Chapter 1

General Introduction
Human papillomavirus and cervical cancer

Persistent infection with high-risk human papillomavirus (hrHPV) is the most important risk factor for the development of cervical cancer. The causal relationship is illustrated by the fact that viral DNA can be detected in virtually all cervical cancers (1-3). Transmission of HPV is associated with sexual intercourse and 80% of women are likely to acquire infection with HPV in their lifetime (4). Despite this high incidence rate of HPV infections most women clear the virus without any symptoms. This indicates that the development of cervical cancer is a rare complication, which can occur only after persistence of the infection and for which acquisition of additional alterations in the host cell are indispensable. These alterations may be genetic or epigenetic, ultimately affecting the expression of oncogenes and tumor suppressor genes.

The cervical transformation zone is thought to be most susceptible to hrHPV-induced transformation. The cervical transformation zone, or squamocolumnar junction, is the border between the squamous epithelium of the ectocervix and the columnar epithelium of the endocervix. This border shifts from puberty onwards during adolescence from the outer part of the cervix to the endocervical canal. During this process the cylindrical epithelium of the endocervix is replaced by squamous epithelium.

Cervical cancer remains a major cause of cancer related deaths in the world, although the incidence in the developed world is declining due to organized screening programs. The most common histotype of cervical cancer is squamous cell carcinoma (SCC) which accounts for ~80% of cases. An additional 15-20% of cases are adenocarcinomas (AdCA). Squamous cell cervical carcinoma develops through a series of well defined precursor lesions named cervical intraepithelial neoplasia (CIN). These CIN lesions are classified according to the degree of atypia of the epithelial cells, ranging from less than one third of the epithelium in CIN1 up to the whole epithelial layer in CIN3 (Figure 1). Whether these lesions always develop through succession is currently under debate, since high-grade precursor lesions (CIN2-3) are likely to develop rapidly following an hrHPV infection without an apparent CIN1 intermediate state (5). At present, CIN1 and especially a subset of CIN2 lesions are increasingly considered as histological manifestations of a productive viral infection. The risk of progression of CIN lesions to invasive cervical cancer is associated with the degree of the CIN lesion (6). The time of progression also
appears to be related to the degree of the CIN lesion, being shortest for CIN3. Notwithstanding, while high-grade CIN may develop within 3 years, eventual progression to invasive cancer may take another 10-30 years (3, 5, 7-9). Conversely, all CIN lesions have the potential to regress to a previous stage, although the chance declines considerably with increasing severity of the lesion. Adenocarcinoma of the cervix supposedly also develops through a series of precursor lesions, including adenocarcinoma in situ (ACIS), but these are less well defined.

Figure 1. Schematic representation of cervical cancer development. Via micro trauma to the epithelium at the cervical transformation zone, HPV particles can access the basal layer and infect the epithelium. CIN1 and a subset of CIN2 lesions are thought to be histological manifestations of productive viral infections. When viral oncogene expression becomes deregulated, the infection induces cellular transformation manifested as CIN3 lesions, which only after additive host cell alterations can progress to invasive cancer (adapted from Woodman (10)).

**Human papillomaviruses**

HPVs comprise a large family of related virus types belonging to the Papillomaviridae. They can be divided in five evolutionary groups; the Alpha, Beta, Gamma, Mu and Nu genera (11), with a subset of types belonging to the Alpha genus being linked to cervical cancer. Based on their site of infection, HPVs can also be divided in cutaneous types, such as HPV8 and HPV38, and mucosal types such as the well known types HPV16 and HPV18. Mucosal types infect mucosa of the lower anogenital tract and oral/oropharyngeal cavity and
are further subdivided into low-risk and high-risk types. Low-risk types like HPV6 and HPV11 are primarily linked to genital warts and low-grade CIN lesions. To date, 15 high-risk types have been defined based on their association with cervical cancer with HPV16 and HPV18 accounting for 70% of cervical cancers (1). In addition infection with hrHPV, predominantly HPV16, has been linked to a subset of cancer at other anogenital sites as well as head and neck cancers.

**The viral life cycle**

An HPV infection requires access of viral particles to cells of the basal layer, which is facilitated by micro trauma of the epithelium. The virus can attach to the epithelium by binding to heparin sulphate proteoglycans on the cellular membrane. Upon endocytic uptake the virus is uncoated and the DNA is transferred to the nucleus of the epithelial cell (12).

HPV is a small DNA virus (~8kb), containing six early open reading frames (E1, E2, E4, E5, E6, E7) and 2 late genes (L1, L2), which are under control of the long control region (LCR) (Figure 2A). The LCR contains viral promoter and enhancer sequences that are regulated both by viral proteins encoded by the E2 open reading frame and cellular transcription factors, such as Sp1 and AP-1. The normal life cycle of HPV is tightly related to the differentiation program of the infected host cells (reviewed by Doorbar (13)). During normal epithelial differentiation, keratinocytes entering the suprabasal layers stop replicating and undergo terminal differentiation as they move upwards through the epithelium. Viral replication and virion production takes place in these non-dividing differentiating cells.

Following infection, the early genes E1, E2, E6 and E7 are expressed at low levels in the undifferentiated basal cells. In these cells low copy numbers of the virus are maintained with help of the viral proteins E1 and E2. The early genes E1 and E2 are involved in replication of the viral DNA and are responsible for maintaining the episomal state (14, 15). E1 is a helicase essential for initiation and elongation of viral DNA replication. E2 binds E1 to facilitate initiation of DNA replication and also functions as a transcriptional repressor of the viral promoter that drives transcription of E6 and E7 oncogenes (16, 17). E2 is also necessary for the interaction between viral DNA and mitotic chromosomes to ensure that each daughter cell receives a copy of the viral DNA (18). The host cell replication machinery is reactivated in differentiated cells by actions of the
viral proteins E6 and E7 in order to allow viral DNA replication without inducing apoptosis. E7 promotes S-phase re-entry in differentiated cells by destabilization of p130 (19). Following S-phase re-entry E7 was found to be sufficient to induce a prolonged G2 arrest in differentiated keratinocytes (20). Although a G2/M arrest was previously attributed to the E1^E4 fusion protein, recent findings indicate that the E1^E4 G2/M arrest function is not essential for viral genome amplification and virus assembly in differentiated cells (21). E4, which together with E5 also becomes upregulated in the upper differentiated strata, is shown to enhance the release of viral particles by the interaction with cytoskeletal proteins (22). Once the cells reach terminal differentiation, E2 represses E6 and E7 expression, while the late genes L1 and L2 are expressed to provide the viral capsid. Viral assembly occurs in the stratum spinosum and granulosum of the squamous epithelium. Thousands of new virus particles are shed with the exfoliating epithelial cells. The HPV life cycle described above is fulfilled in normal infected epithelium and in low-grade cervical lesions.

Figure 2. A. The genome of HPV16 is 7.9 kB in size and is divided in 8 open reading frames encoding the viral early genes E1, E2, E4, E5, E6, E7 and the viral late genes L1 and L2, which are under control of the long control region (LCR). B. The full length E6/E7 transcript and the common spliced E6*I/E7 transcript which is further described in the section E6* below. (Figure adapted from Rosenberger (23)).
Transforming viral infection

High-grade cervical lesions are characterized by a transforming viral infection resulting from deregulated viral gene expression. Deregulation of the normally tightly regulated expression of oncogenes E6 and E7 alters the above described, to a transforming infection in which E6 and E7 are being overexpressed in dividing cells.

The mechanism underlying the switch in E6/E7 expression from mainly differentiating to dividing cells is not completely understood. A possible contribution to uncontrolled E6 and E7 expression is integration of the viral DNA in the host genome. Viral integration may lead to the disruption of the E2 open reading frame, resulting in loss of the repressing function of E2 on E6 and E7 transcription (24). Contribution of loss of E2 expression to increased E6 and E7 expression is supported by the finding that re-introducing E2 into cervical cancer cells leads to E6 and E7 silencing and reactivation of p53 and pRB pathways, resulting in irreversible senescence (25). However, integrated copies are not present in all carcinomas, and many carcinomas have episomal copies retained. Moreover, recent data suggest that deregulated viral oncogene expression occurs prior to viral integration. Deregulated expression of E6 and E7 contributes to a genetically instable environment. It is therefore likely that viral integration is a consequence of this chromosomal instability rather than the cause. Additionally, viral integration rates are found to differ between HPV-types and are suggested to be related to the extent to which the different hrHPV types cause chromosomal instability (26). Recently, it has been suggested that a coexistence of integrated and episomal copies may be beneficial for transformation. The proteins E1 and E2 from the episome can aid in initiating DNA replication from the integrated virus (27).

Other mechanisms could also contribute to deregulated viral gene expression, such as methylation of viral DNA, which has been demonstrated to increase during the course of carcinogenesis (28). The LCR of high-risk HPVs contains four E2 binding sites (E2BS) located near DNA binding sites for transcription factors such as Sp1 and TATA-binding protein. Methylation of the E2BS is known to interfere with E2 binding and is considered an alternative to interfere with E2-mediated regulation of E6 and E7 expression. Methylation of E2BS varies depending on the differentiation status of the cell (29). The methylation of the LCR region specifically at E2BS number 2 increases with progressive disease (30, 31).
E5
The viral protein E5 supports the immortalization potential of E6 and E7, although it is not essential for transformation in vitro (32). Nevertheless, a recent study demonstrated that prolonged estrogen treatment of E5 transgenic mice resulted in increased cancer rates (33). By inhibiting degradation of the EGF-receptor, E5 enhances EGF signaling (34). E5 has also been described to be able to inhibit apoptosis (35, 36), extend the lifespan of keratinocytes and stimulate cJun, JunB, cFos expression (37, 38).

E6
The viral oncoprotein E6 is best known for its ability to prevent cells from undergoing p53-mediated events, such as apoptosis. Association of E6 with E6AP, a specific ubiquitin ligase, marks p53 for proteolytic degradation (39, 40). This results in degradation of p53 and also in abolishment of transcription of p53 target genes such as p21. Inactivation of the pro-apoptotic protein Bak by E6 also contributes to inhibition of apoptosis (41). This ultimately allows cells with genetic alterations to survive, thereby contributing to genetic instability. Additional functions of E6, which may contribute to transformation include binding and targeting PDZ domain containing proteins, such as Dlg, Scribble, MAGI and Par polarity complexes, for proteasome-mediated degradation (reviewed by (42)). In mice it has been shown that the ability of E6 to cooperate with E7 in malignant transformation is dependent on its PDZ binding domain (43-45). The PDZ binding motif has been shown to be essential for the normal viral life cycle, suggesting that these interactions are also critical for viral replication (46). Type-specific functioning of this motif is still under investigation. Furthermore, E6 contributes to transformation by induction of telomerase activation upon stimulating the expression of its catalytic subunit hTERT (47). The activation of telomerase is essential for immortalization (see also section immortalization) and cancer development.

E6*
The genes E6 and E7 are expressed from a bicistronic mRNA which is generated from the same promoter P97 (See Figure 2B) (48, 49). Splicing of the HPV16 E6/E7 transcript leads to several truncated versions of E6 named E6* (I-IV) and E7. Unspliced transcripts result in mainly E6 expression and little E7 expression. Conversely, E7 expression is increased upon splicing of E6 (50).
The combination E6*I with E7 is abundantly found in cervical carcinomas and HPV16 positive cervical cancer cells (51). So far, these splicing events are only described for high risk types (52). Also other viral splice products are present in cervical cancer and recent analysis suggests that splice variant analysis may aid in improving the specificity of HPV DNA testing as the proportion of E6*I*I and E1C splice products is markedly increased in high-grade CIN lesions and SCC compared to hrHPV infected normal cells (53).

The function of various splice variants is not completely understood. E6*I of high-risk types HPV16 and HPV18 binds to E6 and E6AP leading to inhibition of E6-E6AP binding and concomitant p53 degradation as well as reduced binding of E6 to other targets (54, 55). Like E6, E6* was also shown to target several cellular proteins such as PDZ-domain containing proteins (e.g. Dlg, MAGI) for proteasome mediated degradation, without direct interaction (56). Additional functions may include targeting of proteins for degradation which are inaccessible to full-length E6, as for instance PKB/AKT. Hence, E6* proteins are likely to exert their effect by interference with protein turnover. Besides inhibition of E6-E6AP binding, the splice product may also enhance the function of E6 in certain instances by creating heterodimers of E6-E6*, as is described for targeting of PDZ domain containing proteins (56).

E7

The E7 protein is involved in disturbing normal cell cycle regulation by inactivating G1/S checkpoint regulators. Most notably is the interaction with pocket protein family members pRb, p130 and p107 (57, 58). Interaction with pRb promotes proteasomal degradation (59, 60) and disturbs pRb from inactivating transcription factors of the E2F family. Normally, release of E2F from the inhibitory interaction with pRb would only occur upon phosphorylation of pRb by cdk4/6 and cdk2 in the G1 phase to induce S phase entry. The release of E2F results in transcription of genes involved in DNA replication. Therefore, E7 activity in differentiating cells ensures S phase-entry and expression of genes necessary for viral vegetative replication (61, 62). A consequence of E7 deregulation in proliferating cells is a strong increase in expression of the cdk4 inhibitor p16\textsuperscript{INK4A}, most likely as a result from interference with a negative feedback mechanism of pRb to p16\textsuperscript{INK4A} (63). Though this is without an apparent effect, since downstream pocket proteins are targeted by E7, p16\textsuperscript{INK4A} overexpression is nowadays considered a valuable marker for transforming hrHPV infections (64). Another recently published
mechanism, at least for HPV16, illustrates that E7 induces transcriptional activity of histone demethylase KDM6B which results in p16^{INK4A} upregulation independent of pRb-binding (65).

In addition, E7 can abrogate the growth inhibitory functions of cyclin-dependent kinase inhibitors p21 (60, 66) and p27 (67). However, it has also been reported that E7 stabilizes p21 protein expression especially in differentiated cells (68, 69). Expression of E7 also contributes to genomic instability as it induces centrosome duplication errors leading to numerical chromosomal abnormalities (70). Moreover, E7 has been shown to enhance DNMT1 and indirectly bind to histone deacetylases (HDACs) thereby influencing chromatin remodeling and accessibility of promoters for E2F activity (71).

Like for E6*, also a link between E7 and PKB/AKT activity is described. E7 upregulates activity of PKB/AKT in raft cultures of primary keratinocytes, which is attributed to its pRb-binding ability. Phosphorylation of the kinase PKB/AKT is increased in cervical lesions compared to normal tissue and this upregulation is also shown in raft cultures of HPV-immortalized keratinocytes (72).

E7 can also interact with the Activator Protein 1 (AP-1) family member cJun, thereby causing a direct increase in cJun transactivating activity (73). The transcription factor complex AP-1 has an essential role in transcriptional regulation of the viral LCR and will be discussed below in more detail.

**In vitro models of productive and transforming HPV infections**

In order to gain a better understanding of the molecular background of the productive viral life cycle as well as HPV-mediated transformation, the use of *in vitro* model systems of primary human keratinocytes have proven to be very useful. Two different systems used for studying productive and transforming infections, respectively, will be discussed below.

**Productive infection in vitro**

Culturing epithelial cells in organotypic rafts yields an orderly stratification and differentiation of cells, which is reminiscent of squamous epithelium *in vivo* (74). This is achieved by mixing fibroblasts with collagen matrix as dermis, onto which keratinocytes are seeded and grown to confluence. Nutrients in the medium are taken up via capillary action through the dermal equivalent. The
raft structure is then lifted to liquid-air interface after which cells are allowed to differentiate for approximately 10 days. This system of orderly stratification of the epithelium enables HPV to fulfill its life cycle. A few raft systems have been shown to produce virions using tissue explants or immortalized cell lines (75-79). Wang et al. have recently achieved virion production in primary cells. Using supercoiled circular plasmids a high transfection efficiency was obtained in primary keratinocytes which, combined with Cre-loxP recombination, resulted in efficient generation of circular HPV18 genomes. This resulted in much higher viral titers as previously obtained in immortalized cells, allowing infections of new primary cultures. This system will be very useful to gain more insight in virus-host interactions (80).

Transforming infection *in vitro*
Expression of both E6 and E7 is essential for maintenance of a transformed phenotype. These proteins are the only two viral proteins that are constitutively expressed in HPV positive cervical carcinomas. This is supported by the finding that repression of either oncogene by E2 overexpression or silencing by siRNAs resulted in growth arrest, senescence and/or apoptosis in cervical cancer cell lines (81-87).
Moreover, E6 and E7 were sufficient for *in vitro* immortalization of primary keratinocytes (58, 88). While in primary human foreskin keratinocytes E6 and E7 are both required for immortalization, in other cellular systems, such as mammary epithelial cells, E6 or E7 alone can induce growth over 400 population doublings, indicative of immortalization (89, 90). E6 and E7 can immortalize keratinocytes from various origins such as prostate (91, 92), oral cavity (93), tonsil (94), bronchus (95), foreskin (96) and cervix (97).
Examination of human foreskin keratinocytes transfected with hrHPV revealed at least four consecutive stages of transformation, characterized as extended lifespan, immortalization, anchorage independent growth and tumorigenicity in nude mice (98). Whereas an extended lifespan is a direct and invariable consequence of hrHPV E6 and E7 expression, progression through the other stages requires the acquisition of additional (epi)genetic events in host cell genes. Complementation studies have revealed that progression to the various transformation stages are recessive processes with at least four complementation groups being described for the transition to immortalization. For progression towards anchorage independent growth a single complementation group has been described and for tumorigenicity two
complementation groups (98, 99). These distinct stages of transformation are discussed below.

**Extended Lifespan, immortalization and telomerase activation**

Like other somatic cells, primary human keratinocytes have a finite replicative lifespan ranging from 50-100 population doublings before they enter a non-proliferative state called replicative senescence (100). This is caused by telomere erosion resulting from the inability of DNA polymerase to completely replicate the chromosome ends, which are normally structured in such a way that protection from a DNA damage response is ensured. When chromosome ends become critically short this is recognized as DNA damage, upon which a.o. p53 is activated and the cells enter growth arrest. Senescent cells fail to proliferate but remain metabolically active. Senescence can be characterized by morphologically enlarged and flattened cells, which stain blue upon beta-galactosidase staining at pH6. Besides telomere erosion, senescence can also be induced by oncogenic stress, termed oncogene induced senescence (OIS) (reviewed by Mooi et al (101)).

Expression of HPV oncogenes E6 and E7 allows cells to bypass the senescence barrier by effectively inactivating p53 and pRb. In these extended cell divisions, cells may eventually reach a state of massive cell death as a result of further telomere shortening up to a critical point, which is called crisis. At this stage chromosomal aberrations such as end-to-end fusions can be observed. Bypass of crisis, mostly occurring at a low frequency, leads to immortalization and has been associated with the activation of telomerase (see below) (102, 103).

Next to the activation of telomerase, HPV-immortalized cells have acquired many chromosomal alterations, with gains of chromosomes 1, 3q, 20q and losses of chromosome 3p, 11q and 22q being most common (104-107). Most of these alterations are also common in cervical cancer cell lines as well as in high-grade lesions and squamous cell carcinomas of the cervix (107). In addition to genetic alterations, immortalized cells may have acquired epigenetic alterations, which may result in changes in expression of both protein encoding genes as well as non-coding microRNAs.

Telomerase is a ribonucleoprotein complex which consists of an RNA component (hTR) serving as the template to generate TTAGGG repeats, and a catalytic subunit (hTERT). While hTR is universally expressed, expression of the catalytic subunit hTERT is restricted to a small subset of somatic cells (e.g. stem cells, sperm cells), as well as most cancer cells. Normal cervical tissue and
low grade precursor lesions possess little to no hTERT expression and telomerase activation, whereas telomerase activity and elevated hTERT expression is detected in 40% of CIN3 lesions and most cervical squamous cell carcinomas (90%) (108). hrHPV E6 has also been demonstrated to directly activate telomerase, either by myc-mediated upregulation of hTERT (109) and/or by inducing degradation of the transcriptional repressor NFX1-91 (110). Besides interference hTERT promoter regulation, E6 can also physically interact with the hTERT protein (111).

Anchorage independent growth and tumorigenicity
Upon further passaging, HPV-immortalized cells may become resistant to anoikis, i.e. induction of apoptosis when contact between cells and matrix is disrupted (112). They overcome the requirement of attachment to extracellular matrix, represented by the coated culture dish in vitro. The phenotype of growth without attachment, anchorage independent growth, is characterised by the ability to form colonies in semi-solid agarose.

The acquisition of an anchorage independent phenotype results from the further accumulation of host cell alterations. For example loss of tumor suppressor genes on chromosome 11 has been shown to be involved in the acquisition of anchorage independence and tumorigenicity of HPV containing cells. In chromosome transfer studies, introduction of chromosome 11 was associated with an inhibition of tumorigenicity (113). One of the tumor suppressor genes located on chromosome 11q23 that has been further characterized is TSLC1/CADM1. This gene is silenced in cervical cancer cell lines, and ectopic expression of CADM1 in the cervical cancer cell line SiHa reduced the colony forming capacity and tumorigenicity (114). Another candidate gene on chromosome 11, encodes Fos Related Antigen 1 (Fra-1), a Fos gene family member and possible dimerization partner of the AP-1 transcription factor complex, which will be discussed later in this introduction.

Pathways involved in HPV-mediated transformation

Candidate genes involved in cervical cancer development may be identified by genetic and expression profiling of high-grade CIN lesions and cervical carcinomas. For example the long arm of chromosome 3 is often overrepresented in CIN and cervical carcinomas (105, 107, 115). A well known oncogene located on chromosome 3q26 is PIK3CA. This gene encodes for the
catalytic subunit of the enzyme PI3-kinase which is part of a complex signaling pathway that regulates many cellular processes such as proliferation, growth and apoptosis (116, 117). There is some evidence linking this pathway to cervical carcinogenesis both from genomic studies, showing PIK3CA copy number increases (105, 107, 115), as well as immunohistochemical data, showing increased protein expression in cervical carcinomas (72, 118, 119). In vitro studies illustrate the stimulatory effect of PI3-kinase signaling on the proliferation and kinase activity in cervical cancer cell lines (115, 120). Moreover, E7 can upregulate of PKB/AKT activity, which appears to be pRb dependent (72). The exact functional contribution of this signaling pathway to cervical cancer development needs to be further dissected.

Next to the PI3-kinase pathway also Notch signaling has been implicated in cervical carcinogenesis. The Notch signaling pathway is important in cell fate and determination (reviewed by Radkte (121)). Evidence exists in support of both a tumor suppressive as well as an oncogenic contribution of Notch to cervical carcinogenesis (122-127). Interestingly, AP-1 has been identified as one of the downstream targets of Notch signaling. Hence, further studies are warranted to clarify the involvement of this pathway in cervical cancer.

The AP-1 transcription factor complex

Activator protein 1 (AP-1) is a transcription factor complex consisting of members from Jun and Fos families that bind to the consensus sequence 5’-TGA(G/C)TCA-3’ termed TPA response element (TRE) (128). Jun family members (cJun, JunB and JunD) are able to form homodimers, or heterodimers with Fos family members (cFos, FosB, Fra1 and Fra2). The composition of AP-1 depends on the expression of family members, which differs between cell types, developmental states and environmental influences, such as growth factors. The various Jun members possess different DNA binding activities while Fos family members differ in their transactivating capabilities, leading to a broad range in AP-1 functioning.

The transcription factor AP-1 plays a major role in many crucial cellular processes like proliferation, differentiation, apoptosis and neoplastic transformation (reviewed by (129-131). The viral upstream regulatory region (URR) of HPV has two binding sites for AP-1, one in the enhancer and one in the promoter region (132-134). As AP-1 positively or negatively controls the expression of E6 and E7 of most of the hrHPV-types, it is an important factor for HPV-induced carcinogenesis. Upon anti-oxidant or TNFα treatment, AP-1
General Introduction

stoichiometry changes which results in inhibition of E6/E7 transcription (135, 136).
The AP-1 complex composition is suggested to change during HPV-induced carcinogenesis. *In vitro* experiments have shown that in HPV immortalized, non-tumorigenic keratinocytes the AP-1 complex primarily consists of cJun/Fra1 dimers while cFos is barely expressed. In cervical cancer cell lines on the other hand, the complex is predominantly composed of cJun/cFos dimers (136, 137). These findings correspond to data obtained from normal cervical epithelium and cervical cancer. Jun member expression is confined to lower layers in low-grade CIN, while expression is detected throughout all layers in high-grade CIN and invasive cancer (134). Expression of Fra1 is high in normal epithelium and in low grade lesions, while cFos expression increased in high-grade lesions and cancer at the cost of Fra1 (138).

In esophageal cancer the composition of the AP-1 complex was correlated to the presence of HPV. While in normal tissue Jun, Fra1 and Fos were all seen in complex, HPV-positive tumors primarily showed a complex of Jun/Fos while HPV-negative tumors still possessed Fra1 (139). The oncogenic function of cFos stems from its transactivating domain while Fra1 does not harbor this region. In contrast to their suggested tumor suppressive role in cervical cancer, Fra1 and Fra2 have oncogenic properties in breast and colon cancer cell lines (140, 141).

Outline of this thesis

In this thesis an *in vitro* model system was studied to gain more insight in the molecular events that may contribute to malignant transformation of HPV-immortalized cells. Next to the identification of genes targeted by DNA methylation during HPV-induced transformation and the functional consequences thereof, the potential signaling pathways involved were analysed. We have previously transfected primary human keratinocytes with full-length HPV 16 or 18 DNA, resulting in four immortal keratinocyte cell lines, i.e. FK16A and FK16B containing HPV16, and FK18A and FK18B containing HPV18 (103). With increasing passage these cell lines revealed increasing severity of dysplastic features in organotypic cultures that are reminiscent of the various stages of pre-malignant cervical lesions (69). Moreover, consecutive passages showed accumulation of chromosomal alterations overlapping with those found in cervical (pre)malignant lesions (107, 142) and shared altered
expression of certain genes with these (pre)cancer stages (103, 143, 144). Figure 3 shows the alignment of cervical carcinogenesis in vivo and the process of in vitro transformation of HPV16 and HPV18 transfected keratinocytes.

Figure 3. Schematic alignment of cervical carcinogenesis in vivo and HPV-mediated transformation in vitro. For a more elaborate explanation of cervical carcinogenesis in vivo we refer to Figure 1 of this chapter. The in vitro model of HPV-mediated transformation, as examined in this thesis, consists of primary keratinocytes transfected with full length hrHPV. Upon introduction of hrHPV primary keratinocytes obtained an extended lifespan following which they acquired an immortal phenotype characterized by telomerase activation. Further passaging of immortal cells resulted in the acquisition of an anchorage independent phenotype, characterized by colony formation in soft agarose. Tumorigenicity in nude mice is represented by cervical cancer cell lines as HPV transfected cell lines have not yet reached this stage. Shared host cell alterations that occur in both in vivo and in vitro situations, such as genetic and epigenetic events as well as telomerase activation are depicted between the longitudinal bars. A shift in AP-1 complex composition has so far only been demonstrated in vitro.

To identify putative tumor suppressor genes that are silenced by DNA methylation during HPV-induced transformation we analyzed promoter methylation of 29 tumor suppressor genes, which is described in Chapter 2. In this chapter, various passages of four HPV-transfected keratinocyte cell lines (FK16A, FK16B, FK18A, FK18B) representing the different stages of transformation in vitro were analyzed for DNA methylation using Methylation Specific Multiplex Ligation dependent Probe Amplification (MS-MLPA). This allowed the identification of a sequence of 8 methylation events during HPV-mediated transformation in vitro. Methylation of these genes also corresponded to DNA methylation events found in the cervical carcinomas analyzed.
General Introduction

One of the genes included in the MS-MLPA screen, i.e. CADM1, has previously been shown to be functionally involved in HPV mediated transformation in vitro (114). In Chapter 3 the extent of DNA methylation of CADM1 was further analyzed using Methylation-Specific PCR in HPV containing cell lines and cervical tissue specimens. Density of DNA methylation increased with acquisition of the anchorage independent phenotype in vitro as well as with the severity of cervical disease in vivo. Dense methylation (defined as more than 2 methylated regions) was associated with reduced CADM1 protein expression.

In Chapter 4 a newly identified candidate gene, which was previously found to be most significantly downregulated in cervical carcinomas compared to normal tissue (145), was investigated both functionally and in cervical squamous lesions. MAL was shown to be silenced by DNA methylation in HPV immortalized cell lines and up to 93% in cervical carcinomas. Overexpression of MAL in the cervical cancer cell line SiHa resulted in a reduction in proliferation, migration and anchorage independent growth further illustrating its tumor suppressive function. Methylation of MAL in cervical scrapes was shown to correlate to a significantly reduced mRNA expression and to reflect the presence of underlying high grade lesions.

Another potential candidate involved in HPV-mediated transformation, PIK3CA, is located on the most commonly gained chromosomal region in cervical cancers, the long arm of chromosome 3. In Chapter 5 the role of PIK3CA and PI3-kinase signaling in HPV-mediated transformation were analyzed. Expression of the catalytic subunit PIK3CA and subsequent phosphorylation of downstream PKB/AKT was increased with increasing stages of transformation. Pathway inhibition using either chemical inhibition or siRNAs against PIK3CA inhibited proliferation, migration and anchorage independent growth of HPV16 transfected keratinocytes. In organotypic raft cultures of HPV16- and HPV18-immortalized cells, phosphorylated PKB/AKT was primarily seen in differentiated cells staining positive for cytokeratin 10 (CK10). Upon PI3-kinase signaling inhibition, a severe impairment in epithelial tissue development as well as a dramatic reduction in p-PKB/AKT and CK10 was seen. Hence, PI3-kinase signaling also appears involved in differentiation of HPV-immortalized cells.
Notch signaling and downstream AP-1 involvement have been of interest in the HPV field for many years. However research has not yielded uniform results regarding the functional role of this pathway. In **Chapter 6** this pathway was further functionally dissected by using two levels of Notch expression which according to literature may have opposing effects. Introducing moderate Notch levels in the cancer cell line SiHa resulted in an increased proliferation and anchorage independent growth, while high levels of Notch in this cell line led to inhibition of anchorage independent growth without affecting proliferation rates. A likely important switch associated with phenotypical outcomes involved the AP-1 complex composition. SiHa cells possess an AP-1 complex composed of cJun/cFos dimers, which is reinforced by moderate Notch expression. In the case of high level Notch overexpression, however, a shift was observed towards cJun/Fra1 composition, which is characteristic of non-tumorigenic cells. Thus, Notch expression influenced AP-1 complex composition, which likely contributes to the phenotypical outcome seen after manipulation of this signaling pathway.

In **Chapter 7** a summary and discussion are presented based on all data presented in this thesis. We show that our results have further improved the knowledge on HPV mediated transformation *in vitro*.

**Reference List**


General Introduction


General Introduction


General Introduction


Chapter 1


General Introduction


Chapter 1


