Functions of Human Papillomavirus E5 oncogene in epithelial cells and in the onset of cervical cancer

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ACADEMIC DISSERTATION

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To my family,
Jouko and Matias

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List of original publications

This thesis is based on the following original research articles, which are referred to in the text by their assigned Roman numerals.

I Kivi N, Greco D, Auvinen P, Auvinen E (2008). Genes involved in cell adhesion, cell motility and mitogenic signaling are altered due to HPV 16 E5 protein expression. *Oncogene*. 27(18):2532-41.

II Greco D*, Kivi N*, Qian K, Leivonen SK, Auvinen P, Auvinen E (2011). Human Papillomavirus 16 E5 modulates the expression of host microRNAs. *PLoS ONE*. 6(7):e21646.
* equal contribution

III Auvinen E, Kivi N, Vaheri A (2007). Regulation of ezrin localization by Rac1 and PIPK in human epithelial cells. *Experimental Cell Research*. 313(4):824-33.

Abbreviations

ANOVA	analysis of variance
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
Ct	cycle treshold
D-MEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERM	Ezrin-Radixin-Moesin
FAK	focal adhesion kinase
FCS	foetal calf serum
GO	gene ontology
HLA	human leukocyte antigen
HPV	human papillomavirus
IF	immunofluorescence
IHC	immunohistochemistry
kDa	kilodalton
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MEM	minimum essential medium
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
pAb	polyclonal antibody
PBS	phosphate-buffered saline
qPCR	quantitative polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
pRb	retinoblastoma protein
RNA	ribonucleic acid
SIL	squamous intraepithelial lesion
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

Abstract

Human papillomaviruses (HPVs) are the causal agents of cervical cancer, which is the second most common cancer among women worldwide. Cellular transformation and carcinogenesis depend on the activities of viral E5, E6 and E7 proteins. Alterations in cell-cell contacts and in communication between epithelial cells take place during cervical carcinogenesis, leading to changes in cell morphology, increased cell motility and finally invasion. The aim of this thesis was to study genome-wide effects of the HPV type 16 (HPV-16) E5 protein on the expression of host cell messenger RNAs (mRNAs) and microRNAs by applying microarray technology. The results showed that the HPV-16 E5 protein alters several cellular pathways involved in cellular adhesion, motility and proliferation as well as in the extracellular matrix. The E5 protein was observed to enhance wound healing of epithelial cell monolayers by increasing cell motility *in vivo*. HPV-16 E5-induced alterations in the expression of cellular microRNAs and their target genes seem to favour increased proliferation and tumorigenesis. E5 was also shown to affect the expression of adherens junction proteins in HaCaT epithelial keratinocytes. In addition, a study of a membrane cytoskeletal cross-linker protein, ezrin, revealed that when activated, it localizes to adherens junctions. The results suggest that ezrin distribution to forming adherens junctions is due to Rac1 activity in epithelial cells.

These studies reveal for the first time the holistic effects of HPV-16 E5 protein in promoting precancerous events in epithelial cells. The results contribute to identifyinging novel markers for cervical precancerous stages and to predicting disease behaviour.

Tiivistelmä (Summary in Finnish)

Papilloomaviruksen aiheuttama kohdunkaulan syöpä on maailman-laajuisesti naisten toiseksi yleisin syöpä. Papilloomaviruksella on kolme proteiinia, E5, E6 ja E7, joiden vuorovaikutus solun proteiinien kanssa ovat merkittäviä syövän syntyyn vaikuttavia tekijöitä. Keskeisiä tapahtumia syövän ja epiteelimuutosten kehittymisessä ovat epiteelin erilaistumisen, solujen välisen kommunikaation ja solujen tarttumiskyvyn häiriintyminen. Nämä johtavat solumorfologian muutoksiin, soluliikkuvuuden lisääntymiseen ja yhdessä muiden tapahtumien kanssa lopulta invaasioon.

Tämän väitöskirjatutkimuksen tavoitteena oli tutkia laaja-alaisesti mikrosirujen avulla ihmisen papilloomavirus tyyppi 16:n (HPV-16) E5-proteiinin vaikutuksia isäntäsolun lähetti-RNA:iden sekä mikro-RNA:iden ilmentymiseen. Tutkimuksen tavoitteena oli tarkastella solu- ja kudostasolla niiden proteiinien ilmentymistä ja sijoittumista solussa, joiden ilmentymisen havaittiin muuttuneen HPV-16 E5-proteiinin vaikutuksesta sekä tutkia tarkemmin eri soluproteiinien ilmentymisen muutoksia ajan funktiona. Tutkimuksessa selvitettiin myös E5-onkogeenin merkitystä epiteelisolujen kiinnittymiseen ja liikkuvuuteen. Tulokset osoittivat, että E5-proteiini muuttaa mm. useiden solukiinnittymiseen ja solunsisäiseen signaalinkuljetukseen osallistuvien geenien ilmentymistä sekä RNA- että proteiinitasolla. Näitä geenejä pystyttiin ryhmittelemään ja yhdistämään tiettyihin solunsisäisiin signalointireitteihin. E5-proteiinin vaikutus solujen liikkuvuuden lisääntymiseen havaittiin myös eläviä soluja kuvantamalla. Tulokset osoittavat myös, että E5-proteiini pääasiassa vähentää isäntäsolun mikro-RNA:iden ilmentymistä. Havaitut muutokset mikro-RNA:iden ja näiden kohdeproteiinien ilmentymisessä näyttävät edistävän syöpään johtavia tapahtumia solussa. Väitöskirjatyössä selvitettiin myös pieniin GTPaaseihin kuuluvien Rho-proteiinien merkitystä epiteelisolun tukirangan säätelyproteiinin, esriinin, säätelyssä. Rac1-aktiivisuuden osoitettiin olevan tarpeen esriinin sijoittumiseksi vyöliitoksiin.

Työssä pyrittiin myös mallintamaan HPV-16 E5-proteiinin kokonaisvaltaisia vaikutuksia pahanlaatuisten muutosten käynnistämisessä epiteelisolussa. Tutkimus antaa uusia mahdollisuuksia löytää muun muassa merkkiaineita kohdunkaulan syövän esiasteiden toteamiseen ja taudin käyttäytymisen ennustamiseen.

1. Introduction

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that infect epithelial cells. To date, more than 120 HPV genotypes have been identified (Bernard *et al.*, 2010), and these are divided into low- and high-risk types according to their association with malignant tumours. Strong evidence for decades has shown that persistent HPV infection plays a critical role in the pathogenesis of cervical cancer, which is one of the most common malignancies in women worldwide. High-risk HPVs have been detected in up to 50% of all anogenital cancers. HPV also plays a role in squamous cell carcinomas of the head and neck (25-30 %), and the involvement of HPV in skin cancers has been under extensive investigation in recent years (Karagas *et al.*, 2006; Leemans *et al.*, 2011).

Oncogenic high-risk HPV types induce malignant transformation in cervical mucosal epithelia by expressing E5, E6 and E7 oncogenes. The maintenance of the malignant phenotype in the cells requires continued expression of E6 and E7 proteins (Banks *et al.*, 1987; Butz *et al.*, 2003; Goodwin and DiMaio, 2000). Both low- and high-risk HPVs infect and replicate in the same tissues, but considerable differences are present in their pathogenesis and cellular targets. E6 and E7 oncoproteins of the high-risk HPVs are able to transform infected cells, but the same proteins of the low-risk HPVs have no detectable transforming activity (Storey *et al.*, 1988). The role of the E5 oncoprotein in the transformation process is less well understood. HPV E5 localizes to endosomal membranes and the Golgi apparatus, but is also found at the plasma membrane (Conrad *et al.*, 1993; Oetke *et al.*, 2000). The E5 protein is expressed at early stages of infection; however, the gene is occasionally deleted when the HPV genome is integrated into the host genome (Pater and Pater, 1985). Therefore, the E5 protein is unnecessary for the maintenance of malignancy, but it might contribute to early-stage neoplastic proliferation. Expression of HPV oncogenes alters expression of cellular genes as well as the expression of recently discovered microRNAs, resulting in malignancy and cancer.

Loss of epithelial differentiation, cell-cell communication and disturbance of cell adhesion are the key events leading to changes in cell morphology, enhancement of cell motility and, together with other cellular events, finally inducing cancer invasion. Ezrin is a plasma membrane-cytoskeleton cross-linker protein with multiple functions, such as involvement in cell adhesion and motility (Crepaldi *et al.*, 1997; Hiscox and Jiang, 1999). Ezrin has a key role also in tumour metastasis (Endo *et al.*, 2009; Federici *et al.*, 2009; Khanna *et al.*, 2004), and therefore, the effect of HPV-16 E5 protein on ezrin expression was evaluated here. HPVs are known to alter the expression of cadherins and catenins, which are important in cell-cell adhesion and in establishing proper cellular differentiation (Hubert *et al.*, 2005; Wilding *et al.*, 1996; Yasmeen *et al.*, 2010). Investigation of the alterations in cellular gene expression as well as in cell adhesion and motility elucidates these early events of transformation. Among these altered genes, it is possible to find markers for better detection of precancerous lesions and improved prediction of disease behaviour.

2. Review of the literature

2.1. Human Papillomaviruses (HPVs)

2.1.1. Genome and life cycle of HPVs

Human papillomaviruses (HPVs) are small, double-stranded, non-enveloped DNA viruses that belong to the *Papillomaviridae* family. Papillomaviruses are highly host, and tissue-specific, and they are rarely transmitted between species (Mistry *et al.*, 2008). Papillomaviruses typically infect the basal layer of skin or mucosal epithelium of the genital tract, anus, mouth or airways (Doorbar, 2005). Papillomaviruses do not elicit antibody responses due to the infection site within the epithelium being situated away from dermal immune cells (Stanley *et al.*, 2007). Therefore, the classification of HPV types is based on their degree of nucleotide sequence homology within the L1 open reading frame (ORF) (de Villiers, 1997). To date, nearly 120 HPV types have been identified (Bernard *et al.*, 2010), and these are divided into low- and high-risk types. Both high-risk and low-risk types can cause the growth of abnormal cells, but only the high-risk types are able to cause precancerous lesions. In low-grade lesions, the high-risk HPV genomes are present as episomes, while during progression to high-grade lesions or carcinomas, the genome is often integrated into the host cell genome (Jeon *et al.*, 1995).

The papillomavirus genome is a double-stranded circular DNA molecule approximately 8000 base pairs in length. It is packaged within a 60 nanometer capsid composed of viral L1 and L2 late proteins with 72 star-shaped capsomers presenting icosahedral symmetry. Most papillomaviruses contain six early and two late ORFs, and all coding sequences are located on one DNA strand only. The genetic organization of papillomaviruses is presented in Figure 1.

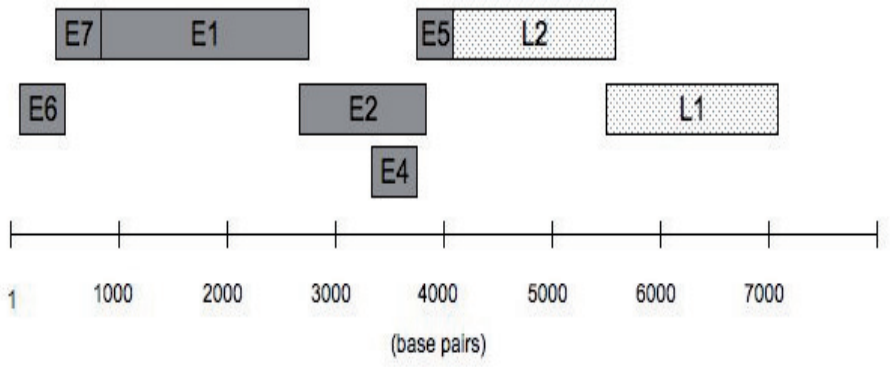

Figure 1. *Linear representation of the HPV genome. E1, E2, E4, and E5 are involved in viral replication and transcriptional control. E5, E6 and E7 are the main oncogenes, and L1 and L2 are the capsid proteins.*

A productive HPV life cycle is closely linked to epithelial differentiation (Howley and Lowy, 2001), as presented in Figure 2. Basal epithelial cells become exposed to the virus via wounds of the stratified epithelium (Howley and Lowy, 2001). Viral DNA replication occurs in the differentiating epithelium during the S-phase of the cell cycle in cooperation with cellular replication proteins (Lambert, 1991).

2.1.2. Role of HPV proteins

2.1.2.1. HPV proteins involved in replication and transcription

The main functions of the early proteins are in regulating transcription and replication (E1 and E2) and causing transformation (E5, E6 and E7). Most of these proteins are expressed throughout the infectious cycle, with reduced expression at late stages. E2 ORF encodes two or three different proteins, which all act as transcription factors and regulate viral transcription (Baker *et al.*, 1987; Bouvard *et al.*, 1994b; Cripe *et al.*, 1987). E1 origin-binding protein and the E1 replicative DNA helicase are encoded to support viral DNA replication (Stenlund, 2003). The E2 proteins bind to E1 and stimulate replication of viral DNA (Chow and Broker, 1994), and E1 is required throughout initiation of replication and elongation. High levels of E2 protein repress the expression of E6 and E7 proteins, and this function is disturbed by HPV genome integration into the host cell genome due to disruption of the viral genome within the E2 ORF. E4 protein is the first viral protein expressed in the late stage of the infection (Doorbar *et al.*, 1997). The function of E4 is unknown, although it is associated with keratin intermediate filaments (Doorbar *et al.*, 1997; Roberts *et al.*, 1997) and induces keratin reorganization (McIntosh *et al.*, 2010).

Late structural capsid proteins, L1 and L2, are expressed when infected host cells become terminally differentiated. Synthesis of the HPV capsid and production of progeny virus are induced in the uppermost layers of the epithelium. The late phase requires differentiation of the stratified epithelium.

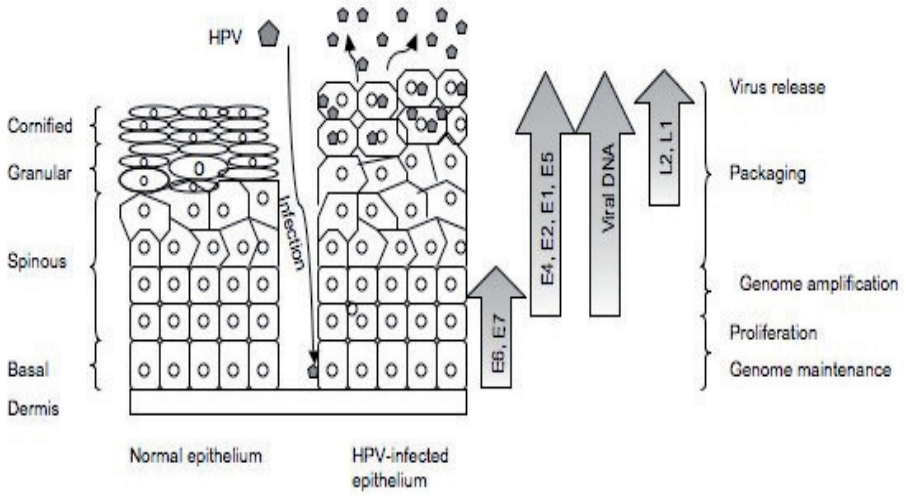

Figure 2. *Human papillomavirus (HPV) life cycle. HPV infects the basal layer of the epithelium. The viral genome is established in the nucleus, and early viral genes are expressed. The viral genome replicates with the assistance of cellular DNA replication machinery. Differentiation of HPV-infected cells triggers the productive phase of the viral life cycle. Arrows indicate the expression of the different HPV genes (adapted from Doorbar, 2006 and Moody and Laimins, 2010).*

2.1.2.2. HPV oncogenes

E5 protein

The E5 protein of high-risk HPV-16 is a small hydrophobic peptide with weak transforming activity (Pim *et al.*, 1992). The lack of antibody against E5 has raised difficulties in characterization of the E5 protein, and therefore, E5 mRNA expression has been used in numerous studies as a measure of E5 gene expression, assuming a correlation between mRNA and protein expression. E5 is expressed both in the early and late stages of the viral life cycle. The E5 protein is associated with cellular membranes (Auvinen *et al.*, 2004; Conrad *et al.*, 1993; Oetke *et al.*, 2000; Suprynowicz *et al.*, 2008). The ORF of E5 is occasionally disrupted in cervical cancer upon integration, but it is potentially

important for the initiation of transformation (Chang *et al.*, 2001; Schwarz *et al.*, 1985). The first evidence of the transforming activity of HPV E5 was discovered in a study where the HPV type 6 E5 protein assisted anchorage-independent growth in murine fibroblasts (Chen and Mounts, 1990). Soon after this, the HPV type 16 E5 protein was reported to cause anchorage independence and tumorigenic transformation of murine fibroblasts and epidermal keratinocytes (Leptak *et al.*, 1991; Leechanachai *et al.*, 1992; Pim *et al.*, 1992). HPV E5 has been suggested to have its primary activity in differentiated cells since the loss of HPV 31 E5 resulted in impaired activation of late viral functions (Fehrmann *et al.*, 2003). HPV 16 E5 has been observed also to have a role during the productive stage because loss of E5 reduced DNA synthesis in human keratinocyte raft cultures (Genther *et al.*, 2003).

E5 protein functions contribute substantially to the transformation process by increasing epidermal growth factor receptor (EGFR) -mediated signalling (DiMaio and Mattoon, 2001) and cell proliferation by activating this pathway (Pim *et al.*, 1992). One major signalling route of the EGFR is the Ras/MAPK (mitogen-activated protein kinases) pathway (Klapper *et al.*, 2000). Activation of the Ras oncogene leads to activation of the MAPKs, ERK1/2 of which is strongly associated with human cancer. Expression of the HPV-16 E5 protein suppresses degradation of EGF-EGFR complexes in endosomes, as was shown in human keratinocytes expressing E5 (Straight *et al.*, 1993; 1995). An increase in EGFR recycling to the cell surface has also been detected (Crusius *et al.*, 1997; Straight *et al.*, 1993). Tomakidi *et al.* (2000) observed increased EGFR expression and activation due to HPV-16 E5 in keratinocyte raft cultures. On the other hand, EGFR-independent pathways have also been suggested in the E5-activated signalling cascade. Crusius *et al.* (2000) observed modulation of the sorbitol-dependent activation of MAPK p38 and ERK1/2 in human keratinocytes through an EGF-independent mechanism. The E5 protein sensitizes human keratinocytes to apoptosis-induced osmotic stress (Kabsch and Alonso, 2002), but it is also able to protect human foreskin keratinocytes from ultraviolet B-irradiation-induced apoptosis

(Zhang *et al.*, 2002) as well as FasL- or TRAIL-induced apoptosis in HaCaT keratinocyte raft cultures (Kabsch *et al.*, 2004).

HPV E5 has been suggested to affect endocytic trafficking (Thomsen *et al.*, 2000) and to inhibit gap-junction-mediated cell-cell communication in keratinocytes (Oelze *et al.*, 1995). The E5 protein is associated with the 16 kDa subunit of the vacuolar ATPase (v-ATPase) (Conrad *et al.*, 1993), a component of gap junctions at the plasma membrane. v-ATPase participates in acidification of cytoplasmic vesicles, leading to a state where receptor-ligand complexes are targeted for degradation and recycling to the cell surface, enabling communication between cells (Clague *et al.*, 1994; Finbow and Harrison, 1997). The interaction between E5 protein and v-ATPase may be responsible for the observed impaired cell-cell communication, although conversely, yeast studies suggest disruption of the v-ATPase complex due to E5 expression (Adam *et al.*, 2000; Briggs *et al.*, 2001).

The papillomavirus life cycle takes place away from dermal immune cells, and the virus does not cause cell lysis to activate an inflammatory response (Stanley *et al.*, 2007). The HPV-16 E5 protein is able to assist persistent infection by modulating immune response of the host. HPV-16 E5 has been observed to reduce major histocompatibility complex (MHC) class I expression on the cell surface (Ashrafi *et al.*, 2005; Campo *et al.*, 2010). Campo *et al.* (2010) discovered a functional impact of the E5-induced reduction of HLA-A2 in decreasing the recognition of E5-expressing cells by HPV-specific CD8+ T-cells. Recent data also reveal the function of HPV E5 in helping HPV-infected cells to evade protective immunological surveillance by decreasing CD1d expression (Miura *et al.*, 2010). Downregulation of CD1d is utilized also by herpesviruses in immune evasion (Raftery *et al.*, 2006; Sanchez *et al.*, 2005).

The E5 protein enhances the immortalization potential of E6 and E7 proteins (Stöppler *et al.*, 1996), and its potency to cause cervical cancer is similar to that of E6 (Maufort *et al.*, 2010). Maufort *et al.* (2010) suggested a role for E5

in increasing the dysplastic environment either alone or cooperatively with other viral oncogenes. Cooperation with E7 to induce proliferation, enhance mortalization and promote anchorage-independent growth has been reported earlier (Bouvard *et al.*, 1994a; Valle and Banks, 1995). Similar roles in supporting DNA synthesis in differentiated cells have been demonstrated for E5 and E7 proteins (Flores *et al.*, 2000; Fehrmann *et al.*, 2003; Genther *et al.*, 2003). Furthermore, E5 and E6 proteins cooperate to induce koilocytosis, structural changes in a cell as a result of HPV infection, in the differentiated squamous epithelium (Krawczyk *et al.*, 2008).

E6 and E7 proteins

E6 and E7 proteins of the high-risk HPV types play important roles as oncogenes in carcinogenesis as well as in the maintenance of the transformed phenotype. The E6 protein binds to a cellular ubiquitin-ligase to form a complex that binds the p53 tumour suppressor protein, resulting in p53 degradation (Scheffner *et al.*, 1990; 1993; Werness *et al.*, 1990). Cells without functional p53 display genomic instability (Werness *et al.*, 1990), which is an important component of carcinogenesis in general. The high-risk HPV E6 proteins also destabilize PDZ domain-containing host proteins that regulate cell polarity and signal transduction (Thomas *et al.*, 2008), degrade GAP proteins involved in G protein signalling (Singh *et al.*, 2003) and transactivate the catalytic subunit of the telomerase gene (hTert) (Klingelhutz *et al.*, 1996).

Normal, uninfected cells exit the cell cycle as they leave the basal layer during differentiation, but in HPV infected cells persist in the cell cycle due to E7 protein actions (Cheng *et al.*, 1995). The E7 protein binds to the retinoblastoma protein (pRb), resulting in activation of genes that regulate cell proliferation (Dyson *et al.*, 1989). The binding of pRb is mediated througha a conserved region present in all high-risk E7 proteins (Phelps *et al.*, 1998). Low-risk E7 proteins are also associated with pRb, but with a much lower affinity (Ciccolini *et al.*, 1994; Oh *et al.*, 2004).

Besides the inactivation of p53 and pRb, the E6 and E7 proteins interact and interfere with various cellular proteins (Balsitis *et al.*, 2006; Shai *et al.*, 2007), and both proteins play a role in cell transformation and immortalization. E6 and E7 together can immortalize human epithelial cells cooperatively (Hawley-Nelson *et al.*, 1989; Munger *et al.*, 1989). The E7 protein causes tumour promotion, whereas E6 acts more strongly during tumour progression and accelerates malignant conversion of benign tumours as shown in E6- and E7-transgenic mice experiments (Song *et al.*, 2000). Functions of all three oncogenes (E5, E6 and E7) are to ensure viral replication and to promote the spread of progeny by regulating cell survival throughout the normal viral life cycle.

2.2. HPVs and disease mechanisms

2.2.1. HPV-related diseases

The majority of HPV infections cause no symptoms, whereas some types cause benign warts, papillomas. Cutaneous HPV types cause common skin warts, in which HPV infection causes rapid growth on the outer layer of the skin. Common warts are most often found on the hands and feet. Mucosal HPV types infect the mucosal surfaces found in, for instance the nose, mouth, anus and genital areas. Multiple infections with several different types are also common. Mucosal HPV types are divided into low- and high-risk categories according to their oncogenicity. Persistent infection with high-risk HPV can lead to premalignant lesions and invasive cancers of the cervix, vulva, vagina and anus in women or cancers of the anus and penis in men (Schiffman and Castle, 2003). The most common HPV-associated diseases are presented in Table 1.

Table 1. *HPV-associated diseases and their predominant HPV types. Adapted from the 'Health Professional's HPV Handbook' (Prendiville and Davies, 2004).*

Disease	**HPV type**
Genital warts	6, 11, 42-44
Skin warts	1-4, 26, 27
Mild genital dysplasia	6, 11, 16, 31, 45
Severe genital dysplasias and cancer	the high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73)
Laryngeal papillomas	6, 11
Head and neck carcinomas	16, 33
Epidermodysplasia verruciformis	5, 8, 12, 15, 20, 24, 38
Skin cancer	20, 38

HPV prevalence in Finland among young women is remarkably high. Auvinen *et al.* (2005) observed a 33% prevalence among first-year university students. Diagnostic laboratory methods are available for the detection and genotyping of HPV. Papillomavirus testing is primarily used as an adjunct to Papanicolau screening to detect cervical premalignant lesions. Most HPV infections clear spontaneously within 6-24 months, especially in young women. Currently, no medication against HPV infection exists. Premalignant lesions can be removed surgically.

2.2.2. Cellular transformation by HPV

In rare cases (approximately 0.1% of all HPV infections), high-risk HPV infection can lead to premalignant lesions and invasive cancer. High-risk HPV types can induce malignant transformation in epithelial cells mainly due to the expression of E6 and E7 oncogenes, which promote tumour growth (Hampson *et al.*, 2001). HPV-induced cancers often involve integration of viral sequences into the genomic DNA. In cooperation, as well as individually, high-risk E6 and E7 proteins immortalize cells and have transforming

activity, contrary to low-risk types (Hawley-Nelson *et al.*, 1989; Pim and Banks, 2010). High-risk E6 and E7 proteins have numerous cellular target proteins and interactions, but concerning transformation, the interaction with cellular tumour suppressors, p53 and pRb, is crucial (Dyson *et al.*, 1989; Scheffner *et al.*, 1990; 1993; Werness *et al.*, 1990). Both p53 and pRb mutations are common in many types of cancers, although they occur very rarely in cervical cancers, suggesting that E6 and E7 oncogene-induced functional inactivation of these proteins has an equally strong effect as mutated p53 and pRb proteins.

The E7 protein is suggested to act primarily in promoting carcinogenesis, while E6 potently accelerates disease progression (Song *et al.*, 2000). Although inactivation of p53 is crucial for immortalization of keratinocytes, some p53-independent functions of E6 may also contribute to this event. HPV E6 protein is known to activate the catalytic subunit of telomerase, hTERT (Klingelhutz *et al.*, 1996). E7 protein has been suggested to induce activation of alternative lengthening of telomeres, which is important in reducing genomic instability and promoting tumour progression in early stages of cancer development (Moody and Laimins, 2010). Furthermore, E7 protein has been reported to be associated with cyclins A and E, as well as with cyclin-dependent kinase inhibitors p21 and p27, disrupting the cell cycle (Longworth and Laimins, 2004).

The transformation process requires additional oncogenic events besides E6 and E7 expression, as suggested by the long latency period between HPV infection and cancer development (Schiffman *et al.*, 2007). HPV-associated cancers usually involve genomic instability and chromosomal imbalance and rearrangements (Koopman *et al.*, 1999; Korzeniewski *et al.*, 2010; Yangling *et al.*, 2007). E6 and E7 oncogenes are able to induce genomic instability independently when studied in normal human fibroblasts (White *et al.*, 1994). E7 alone has been reported to induce centrosome amplification, which correlates with cell division errors (Duensing *et al.*, 2001). Usually cells with abnormal mitoses undergo apoptosis, but during HPV infection, E6 and E7

assist in accumulation of abnormal centrosomes (Patel *et al.*, 2004). E6 and E7 proteins affect several cellular events leading to cancer, e.g. interference of cell differentiation (Alfandari *et al.*, 1999), DNA synthesis (Cheng *et al.*, 1995), cell cycle and inhibition of apoptosis (Woodworth *et al.*, 1992) and mitotic checkpoints (Thomas and Laimins, 1998; Thompson *et al.*, 1997).

2.2.3. HPV and cellular microRNAs

MicroRNAs are post-transcriptional regulators of cellular gene expression expressed in all multicellular eukaryotes (Bartel, 2004). Lee *et al.* (1993) discovered the first miRNA, lin-4, already in 1993, and to date, over 1200 mature human miRNA species are known (miRBase release 16, 2010 http://www.mirbase.org/; Reinhart *et al.*, 2000; Pasquinelli *et al.*, 2000; Kozomara and Griffiths-Jones, 2011). MiRNAs bind to complementary sequences in the 3' UTR of target mRNA transcripts, resulting in destabilization of their target mRNA and/or blockage of its translation. MiRNAs and their target mRNA expression usually correlate inversely, although a direct correlation has also been noted (Huntzinger and Izaurralde, 2011). MiRNAs protect cells from apoptosis, affect the cell-matrix adhesion and interfere with epithelial differentiation, among other functions. Computational predictions suggest that a single miRNA can regulate the expression of more than 200 different target mRNAs (Krek *et al.*, 2005). Therefore, involvement of miRNAs in various diseases has been widely reported, and, indeed, aberrant expression of miRNAs has been observed in many human malignancies (Visone and Croce, 2009; Zimmerman and Wu, 2011). Furthermore, miRNAs seem to have a role in many viral infections in regulating cellular gene expression (Roberts and Jopling, 2010). Among DNA viruses, herpesviruses and polyomaviruses are known to express miRNAs to autoregulate viral mRNA expression (Pfeffer *et al.*, 2005; Seo *et al.*, 2009). Viral miRNAs may help to ensure the accurate expression of the viral genome and to downregulate the expression of host cell transcripts (Seo *et al.*, 2008); however, very little evidence currently exists to support this. Host cell-

encoded miRNAs also play a role in viral infection processes, such as apoptosis as well as adaptive and innate immune responses (Umbach and Cullen, 2009).

Although HPV-encoded miRNAs have not been discovered (Cai *et al.*, 2006), HPV is known to cause alterations in cellular miRNA expression, and these modifications may play a crucial role in HPV pathogenesis. Lui *et al.* (2007) reported downregulation of miR-143 and upregulation of miR-21 in cervical cancer tissue. Similar expression alterations of these microRNAs have been reported in many cancers. High-risk HPV E6 protein causes downregulation of miR-218 (Martinez *et al.*, 2008) and tumour-suppressive miR-34a (Wang *et al.*, 2010). The target protein of miR-218, LAMB3, is known to increase cell migration and motility (Calaluce *et al.*, 2004). These are important events in cancer invasion and metastasis, and HPV would thus enhance these functions by downregulating miR-218. In addition, the HPV E7 protein suppresses the expression of miR-203 (Melar-New and Laimins, 2010), which has a role in decreasing the proliferative capacity of epithelial cells upon differentiation. Thus, the E7 protein contributes to the disturbance of epithelial differentiation.

2.2.4. HPV and cancer

Cervical cancer

Cervical cancer is the third most common cancer in women, with over 500 000 new cases each year worldwide (Jemal *et al.*, 2011). In Finland, approximately 150 cervical cancer cases are diagnosed annually. Cervical cancer is a major cause of cancer-related mortality, especially in developing countries. Persistent infection by one of the high-risk HPV types causes practically all cervical cancers and their immediate precursors (Schiffman *et al.*, 2007; Walboomers *et al.*, 1999). The most frequently encountered types

are HPV 16 and 18, which together cover 60-70% of cervical cancers. Variation in overall HPV DNA detection is minor between continents (Bosch *et al.*, 1995; Clifford *et al.*, 2003), although the proportions of high-risk HPV 16 and 18 infections may vary by region, being highest in Europe and lowest in sub-Saharan Africa (Clifford *et al.*, 2005). The majority of cervical HPV infections are subclinical or resolve spontaneously due to activation of the host immune system, and only a small subset of HPV-induced lesions eventually progress to cancer. In invasive cancers, viral capsids are not formed, but the viral genome can still replicate actively (Doorbar, 2005).

Cervical cancer proceeds from premalignant cervical intraepithelial lesions. These lesions are histologically classified on the basis of abnormal epithelial cells that progressively extend from the lower parabasal layers of the squamous epithelium through the entire thickness of the epithelium, depending on the grade (Thomison *et al.*, 2008). The levels of severity are CIN (cervical intraepithelial neoplasia) 1, in which the non-differentiated cells infiltrate only the first layer of tissue, CIN 2, in which the non-differentiated cells penetrate to the second or third layer of tissue, CIN3 or carcinoma *in situ*, in which the non-differentiatied cells penetrate all epithelial layers. Diagnosis of CIN 1 is not always reliable (Stoler and Schiffman, 2001), and CIN 1 lesions often regress spontaneously, especially among women under 30 years of age (Moscicki *et al.*, 2004). The Finnish Current Care guidelines recommend treatment of CIN 1 after persistence of 24 months and immediate treatment of CIN 2 and CIN 3 lesions because of their high probability of progression (Cervical cancer screening: Finnish Current Care guidelines, 2010). An organized nationwide screening programme was launched in Finland in the early 1970s (Anttila *et al.*, 1999; Hakama and Räsänen-Virtanen, 1976), and mortality rates have subsequently been reduced by 80%.

Other HPV-related cancers

Some cancers of the vulva, vagina, penis, anus, and head and neck (oral cavity and oropharynx) are also associated with HPV infection. HPV involvement has been observed in approximately 25% of oral and 35% of laryngeal cancers (reviewed in Kreimer *et al.*, 2005). HPV infection also plays a role in 85% of anal cancers (Ryan and Mayer, 2000), 60-80% of vaginal cancers (Daling *et al.*, 2002) and 40% of vulvar and penile cancers (Daling *et al.*, 2005; Jones *et al.*, 2005).

2.2.5. Cervical cancer screening

Papanicolaou (Pap) screening is the primary screening method for cervical cancer and its precursors worldwide, and a marked decrease in cervical carcinoma incidence and mortality in developed countries has been achieved due to screening programmes (Hakama *et al.*, 1985). High-risk HPV types are present in more than 99% of cervical cancers and the majority of CIN 2 and CIN 3 cases (Walboomers *et al.*, 1999), whereas CIN 1 is typically associated with low-risk HPV. The progression rate of CIN 3 to invasive carcinoma is approximately 12%, whereas the corresponding rate for CIN 1 is only 1% (Ostör, 1993). Screening for high-risk HPV types has been considered an additional or alternative tool for cytology-based cervical cancer screening (Cox and Cuzick, 2006). Multiple studies have shown higher sensitivity in HPV testing than in cytology for high-grade CIN (Dillner *et al.*, 2008; Kulasingam, *et al.*, 2002; Leinonen *et al.*, 2009). Earlier detection of invasive premalignant lesions and cancer has been reported, thus improving survival. A disadvantage of HPV testing is its low specificity, i.e. it detects all HPV infections rather than existing disease, which results in a high false-positive rate, particularly in young women. In light of this, HPV testing has been suggested for use in primary screening in women aged over 35 years, with cytology reserved for the triage of women positive for high-risk HPV. This

scheme has also been presented in Finland (Nieminen *et al.*, 2010; Tarkkanen *et al.*, 2007), and the recently updated Current Care Guidelines include this option.

2.2.6. Vaccines against HPV infection

Worldwide, several approaches have been applied for the prevention of cervical cancer. Two prophylactic vaccines against two or four HPV types have been commercialized in recent years, and these vaccines are already in use in some countries. Both vaccines are composed of virus-like particles, which resemble a virus without any viral genetic material. Cervarix (GlaxoSmithKline) has been shown to have high efficacy against both HPV 16 and 18, and it also provides some protection against the other three most common cancer-causing types (HPV 31, 33 and 45) (Paavonen J *et al.*, 2009). Cross-protection against HPV 45 has been observed, which may be important in the prevention of cervical adenocarcinoma (Szarewski, 2010). Gardasil (Merck & Co.) HPV vaccine is effective against common squamous cell cancer-causing HPV types 16, 18 and 31 (Future I/II Study Group *et al.*, 2010). In addition, Gardasil protects from HPV 6 and 11 infections, which cause genital warts. The efficacy of Cervarix has been proven for approximately 6 and a half years and that of Gardasil for 5 years (Paavonen *et al.*, 2009; Villa *et al.*, 2006). Neither vaccine is therapeutic, and they do not cure existing infections. The best protection is obtained when women are vaccinated before sexual activity commences. Both vaccines prevent up to 90% of new persistent HPV infections and cervical precancerous lesions (Future II Study Group, 2007; Garland *et al.*, 2007; Paavonen *et al.*, 2007).

2.3. Alterations in cell adhesion and cell motility in cancer

Disruption of cell adhesion and breakdown of normal communication between adjacent cells are frequent in cancer. Reduced cell adhesion is critical for cancer invasion and metastasis. Especially E-cadherin and catenins are key components of adherens junctions of epithelial cells (Harris and Tepass, 2010). In cancer cells, the adhesion involving E-cadherin is usually inactivated by various mechanisms, and this event plays a significant role during multistage human carcinogenesis (Makrilia *et al.*, 2009). Formation and disassembly of adhesions facilitate the migration process by activating Rho GTPases, which regulate actin polymerization and myosin II activity (Ridley *et al.*, 2003). Epithelial tissue is the site of more than 80% of human cancers, and epithelial-to-mesenchymal transition (EMT) is an important event during tumour progression and metastasis where epithelial cells lose polarization (Lee *et al.*, 2006; Thiery and Sleeman, 2006). EMT is characterized by reduced expression of E-cadherin and increased N-cadherin.

Migration of cancer cells is regulated by adhesion of cells to the extracellular matrix. Interactions of the cell and matrix as well as epithelial cell polarity are mediated by integrins and other cell surface adhesion receptors (Miranti and Brugge, 2002). Integrin trafficking is known to be important for directional cell motility of transformed cells (Hynes, 2002). Integrins not only mediate adhesion but also participate in regulating cell survival and proliferation (Juliano and Haskill, 1993). Integrins also activate paxillin and focal adhesion kinase (FAK) (Parsons, 2003; Brown and Turner, 2004), leading to enhanced signalling to Rho GTPases, such as Rac, Rho and Cdc42 proteins, involved in regulation of the cytoskeleton (Ridley *et al.*, 2003).

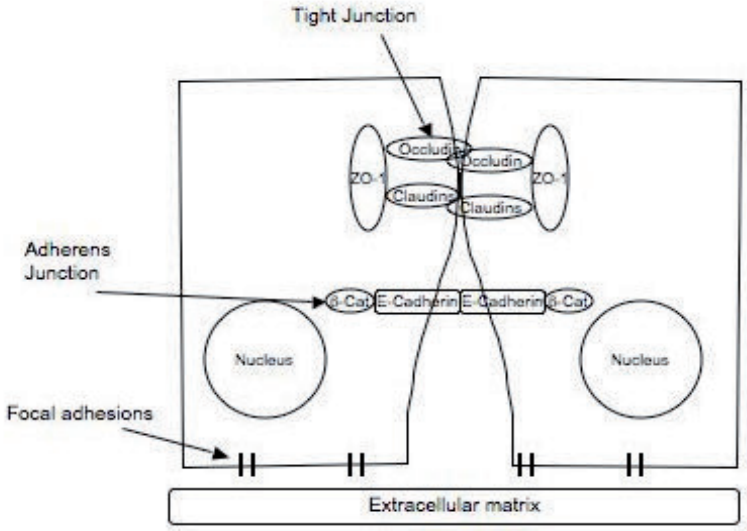

Figure 3. *Epithelial cell adhesion. In all cell junctions, unique adhesion receptors interact either with similar proteins on adjacent cells or with the components of the extracellular matrix through their extracellular domain. Tight junctions are formed by occludins and claudins, which are linked to the actin cytoskeleton through ZO proteins. Tight junctions seal adjacent cells together and function as selective permeability barriers. Adherens junctions are formed by cadherins, which are linked to the actin cytoskeleton through catenins and vinculin. Focal adhesions are formed by integrins.*

In motile cells, early focal adhesions contain integrins, vinculin, talin, α-actin, paxillin and FAK, among other proteins, and, as the leading edge of a migrating cell moves forward, these adhesions disassemble or elongate and grow, depending on the cell type. Paxillin phosphorylation is observed to be a key regulator of adhesion maturation by recruiting vinculin (Pasapera *et al.*, 2010). Rac, RhoA and Cdc42 are activated at the front of migrating cells, and RhoA also at the cell rear (Kurokawa *et al.*, 2005; Machacek *et al.*, 2009). Activation of Rho kinases has been suggested as a factor predicting invasive potential of cancer cells (Parsons *et al.*, 2010).

In cervical cancer, many cell adhesion proteins are degraded or downregulated. The HPV E6 protein disrupts adherens junctions by inhibiting the APC/hScrib interaction, which consequently reduces the negative regulation of entry into the S-phase of the cell cycle (Ishidate *et al.*, 2000; Takizawa *et al.*, 2006). The E-cadherin protein is an important component of cellular adherens and tight junctions, and the gene is most often methylated in cervical cancer. Downregulation of E-cadherin releases β-catenin from the cell membrane into the nucleus, further activating the Wnt signalling pathway (Wang et al., 2010). A decrement of E-cadherin expression has been observed in cervical preneoplastic lesions (Hubert *et al.*, 2005), and increased invasiveness and downregulation of E-cadherin in HPV E6 and E7-transfected keratinocytes *in vitro* have also been noted (Wilding *et al.*, 1996). The exact roles of HPV oncogenes in these events remain to be determined.

2.4. Role of Ezrin in cell adhesion, cell motility and carcinogenesis

Ezrin (also called cytovillin) is a membrane cytoskeletal cross-linker often observed as a key molecule during onset and progression of the metastatic cascade (Elliott *et al.*, 2004; Endo *et al.*, 2009; Federici *et al.*, 2009; Gould *et al.*, 1989; Sarriò *et al.*, 2006; Turunen *et al.*, 1989). Ezrin is a member of the ezrin, radixin, moesin (ERM) family and has been reported to be involved in tumour progression (Gautreau *et al.*, 2002). ERM proteins are membrane-cytoskeleton linking proteins and are involved in numerous cellular events, including cell motility, signal transduction, cell-cell and cell-matrix interactions, cell growth and apoptosis (Poullet *et al.*, 2001). ERM proteins are regulated by small GTPases, which control actin cytoskeleton remodelling and many cellular activities (Etienne-Manneville and Hall, 2002). An interaction between Rho proteins and ERM family proteins in cell motility and mitogenic signalling in epithelial cells has been suggested based on their

functional similarities (Mackay *et al.*, 1997; Matsui *et al.*, 1998). Rho is known to control cell adhesion and motility through its effects on the actin cytoskeleton and cell morphology. Ezrin is linked to the plasma membrane and cytosolic organelles, resulting in a close relation with such cellular functions as cell adhesion, motility, apoptosis and phagosytosis (Lugini *et al.*, 2003; Luciani *et al.*, 2004; Killock *et al.*, 2009). Brambilla and Fais (2009) suggested that in tumour cells ezrin may act as a mediator and a linker for metastasis-associated cell-surface proteins and other intracellular molecules. Ezrin has been reported to be involved in the metastatic phenotype of osteosarcoma, melanoma and ovarian carcinoma (Federici *et al.*, 2009; Khanna *et al.*, 2004; Köbel *et al.*, 2006). Ezrin involvement in breast cancer metastasis has been observed as well (Elliot *et al.*, 2005; Li *et al.*, 2008).

In breast cancer cells, ezrin was shown to function as an organizer of cellular adherens junctions, and ezrin silencing was observed to induce E-cadherin increment, leading to inhibition of cell migration and invasion (Li *et al.*, 2008). Ezrin has also been demonstrated to contribute to EGF-stimulated migration of cervical cancer cells (Chiang *et al.*, 2008). Yu *et al.* (2004) and Khanna *et al.* (2004) suggested that ezrin likely functions at the intersection of multiple signalling pathways. In summary, ezrin has both structural and regulatory interactions in the cellular cytoskeleton as well as a significant role in cancer development and progression.

2.5. Molecular profiling by array technologies

Now that the sequencing of the human genome has been completed and all genes have been identified, microarray technology has provided much new information about gene expression and function. Microarrays have a wide range of applications in biomedical research and diagnostics, especially in cancer research (Ewis *et al.*, 2005). The technology for DNA microarray was developed in the early 1990s (Fodor *et al.*, 1991; Schena *et al.*, 1995). The

advantage of the methodology is the ability to measure changes in the levels of multiple biomolecules simultaneously. DNA microarrays may contain thousands of DNA fragments, e.g. oligonucleotides or cDNA clones synthesized onto a solid support. Microarrays can be used to measure RNA, DNA or protein levels from cells or tissues on a genome-wide scale, providing an excellent tool for measuring relative differences in RNA levels between samples. The challenge of the microarray experiments is that they are highly capable of generating long lists of genes with altered expression, but they provide little clue as to which of the changes are important in a given phenotype. It is tempting to look for genes that confirm pre-existing knowledge, so careful experimental design is critical.

DNA microarray technology is based on the complementary base pairing property of nucleic acids. The labelled sample RNA is hybridized with the probe onto the surface. Gene expression microarrays can be categorized as cDNA and oligonucleotide arrays according to probe type (Fodor *et al* 1991; Schena *et al.*, 1995). Depending on the platform, comparison of the expression of two samples on the same array or only one sample per array can be made. The result is then presented either as a ratio between the expression levels of two samples or as an estimate of transcript levels in one sample (Hardiman, 2004). The results can be further analysed by different softwares. To obtain cDNA microarrays, a collection of characterized and well-annotated cDNA clones is amplified by polymerase chain reaction (PCR) and spotted onto a coated glass slide. Oligonucleotide arrays can be manufactured in a similar fashion, or probes can be synthesized *in situ* nucleotide by nucleotide on a solid support (Southern *et al.*, 1999). In the direct two-colour labelling method, extracted RNA is reverse-transcribed into cDNA and labelled with fluorochrome dyes, such as cyanine 3 (Cy3) and cyanine 5 (Cy5). The use of different dyes allows mRNAs from two different cell populations or tissues to be labelled in different colours, mixed and hybridized to the same array, which results in competitive binding of the target to the sequences in the array. To remove non-biological variation, such as dye bias or experimental conditions, from the microarray data, mathematical normalization is

performed. Many of the normalization methods assume that some subset of housekeeping genes or a set of exogenous controls have constant expression values. The normalization factor is then calculated to adjust the data (Hariharan, 2003; Quackenbush, 2002; Yang *et al.*, 2002). The normalized data is analysed to identify differentially expressed genes between samples using fold change. Microarray results always need additional validation, such as real-time RT-PCR or protein analysis, to rule out false-positive results (Canales *et al.*, 2006; Huang L *et al.*, 2011; Martin *et al.*, 2009). To validate the functional relevance of the gene expression microarray results, protein-level studies using Western blotting, immunohistochemistry or RNAi experiments can be performed (Choi *et al.*, 2005; Liao *et al.*, 2011; Martin *et al.*, 2009).

cDNA and oligonucleotide microarrays are two leading types of arrays used to quantify gene expression. In oligonucleotide microarray (such as those produced by Agilent, used and described in detail in Study III), the quantitation value is based on the difference in fluorescence between the match and mismatch probe sets, whereas the cDNA microarray technology uses the hybridization signal from a single clone spotted in duplicate. cDNA microarrays are less sensitive to single base pairs changes in the probe sequence, but they are more open to cross-hybridization since the clones are usually not completely sequenced. cDNA probes may also be affected by printing effects and PCR amplification efficiency, whereas oligonucleotide arrays are directly synthesized on glass under controlled reaction conditions (McGall *et al.*, 1996). Overall, oligonucleotide microarray platform has clearly better specificity, reproducibility and statistical significance than the cDNA microarray platform (Hughes *et al.*, 2001). When validating the microarray results by quantitative RT-PCR, the concordance between qRT-PCR and oligonucleotide microarray results is approximately 70-80% (Morey *et al.*, 2006), whereas it is approximately 50-60% between qRT-PCR and cDNA microarray (Chuaqui *et al.*, 2002; Rajeevan *et al.*, 2001).

One of the best applications for microarrays is in analysing cellular functions as global gene expression patterns, rather than in identifying a single critical gene. In large-scale analyses of changes in gene expression, genes with similar patterns are clustered. Several software tools for cluster analysis have been developed and are also available from public sources.

Microarrays hold much promise for the analysis of diseases. Numerous individual gene products that are expressed in tumour cells but not in normal tissue have been identified with the help of microarrays, opening the possibility of finding new tumour biomarkers. Tissue microarrays provide a tool for identification of tumor markers from whole solid tumours (Kononen *et al.*, 1998). Gene expression profiling has also been used to reveal altered signalling pathways in specific diseases or tumours, such as in the metastatic potential of melanoma cells or in HIV infection (Clark *et al.*, 2000; Geiss *et al.*, 2000). Gene expression is regulated at many levels and the regulation of mRNA stability contributes significantly to the measured gene expression changes in microarrays (Cheadle *et al.*, 2005). Despite its limitations, the DNA microarray is a powerful tool to gain genome-wide understanding of gene expression changes and to identify target candidates for diagnosis and treatment of many different types of diseases.

3. Aims of the study

Human papillomavirus (HPV) has three oncogenes: E5, E6 and E7. HPV oncogene-encoded proteins are known to interact with many cellular proteins, causing events leading to carcinogenesis. The aim of this study was to model the holistic effects of the E5 protein in triggering malignant changes in epithelial cells, thereby contributing to earlier and more exact recognition of the events of papillomavirus carcinogenesis.

Detailed aims where as follows:

- to examine the effects of the HPV-16 E5 oncogene on cellular gene expression in a genome-wide gene expression microarray

- to further analyse cellular level alterations in gene and protein expression due to expression of HPV E5 in epithelial cells

- to evaluate the role of cellular microRNAs in mediating the effects of E5 on changes in gene expression

- to investigate the mechanisms of cell adhesion and cell motility of epithelial cells in relation to carcinogenesis

4. Materials and methods

4.1. Cell culture (I-III)

An immortalized HaCaT human keratinocyte cell line stably transfected with HPV 16 E5 (HaCaT-E5) gene expressed from a dexamethasone-inducible promoter, as well as a control cell line transfected with the empty vector (HaCaT-pMSG) were provided by Professor Angel Alonso and are described in Oelze *et al.* (1995). HaCaT-E5 cells and HaCaT-pMSG control cells were used in microarray analyses (I and II), three-dimensional raft cultures (II) and cell motility assays (I). An HPV 18-positive HeLa human epithelial cell line was obtained from the ATCC (Manassas, VA) and used in ezrin localization studies (III).

HaCaT cell lines were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle´s medium (D-MEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. The HeLa cell line was grown in similar conditions in minimum essential medium (MEM).

HaCaT-E5 and –pMSG cells were serum-starved for 24 h and induced with 1 μM dexamethasone (Sigma-Aldrich Inc., Saint Louis, MO) for different times for analysis. Comparisons were performed between E5 and control cells treated in a similar manner.

Three-dimensional cell cultures mimicking layered epithelium were prepared using HaCaT-E5 and -pMSG cell lines in collagen raft cultures (modified from Lambert *et al.*, 2005). Raft cultures were fixed embedded in paraffin, and 4- to 5-μm sections were used in immunohistochemical stainings (II).

4.2. RNA extraction (I, II)

Total RNA was isolated from confluent HaCaT-E5 and –pMSG cell cultures that had been serum-starved and induced with dexamethasone (Sigma-Aldrich Inc.) using TriPure reagent (Roche Applied Science, Indianapolis, IN) (I) or mirVana™ miRNA Isolation Kit (Ambion) (II) after different induction times. RNA was quantitated and quality confirmed for microarray experiments using an Agilent 2100 Bioanalyzer (Agilent Technologies, Rockville, MD). For quantitative RT-PCR of cellular mRNA, total RNA (I) or the large RNA fraction of the mirVana™ isolation product (II) was used. In Taqman microRNA assays, total RNA extracted with mirVana™ miRNA Isolation Kit (Ambion) was used.

4.3. Microarrays (I, II)

The RNA samples were indirectly labelled using the T7 amplification method (Amino Allyl MessageAmp™ II aRNA Amplification Kit; Ambion, Austin, TX) according to the manufacturer's instructions. Purified antisense RNA was labelled using monoreactive Cy3 and Cy5 dyes (GE Healthcare, Buckinghamshire, UK) (I and II) or monoreactive Alexa 488 (Invitrogen, Gaithersburg, MD) (II) followed by purification according to the manufacturer's instructions. Labelled aRNAs were hybridized either onto Agilent Whole Human Genome 4x44K human slides according to the manufacturer's recommendations (II) or onto cDNA microarrays from the Finnish DNA-Microarray Centre (Turku, Finland) (I). RNA samples for miRNA microarray were labelled using Agilent miRNA labeling kit (Agilent Technologies, Rockville, MD). Labelled samples were hybridized onto Agilent Human miRNA Microarray V1 slides according to the manufacturer's instructions. All slides were scanned using an Axon GenePix 4200AL scanner (Molecular Devices Corporation, Sunnyvale, CA).

4.4. Quantitative RT-PCR (I, II)

Quantitative RT-PCR (qRT-PCR) was performed in triplicate PCR reactions using RNA extracted from HaCaT-E5 and -pMSG cells. For RT-PCR, SYBR® Green PCR Master Mix and RT-PCR kit (Applied Biosystems, Foster City, CA) and a sequence detector ABI PRISM® 7700 (Applied Biosystems) were used. Gene specific primers were designed using PrimerExpress program (Applied Biosystems) and specificity of the PCR amplification was confirmed in both agarose gel and dissociation curve analysis of the amplification plot. For microRNA Taqman assays, total RNA was reverse-transcribed using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems), and the cDNA was amplified using Taqman® MicroRNA assays (Applied Biosystems) for selected miRNAs. The comparative threshold cycle (C_T) method was used in relative quantification of transcript fold-changes in E5-expressing cells as compared with control cells. β-actin transcript expression level was used to normalize for the abundance of the tested transcripts.

4.5. Western blotting (I, II)

Total protein lysates were obtained from pretreated HaCaT-E5 and -pMSG cells using lysis buffer (150mM NaCl; 50mM N-2-hydroxyethylpiperazine-N0-2-ethanesulphonic acid, pH 7.4; 0.1% Igepal; 5mM EDTA) containing proteinase inhibitors (cOmplete Proteinase Inhibitor Cocktail Tablets; Roche Applied Science). Total protein was quantitated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts (40 μg) of total protein were separated in 7.5% or 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% non-fat milk in phosphate-buffered saline,

followed by incubation with primary antibodies in 5% nonfat milk in PBST overnight at 4°C. Two-colour Western detection was performed by blotting each membrane simultaneously for the protein of interest and for β-actin (Sigma-Aldrich) to control for loading. Similarly treated E5 and control cells were compared. The expression of each protein was normalized against the expression of β-actin (loading control).

Proteins were detected with the following antibodies: PI3KR3 (Abgent, San Diego, CA, USA), Akt (Cell Signaling Technology, Beverly, MA, USA), P-Akt (Ser473; Cell Signaling Technology), paxillin (Cell Signaling Technology), P-paxillin (Tyr118; Cell Signaling Technology), PKC-d (BD Biosciences, San Jose, CA), lamin A/C (BD Biosciences), E-Cadherin (BD Biosciences), N-Cadherin (Zymed Laboratories, San Francisco, CA), β-Catenin (BD Biosciences), Claudin-1 (Zymed Laboratories), Integrin αV (BD Biosciences) and p63 (Labvision). Subsequently, the membrane was incubated with fluorescent secondary antibodies: IRDye 800CW goat anti-mouse (LICOR Biosciences, Lincoln, NE, USA) and IRDye 680 goat anti-rabbit (LI-COR Biosciences). β-actin (Sigma-Aldrich Inc.) was used as a loading control and all quantitations were normalized against β-actin in each lane. Images were acquired with the Odyssey infrared imaging system (LI-COR Biosciences) and analysed using the software programme provided by the manufacturer.

4.6. Wound healing assay (I)

HaCaT-E5 and –pMSG cells were grown to 90% confluency on a 24-well plate and pretreated as described previously. Epithelial cell monolayers were scratch-wounded with a pipette tip. Wound closure was followed with real-time microscopy, using a Cell-IQ cell culturing platform (Chipman Technologies, Tampere, Finland) equipped with a phase-contrast microscope and a camera. Images were captured at one-hour intervals for 48 h. Images were analysed with Imagen software (Chipman Technologies). Cell counting

from images and analysis of variance test were performed to determine whether the observed cell movement was due to cell migration and/or cell proliferation.

4.7. Immunohistochemistry (II)

Tissue sections from HPV-associated cervical dysplasia and collagen raft cultures were stained using the automated Ventana Discovery tissue staining instrument (Ventana Medical Systems, Tucson, AZ). Tissue sections of precancerous lesions were obtained from HUSLAB, Department of Pathology. Monoclonal antibodies for p16 (CINtec Histology Kit, MTM Laboratories AG, Heidelberg, Germany), E-Cadherin (BD Transduction Laboratories), N-Cadherin (Sigma-Aldrich Inc.), β-Catenin (BD Biosciences), Ezrin (clone 3C12; Böhling et al., 1996) and p63 (Thermo Scientific) proteins were used. Ventana DAB Map kit was used for detection, and the sections were counterstained with haematoxylin and post-counterstained with Bluing Reagent (Ventana Medical Systems). Finally, the slides were rinsed and dehydrated before mounting.

4.8. Plasmids and transfections (III)

Full-length human ezrin cDNA as well as deleted and truncated ezrin cDNA sequences were cloned into the pEGFP-C1 expression vector (Clontech, Palo Alto, CA). All constructs were verified by nucleotide sequencing. Constructs for wild-type, constitutively activated Q61L, and dominant negative T17N Rac1 were kindly provided by Dr. Alan Hall (Memorial Sloan-Kettering Cancer Center, New York, NY), GFP-C3 from Dr. Johan Peränen (University of Helsinki, Finland) and wild-type phosphatidylinositol-4-phosphate 5-kinase-alpha (PIPKα) and lipid-kinase negative mutant K138A-PIPKα from

Dr. Petri Auvinen (University of Helsinki, Finland) and kinase-deficient D227A-PIPKα from Dr. Christopher Carpenter (Harvard Medical School, Boston, MA).

HeLa cells were grown on sterile glass coverslips to 30-50% confluency and transfected using the FuGENE transfection reagent (Roche, Mannheim, Germany) (III). For activation of Rac1, cells were also serum-starved for 24 h and stimulated with 40 ng/ml PDGF (R&D Systems, Minneapolis, MN).

4.9. Immunofluorescence (III)

HeLa cells grown and transfected on sterile glass coverslips were fixed with a cytoskeleton-preserving fixative (4% paraformaldehyde, 0.32 mol/l sucrose, 10 mmol/l MES, 138 mmol/l KCl, 3 mmol/l $MgCl_2$, 2 mmol/l EGTA). Coverslips were then incubated with NH_4Cl, permeabilized with 0.5% Triton X-100 in PBS, blocked with 0.2% BSA in Dulbecco's PBS, incubated with primary and secondary antibody diluted in blocking solution, stained for F-Actin (Rhodamine-labelled phalloidin; Molecular Probes, Eugene, OR) and/or nuclei using Hoechst 33342 (Molecular Probes), and mounted.

Antibodies used were to ezrin (clone 3C12, produced in the group of Professor Antti Vaheri and described in Böhling et al. (1996), threonine-phosphorylated ERM proteins (Santa Cruz Biotechnology, Santa Cruz, CA), β-tubulin (Sigma-Aldrich, Saint Louis, MO), paxillin (Zymed, San Franscisco, CA), N-Cadherin (Sigma-Aldrich), E-Cadherin (Transduction Laboratories, Franklin Lakes, NJ), β-catenin (Transduction Laboratories), Rac1 (Santa Cruz Biotechnology), VSVG epitope (Roche Molecular Biochemicals), HA epitope (Roche Molecular Biochemicals) and a polyclonal (Upstate Biotechnology, Lake Placid, NY) or monoclonal antibody (provided by Dr. Petri Auvinen and described in Evan et al., 1985) to the myc epitope.

The stained cells were examined with an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and the fluorescent images were acquired with an Orca digital camera and the software provided.

4.10. Statistical methods (I, II)

All qRT-PCR and microarray analyses were done in triplicate and miRNA qRT-PCR analyses in quadruplicate. In quantitative RT-PCR analyses, Student's *t*-test was employed to statistically evaluate the results, using a *P*-value cut-off of <0.05.

In all microarray analyses, computational segmentation of the absolute foreground intensities and local background estimation from the scanned images were performed with the softwares GenePix Pro v.5.0 (II) or v.6.0 (I) (Axon). The data were then imported into the R software for further statistical analyses (http://cran.r-project.org/). Background correction and quality assessment of the data set were carried out using the BioConductor packages Limma (I and II) (Smyth, 2004) and arrayMagic (I) (Buness et al., 2005). The background-corrected log ratios were then normalized using the LOWESS regression method. Differentially expressed transcripts and micro-RNAs between E5-expressing and control cells at each time-point were studied with a linear model and a moderated t-test (I and II).

Analysis of variance was utilized to find expression patterns with significant alterations throughout the time-points analysed (II). Analysis of variance was also used to detect overall changes in wound healing assay (I).

4.11. MicroRNA target prediction (II)

To analyse altered microRNAs and their potential effect on their target genes, putative targets for each miRNA studied were defined by combining the computational predictions of eight popular algorithms, described in detail in Study II. The predicted target gene mRNA expression levels were then compared with their related miRNA expression to confirm congruency.

5. Results and discussion

5.1. Effect of HPV-16 E5 protein on cellular gene expression (I, II)

E5 is the least studied of the papillomavirus oncogenes; its roles in viral replication and pathogenesis of HPV are not fully understood. The effects of E5 in epithelial cells were evaluated by genome-wide gene expression microarray analyses first using cDNA microarrays (I) and later using oligonucleotide microarrays to obtain better quality data (II). Large-scale screening studies about the effects of E5 on cell membranes have been previously reported (Leykauf *et al.*, 2004; Bravo *et al.*, 2005). To date, only one other microarray gene expression study has been performed on keratinocytes expressing HPV 16 E5. Sudarshan *et al.* (2010) reported that approximately 25 genes were affected due to E5 expression when using primary keratinocytes stably expressing HPV 16 E5. In our studies, a fold-change cut-off was not used under the assumption that even a small difference in gene expression level might be relevant (I, II). By contrast, Sudarshan *et al.* (2010) considered genes significant, that had a fold-change difference higher than 1.5. Study settings differed also concerning E5 expression, cell status and cell type.

HaCaT keratinocyte cell line expressing the E5 protein from an inducible promoter was used as a study model, together with a control cell line without the E5 gene (I, II). First, the focus was on the events at 24 h after induction, when the transcription of E5 RNA was highest (I). Microarray analysis was performed in triplicate and *P*-value of <0.01 was used for statistical significance. Further, a detailed time-scale analysis was performed at 0, 2, 4, 24, 48, 72 and 96 h after induction (II). All time points were studied in triplicate and *P*-value of <0.001 was used to depict statistical significance (II). The microarray studies presented here are the largest studies to date on the effects of HPV E5 on cellular gene expression.

The E5 protein was shown to affect several cellular pathways involved in cell adhesion, cell motility and mitogenic signalling, in agreement with earlier studies reporting changes in cell adhesion and motility due to expression of HPV 16 E5 together with E6 and E7 (Boulenouar *et al.*, 2010). Altogether 117 upregulated and 62 downregulated genes were identified in cDNA microarray analysis in E5-expressing cells as compared with control cells. The identified genes were grouped according to their Gene Ontologies (I: Figure 1). Altered genes from the microarray clustered mainly in the following biological process categories: metabolism, responses to different stimuli and immune and defence responses. Twenty-seven altered genes were selected for qRT-PCR validation (*P*-value <0.05), and in 15/27 (56%) the microarray result was confirmed. To deepen the analysis of the effect of E5 expression on host cell pathways, 34 additional genes from the same Gene Ontology families, or downstream target genes of interest were selected for qRT-PCR validation. A time scale of 0 to 72 h was utilized in qRT-PCR and Western blotting, and E5 expression was shown to affect transcription of cellular genes studied already at time-points earlier than the 24-h time-point used in the microarray analysis.

On the basis of the first observation that E5 expression affects cellular gene expression already in small quantities, Study II was carried out using time-scale analysis up to 96 h after E5 induction. Importantly, for gene expression analysis oligonucleotide microarrays were used, which have better annotation, specificity and reproducibility than the cDNA array. Also in this analysis, genes involved in cell motility, cell adhesion and extracellular matrix were over-represented. In addition, a number of genes of the immune and inflammatory response were found significantly changed at all time-points, confirming the previous microarray results. The E5 protein, together with other HPV oncoproteins, has been reported to modify the host cell immune response against HPV infection (O'Brien and Campo, 2003). qRT-PCR validation of 32 genes selected according to their biological relevance was carried out, and for 72% the alterations could be confirmed. Better

performance of the oligonucleotide arrays contributes to a higher proportion of validated genes. True alterations in gene expression are likely to explain at least partially the oscillation of the transcript levels of several genes over time (I, II).

Despite major differences between these two different microarray platforms, similar patterns in alterations of gene expression were observed. However, data sets from cDNA and oligonucleotide microarrays have substantial variation and poor concordance, and, therefore, they cannot be directly compared (Järvinen *et al.*, 2004; Woo *et al.*, 2004; Zhu *et al.*, 2005).

5.1.1. HPV E5 alters expression of extracellular matrix genes

Interaction of cells with the surrounding extracellular matrix (ECM) affects cell differentiation, proliferation, adhesion and migration (Adams and Watt, 1993). The ECM consists of a number of molecules, including collagens, laminins, fibronectins, vitronectin, tenascins, fibrillins, proteoglycans and elastins (Merker, 1994; Yurchenco and O´Rear, 1994). In this work, alterations in the expression of fibronectin-1, laminin-γ1, laminin-α4, laminin receptor 1, collagen type 16 and cytokeratin-8 were observed (I, II). The integrins αV, -β2 and -β-like 1 were also altered (II). Integrins serve as cell surface receptors and are important in cell adhesion, migration and signalling. The laminin-5 component of the ECM has been suggested to be a transient receptor for HPVs (Broutian *et al.*, 2010; Culp *et al.*, 2006). We did not observe significant alterations in laminin-5 expression. However, upon infection of basal epithelial cells, differences in ECM binding between HPV types have been recently reported (Broutian *et al.*, 2010). Further, an increment of the 67-kDa laminin receptor transcript was detected in E5-expressing cells (I). In agreement with this, an upregulation this transcript has previously been reported in HPV-associated cervical neoplasia (Demeter

et al., 1992). Laminin receptor has also been shown to be involved in tumour invasion and metastasis (Castronovo, 1993).

Here, a modest upregulation of PKC-δ was observed. The PKC family proteins regulate numerous cellular functions such as cell proliferation and survival. The E5 protein has been suggested to have a role in several cellular pathways involving PKC (Brodie and Blumberg, 2003; Chen *et al.*, 1996; Ueda *et al.*, 1996). Furthermore, PKC is also known to phosphorylate matrix metalloproteinases (MMPs), which play a role in tumour invasion and have been demonstrated to degrade ECM. Interestingly, alterations in the mRNA expression of several MMPs were observed in both microarray studies, and downregulation of MMP-2, MMP-7, MMP-12, MMP-13 and MMP-16 was validated by qRT-PCR (I, II). Our unpublished results have also shown expression differences in MMP-7 and MMP-16 between cervical dysplasia and normal tissue. In a larger tumour tissue material, MMP-7 was found to be slightly upregulated in cervical dysplasia, and MMP-16 expression was detected throughout the dysplastic epithelium, while the protein was expressed only in the basal cell layer of normal tissue (unpublished results). These findings are in concordance with the previously reported roles of MMP-7 and MMP-16 in tumour invasion and cancer (Kessenbrock *et al.*, 2010).

5.1.2. HPV E5-induced modifications in the PI3K/Akt pathway and immune response

Several genes of the phosphatidylinositol phosphate kinase (PIPK) pathway appeared altered in the microarray, and they were also validated by quantitative RT-PCR (I). The phosphoinositide-3-kinase (PI3K) pathway is important in diverse cellular mechanisms such as cell proliferation, motility and survival and intracellular trafficking. In the present work, slightly increased expression of phosphoinositide-3-kinase receptor 3 (PI3KR3) was found in E5-expressing cells. Also, transcription of both phosphatidylinositol

transfer protein and inositol polyphosphate-4-phosphatase was upregulated. An increment of class II PI3-kinase transcripts was observed as well (I, II). Increased expression and slightly enhanced activation of Akt, a downstream effector of PI3K, were observed in E5 cells. Akt activation plays an important role in cell proliferation and survival by phosphorylating a variety of substrates (Song *et al.*, 2005). PI3K/Akt signalling has been shown to be frequently disrupted in human cancers. Interestingly, HPV E5 has been reported to protect cells from ultraviolet-B-induced apoptosis and promote survival by activating the PI3K/Akt pathway (Zhang *et al.*, 2002), in agreement with our results. PI3Ks also have important functions in the immune system such as activation of T-lymphocytes (Ward and Cantrell, 2001). Cytokines activate PI3K in immune cells, and, indeed, we found several interleukins to be upregulated in E5-expressing cells. Our microarray data as well as the qRT-PCR results indicate a role for the E5 protein in promoting cell survival by enhancing the activity of the PI3K/Akt pathway.

Genes involved in the immune and inflammatory response were altered in E5-expressing cells throughout the time-frame studied (II). Microarray analysis revealed several interleukins and integrins to be upregulated in E5-expressing cells. From these genes, quantitative RT-PCR validation was performed for integrin-αV, -β2 and -β-like 1 and major histocompatibility complex (MHC) IA, and strong upregulation of integrin-β2 was confirmed. MHC IA mRNA levels oscillated over time, although downregulation was noted at most time-points. Downregulation of MHC I and II has been observed due to HPV E5 oncogene expression, with a subsequent reduction in immune recognition (Ashrafi *et al.*, 2005; Zhang *et al.*, 2003). Strong induction of microRNA-146a (MiR-146a) by the E5 protein was seen in the miRNA microarray (II; Section 5.3.). MiR-146a has been shown to be involved in negative regulation of immune responses and cytokine signalling (Curtale *et al.*, 2010; Taganov *et al.*, 2006). In addition, a slight decrement occured in genes related to TNFα signalling, which may be partially explained by miR-146a upregulation. This was confirmed in E5-expressing cells by TNFα stimulation and miR-146a inhibition, which resulted in significant activation of the downstream protein,

p38 kinase, as well as in ERK1/2 (II). Downregulation of miR-203 was also noted in the miRNA microarray (II; Section 5.3.1.), and with the miR-203 overexpression experiment along with INFγ stimulation, stronger enhancement in p38 kinase in E5-expressing cells than in control cells was seen (II). HPV persistence and cancer development require inactivation of host cell immune defences, and our data thus suggest an important role for HPV E5 in interfering with the innate immune response during infection.

5.2. Epithelial cell adhesion and cell motility (I - III)

5.2.1. Alterations in expression of cell adhesion and motility-related genes due to HPV 16 E5 expression

Cell adhesion molecules, including integrins and cadherins, facilitate cell binding to the ECM and to each other. Epithelial cells adhere to each other via adherens junctions, which require interaction of cadherins with actin filaments by means of α- and β-catenins. Cell motility involves both disintegration and establishment of cellular junctions, when the cells move on the surface of or attach to other cells. We found downregulation of E-cadherin transcription, but protein expression was enhanced in both Western blot and collagen raft staining. Stronger staining of E-cadherin was seen in E5 three-dimensional collagen raft cultures than in control raft cultures (II). Upregulation of N-cadherin and β-catenin proteins was also observed in E5-expressing cells as seen in Western blotting (II). In collagen raft cultures, stronger staining for β-catenin was detected in E5 cultures than in control cultures, whereas no differences were noted when staining N-cadherin (II). In cervical dysplasia, expression of E-cadherin, N-cadherin and ezrin was localized to cellular junctions throughout the epithelia in contrast to normal epithelia, where staining was restricted to the bottom layers (II). The result is hardly surprising since cell-cell adhesion is known to be reduced in human cancers. Carcinogenesis involves downregulation of E-cadherin and

disruption of E-cadherin – β-catenin complexes in adherens junctions, the stability of which is regulated by ezrin (Hiscox and Jiang, 1999). The epithelial-to-mesenchymal transition, an important event during tumour invasion and metastasis, is characterized by reduced levels of E-cadherin and enhancement in N-cadherin expression (Hazan *et al.*, 2004). On a post-translational level, degradation of E-cadherin is mediated by MMPs (Munshi and Stack, 2006), which are generally downregulated in E5-expressing cells. The E5 protein seems to have effects at cellular junctions, and the alterations observed in our study suggest functions in cell motility rather than in carcinogenesis directly. The strong downregulation of MMPs due to E5 expression cannot be fully explained, but E5 may reduce MMP expression indirectly by inhibiting signal-transduction pathways that induce MMP transcription. The E5 protein alone may be insufficient to trigger carcinogenic changes in the host, but instead it functions together with the E6 and E7 oncoproteins. The effects that the HPV-16 E5 protein has on these key adhesion molecules confirm its important role in the initiation of the carcinogenic process.

Epithelial tight junctions seal the cells to each other, preventing the passage of molecules and ions through the space between the cells. In this work, modest upregulation of the tight-junction component claudin-1 was found on an mRNA level, whereas little effect was seen in claudin-1 protein expression (II). Furthermore, several integrins were enhanced in the oligonucleotide microarray, and the increment in integrin-β2 expression in E5-expressing cells was confirmed by qRT-PCR (II). Integrin-β2 is an important protein involved in cell adhesion and cell-surface-mediated signalling. Cell adhesion and signal transduction-associated integrin-αV mRNA and protein levels were also altered in E5 cells, although oscillation over time occurred. The HPV E5 protein has been reported to reduce the adhesiveness of trophoblastic cells to the tissue culture plastic and to endometrial cells (Boulenouar *et al.*, 2010). The E5 protein seems to be involved in reduction of cell adhesion, which is an important phenomenon in cell motility and also in cancer. This is further supported by the observed upregulation of miR-146a, which targets

several genes involved in cell adhesion (see Section 5.3.). In addition, we observed downregulation of miR-203, which could lead to enhancement of its targets, to be involved in cell migration and motility.

Data from the microarray analyses suggest that cell adhesion and motility are among the major cellular events affected by the E5 protein. To study this phenomenon further, we analysed the expression of focal adhesion proteins FAK and paxillin by Western blotting (I). Indeed, increases in both total and phosphorylated paxillin were detected in E5-expressing cells, whereas FAK protein levels remained unaffected. Paxillin localizes to focal adhesions, and when activated by phosphorylation, it enhances cell adhesion and motility (Petit *et al.*, 2000). Increased activity of FAK and paxillin has been implicated in cervical carcinogenesis by HPV E6 and E7 oncogenes (McCormack *et al.*, 1997). Our results suggest that E5 also contributes to the pathogenesis of HPV by enhancing cell motility.

The stability of E-cadherin – β-catenin complexes in adherens junctions between epithelial cells is regulated by ezrin. Ezrin is a protein linking the actin cytoskeleton to the plasma membrane, and it has an important role in regulating cell morphology and motility. We used HeLa cells to investigate epithelial cell adhesion, particularly the role of ezrin. HeLa cells originate from a human cervical adenocarcinoma, and they naturally contain parts of the HPV-18 genome. We found co-localization of ezrin and N-cadherin in adherens junctions, but no expression of E-cadherin (III), confirming the switch from E-cadherin to N-cadherin in cancer cells. Ezrin may possess a role in HPV-associated carcinogenesis, and, indeed, our study revealed an increase in N-cadherin protein expression and a slight decrease in ezrin mRNA levels in the HPV E5-expressing epithelial cells, although no differences were detected in ezrin expression levels between E5 and control cells using collagen raft cultures (II). These phenomena contribute to decreased cell adhesion and enhanced cell motility; we did not, however, study their consequences on cell adhesion or motility *in vivo*.

5.2.2. Regulation of ezrin localization and function in epithelial cells (III)

Ezrin, along with other ERM-protein family proteins, regulates cell morphology and motility. Inactivation of ezrin has been reported to alter cell-cell and cell-matrix adhesion and increase cell motility (Hiscox and Jiang, 1999; Takeuchi *et al.*, 1994), or depending on the tumour cell type, suppress migration and metastasis (Rossy *et al.*, 2007). In cancers, the role of ezrin is complicated. In ovarian carcinomas, the loss of ezrin expression is associated with poor survival (Moilanen *et al.*, 2003), conversely in uveal malignant melanoma (Mäkitie *et al.*, 2001) and pancreatic adenocarcinoma (Akisawa *et al.*, 1999) the presence of ezrin is a poor prognostic indicator.

Ezrin expression and localization were investigated in HeLa epithelial cells by co-expression experiments (III). Endogenous ezrin was found in the cytoplasm and microvilli. We evaluated the effect of a small GTPase Rac1 on ezrin localization. In the presence of constitutively activated Rac1, ezrin was redistributed to intercellular adherens junctions and colocalized with N-cadherin. However, ezrin localization at adherens junctions was reduced by expression of dominant negative Rac1. The role of Rac was further studied by overexpressing Rac1 or stimulating the cells with platelet-derived growth factor (PDGF). In both experiments, endogenous ezrin was relocalized to cell-cell adhesions, confirming the role of Rac1 in ezrin redistribution.

We also studied the role of other GTPases. Participation of RhoA either upstream or downstream of Rac1 in the same pathway has previously been reported (Kaverina *et al.*, 2002; Rolli-Derkinderen *et al.*, 2010). In this system, we showed that RhoA is crucial for the role of Rac1 in ezrin relocalization, as inhibition of RhoA by C3 exoenzyme abolished ezrin localization at cell junctions (III). Phosphoinositide kinase (PIPK) was also found to be involved in this process when using transient cell transfections. Further, co-transfection experiments using wild-type phosphoinositide kinase alpha (PIPKα) and dominant negative Rac1 placed Rac1 downstream of

PIPKα in the pathway leading to ezrin relocalization. This was further substantiated by the finding that constitutively activated Rac1 restored ezrin localization at adherens junctions when co-transfected with kinase-deficient PIPKα. Cells expressing the pseudoactivated form of ezrin have also been shown to possess activated Rac, but not activated Rho (Pujuguet *et al.*, 2003). These results confirm the function of ezrin as an organizer of adherens junctions in epithelial cells.

5.2.3. HPV 16 E5 enhances cell motility *in vivo* (I)

Cell migration and motility are critical in tumour invasion and metastasis. Essential for cell migration is actin reorganization, which is regulated by Rho family small GTPases. In microarray analysis, several genes involved in cell motility were found to be altered in the E5-expressing cells, and increased protein expression levels of activated paxillin and Akt were also detected (I). These findings strongly suggest enhanced cell motility and reduced cell adhesion. A wound healing assay was therefore performed using confluent monolayers of HaCaT-E5 and control cells to study directional movement of a population of epithelial cells with the help of intracellular junctions *in vivo*. Wound closure in E5-expressing cells was monitored by live-cell imaging, and a higher rate of wound closure was observed relative to controls. Wound closure was shown to occur due to enhanced cell movement rather than to increased proliferation, as statistical analysis revealed no temporal differences in cell number in the wounded area. The major pathways activated during cellular motility and metastasis are the PI3K/Akt pathway and the FAK/Src signalling pathway, which operates via paxillin. These pathways are in close relation with each other; Scr activation seems to be required for PI3K activation, which is in turn required for the activation of FAK and Akt (Thamilselvan *et al.*, 2007). The E5 protein level increased and activated Akt and paxillin protein expression in both pathways. Regulation of cancer cell adhesiveness appears to involve Src and PI3K interaction to activate FAK in a

complex manner that also requires Akt and paxillin activation (Basson, 2008). Previous studies have implicated HPV E5 in cell movement. Thomsen *et al.* (1999) reported reduced cell motility in individual cells due to E5 expression in mouse embryonic fibroblasts. On the other hand, Boulenouar *et al.* (2010) recently observed increased cell migration and invasion due to HPV E5 in trophoblastic cells. Regulation of these pathways, including alterations in adhesion-related proteins, suggests an important role for the E5 protein in the early stage of carcinogenic progression.

5.3. MicroRNA profiling in epithelial cells expressing HPV-16 E5 oncogene (II)

The importance of cellular microRNAs (miRNAs) in cancer as well as in viral infections has been stressed in numerous studies. Therefore, the effect of HPV-16 E5 expression on cellular miRNAs was investigated here using large-scale microarray analysis. MiRNA expression in HaCaT-E5 and control cells was profiled in uninduced cells as well as after induction for 24, 48, 72 and 96 h. The number of differentially expressed miRNAs varied from eight to 18 depending on the time-point of E5 expression (*P*-value <0.01). For all 13 miRNAs for which a Taqman miRNA assay was available at the time of the study, alterations were validated by quantitative RT-PCR (III: Table 2). Evidence shows that miRNAs influence gene expression by causing the degradation of their target mRNA (Cheng *et al.*, 2008; Lim *et al.*, 2005), although it has recently been observed that also a positive correlation between miRNAs and mRNA exists (Nunez-Iglesias *et al.*, 2010; Tsang *et al.*, 2007; Wang *et al.*, 2009). The data from the mRNA and miRNA microarrays were integrated, following the assumption that gene expression is inversely correlated. After the integration, known targets of validated microRNAs were sought among the validated mRNA microarray transcripts (Table 2). Observed alterations in the expression of several miRNA target genes, such as

N-cadherin, integrin αV, claudin-1 and β-catenin, were in agreement with miRNA alterations.

Table 2. *Integration of microRNA and mRNA data revealed alterations in several microRNA targets studied by qRT-PCR.*

Regulator miRNAs	**Target gene**	**Gene Symbol**	**Gene ID**
hsa-miR-146a, hsa-miR-214	cadherin 2, type 1, N-cadherin (neuronal)	CDH2	1000
hsa-miR-146a, hsa-miR-200c	fibronectin 1	FN1	2335
hsa-miR-203, hsa-miR-30a-5p, hsa-miR-23b	PDZ domain containing 2	PDZD2	23037
hsa-miR-30a-5p, hsa-miR-106a	junB proto-oncogene	JUNB	3726
hsa-miR-19a	tumour protein p53	TP53	7157
hsa-miR-200c, hsa-miR-433, hsa-miR-624	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	3685
hsa-miR-539	claudin 1	CLDN1	9076
hsa-miR-624, hsa-miR-214	catenin (cadherin-associated protein), beta 1, 88 kDa	CTNNB1	1499
hsa-miR-107	BCL2/adenovirus E1B 19 kDa interacting protein 3	BNIP3	664

All validated microRNAs, excluding miR-146a and miR-324_5p, followed a similar expression trend in qRT-PCR. For the microRNAs studied, the expression was enhanced during the first 48 h and finally at the 72-h time-point the expression was reduced. MiR-146a was found to be constantly upregulated and miR-324_5p downregulated at all time-points evaluated. MiR-203 downregulation was observed at late time-points in the microarray analysis. For these three selected miRNAs, putative target genes, in addition to confirmed targets, were predicted using eight distinct algorithms. Genes involved in cell migration, cell motility and cell junctions were enriched in the miR-203 targets, while targets of miR-146a were involved in cell adhesion and cell cycle. Several cell adhesion and cell death-associated genes were found among the predicted targets of miR-324_5p.

Interestingly, both miR-146a and miR-203 are implicated in a number of cancers. Wang *et al.* (2008) have earlier reported that miR-146a expression is increased in cervical cancer tissues, and it promotes cell proliferation in cell lines, suggesting a role in cervical carcinogenesis. Enhanced miR-146a expression has been detected also in many other cancers (He *et al.*, 2005; Volinia *et al.*, 2006). We found decreased mRNA expression but increased protein expression of E-cadherin, a putative target of miR-146a. Increased half-life due to catenins or other components of cellular junctions may explain this observation (Ireton *et al.*, 2002; Lozano and Cano, 1998). A significant increase was observed in gene as well as in protein expression levels of N-cadherin, another putative target of miR-146a. This is contrary to the classical concept of decreased target expression in the presence of upregulated miRNA. Possibly, miR-146a downregulates an unknown target upstream of these cadherins in the same pathway, resulting in upregulation of protein expression.

Expression of a less studied miRNA species, miR-324_5p, was downregulated throughout the experiment. Downregulation of miR-324_5p has been reported to contribute to tumour cell proliferation and carcinogenesis (Ferretti *et al.*, 2008), which suggests a potential role in the oncogenic functions of the E5 protein.

The E5 protein of HPV-16 induced alterations in the host cell gene. Protein expression may take place through several different mechanisms; in some of cases, E5 may directly affect cellular gene and protein expression, but it may also alter microRNAs. Although almost all human cancers display a certain level of aberrant miRNA expression, very little is known about the cause of the aberrant expression and its role in cancer.

5.3.1. HPV E5 is involved in pathogenesis of HPV infection by repressing miR-203 (II)

We observed downregulation of miR-203 and also a slight increase in its target protein, p63, in E5-expressing cells. p63 is rarely mutated in cancers, although overexpression of the isoform ΔNp63 has been observed in squamous cell carcinomas (Nylander *et al.*, 2000). No clear difference in the intensity of p63 expression was detected in E5 raft cultures compared with control cultures. However, cell nuclei in the differentiated cell layers of E5-expressing cultures lacked p63, while differentiated control cell nuclei stained for p63. MiR-203 is known to promote proliferation and Lena *et al.* (2008) reported miR-203 to repress stem cell-like properties of epithelial cells by downregulating p63. Mir-203 downregulation has been observed in many tumours (Craig *et al.*, 2011; Furuta *et al.*, 2010). The p63 transcription factors are important in maintaining the proliferation of basal epithelial cells (Truong *et al.*, 2006). A recent report stated that another HPV oncogene, the E7 protein, also downregulates miR-203, which causes an increment in p63 expression in HPV-infected cells (Melar-New & Laimins, 2010). This suggests that both E7 and E5 proteins contribute to the pathogenesis of HPV infection through miR-203 by maintaining high levels of p63 proteins, which uphold an active cell cycle. Reduced levels of p63 are critical for normal epithelial differentiation, which is disturbed during HPV infection. We observed abolishment of p63 when miR-203 was overexpressed in HaCaT keratinocytes. Thus, HPV-mediated miR-203 downregulation seems to have a crucial role in interfering with epithelial cell differentiation and subsequently contributing to carcinogenesis.

6. Concluding remarks

Genome-wide effects of the HPV-16 E5 oncogene were studied as reflected in cellular gene expression as well as in microRNA expression in human keratinocytes. A number of altered genes were identified using two different microarray platforms, and these alterations were concentrated in genes affecting cellular adhesion and motility as well as the stucture of the extracellular matrix. The E5 protein was observed to modulate the expression of numerous cellular microRNAs likely to contribute to viral pathogenesis. Alterations in microRNA target proteins were also observed. To date, only a few reports have emerged on the regulation of cellular miRNA expression by virus infection. Elucidation of the roles of the miRNAs as well as functional studies of their targets in the HPV life cycle will be important in future analyses.

Enhancement of cell motility was detected in live-cell imaging studies. In addition, regulation of ezrin localization by Rac1, PIPKα and RhoA in epithelial adherens junctions was observed. Disruption of cell adhesion and increased cell motility are involved in carcinogenesis. Ezrin is one of the regulators of E-cadherin - β-catenin complexes in adherens junctions. In this study, alterations in several junction components were detected, possibly contributing to decreased cell adhesion and increased cell motility. Indeed, enhanced cell motility was observed in HPV E5-expressing cells. Further studies of the HPV E5 protein are likely to reveal its functions in viral replication, cellular signal transduction, cellular transformation and, finally, carcinogenesis.

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