionally, the E5 ORF was also cloned into pL2, a plasmid expressing the inserted gene under the control of the epithelial cellspecific promoter of the EBV LMP2 gene (Nakagawa et al., 1997), giving rise to HaCaT-pL2-16E5 (further cloning details are as in Ashrafi et al., 2003).

**Cell lines.** HaCaT human keratinocytes expressing full-length 16E5 or its mutants, together with control cells harbouring empty pCI-neo were grown in monolayers in Dulbecco’s modified Eagle’s medium (DMEM) high glucose without calcium chloride (Life Technologies), supplemented with 10% fetal calf serum (FCS; Gibco) in 5% CO2 at 37 °C. All cells lines have been shown to express comparable amounts of E5 and mutant mRNAs (Ashrafi et al., 2006).

**Organotypic raft cultures.** Cells were grown in organotypic raft cultures basically as described before (Aasen et al., 2003). Briefly, 1 x 10^6 HaCaT keratinocytes were seeded on a contracted lattice of collagen type I containing primary human epidermal fibroblasts (3 x 10^5 cells per ml collagen) in 3:1 DMEM: Ham’s F12 medium containing 10% FCS, 0.1 mg L-glutamine ml^-1, 0.4 mg hydrocortisone ml^-1, 0.1 nM chola toxin, 5 μg insulin ml^-1, 180 μM adenosine, 5 μg transferrin ml^-1 and 0.2 ng EGFR ml^-1. The lattice was lifted to the air-liquid interface after 3 days and fixed in 4% (v/v) formaldehyde after 12–14 days of stratification (Aasen et al., 2003). Sections were paraffin embedded and processed for routine H&E. Two clones each of HaCaT-pCI-neo, HaCaT-16E5, three clones each of HaCaT-ES5A54, HaCaT-ES5R30 and HaCaT-ES5Del1, and one clone of HaCaT-16E5R79 were grown in raft cultures and each cell clone was analysed at least two times. In addition, one clone each of HaCaT-pL2 and of HaCaT-pL2-16E5 was also analysed.

Both in rafts of HaCaT-pCI-neo-16E5 and HaCaT-pL2-16E5, E5 has been shown to be expressed throughout the thickness of the epithelium, resembling the expression of E5 in CIN lesions (Araibi et al., 1996).

**Immunohistochemistry.** This procedure was performed by the Pathological Services of the Institute of Comparative Medicine. Briefly, sections were de-paraffinized, heated in a pressure cooker for antigen retrieval for Ki67, mcm5, MMP1 and MMP9 detection or treated with Proteinase K (Dako) for cytokeratins 14 and 1 detection. Endogenous peroxidase was blocked using peroxidase block (Envision kit; DakoCytomation) and then the sections were washed first with Tris/Tween then twice with water. The appropriate primary antibody was added and after washing with Tris/Tween the secondary antibody conjugated with horseradish peroxidase was applied; dual anti-mouse/rabbit (Dako) was used for all antibodies, with the exception of MMP9, which required a rabbit anti-goat antibody step (Dako) before the addition of the conjugated secondary antibody. Sections were washed as above and 3,3′-diaminobenzidine (DAB) was added (Envision kit; DakoCytomation). Then sections were counter-stained with haematoxylin, de-hydrated and mounted. The stained sections were analysed using a Zeiss Axioskop 2 microscope and images were digitally captured with the accompanying camera and software KS300 version 3. Analysis of primary antibodies resulted in no staining in all cases (data not shown).

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