

Chapter 8

Summary and Discussion

Summary

Although a transforming infection with hrHPV is necessary for the development of cervical precancerous lesions, additional alterations in the host cell genome are required for progression to invasive cancer. The number of host cell alterations found in cervical carcinomas is rapidly increasing, particularly resulting from recent developments in genome-wide high resolution profiling techniques and the discovery of new regulators, such as non-coding RNAs. However, it is still largely unknown which of these alterations contribute actively to HPV-induced carcinogenesis and can be classified as driver events. In order to gain better understanding of the molecular background of HPV-mediated transformation, the use of *in vitro* model systems has proven very useful. Examining 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 (1). Whereas the acquisition of an extended lifespan is related to viral functions itself, progression to the latter three stages requires the acquisition of additional (epi)genetic events in host cell genes.

In this thesis a number of alterations was investigated that may functionally contribute to HPV-induced transformation, with a focus on those related to the transition from immortal to anchorage independent growth. Therefore we studied both cell lines, including those of a longitudinal *in vitro* keratinocyte model system representing the consecutive stages of HPV 16- or HPV 18-induced transformation and cervical (pre)malignant lesions. This combined *in vitro* and *in vivo* analysis provides a broad picture on both the functional and clinical relevance of the host cell alterations studied and may aid the development of novel screening or treatment modalities.

The analysis of 29 (candidate) tumor suppressor genes for promoter methylation using methylation specific multiplex ligation-dependent probe amplification (MS-MLPA), as described in **Chapter 2**, revealed a cumulative sequence of methylation events during HPV-induced transformation *in vitro*. DNA methylation of TP73 and ESR1 became apparent in early immortal cells followed by RAR β and DAPK1 in late immortal cells. Methylation of MGMT was related to anchorage independent growth. Cervical cancer cell lines revealed additional frequent methylation of CADM1, CDH13 and CHFR. These 8 recurrent methylation events assigned to different stages of transformation *in*

vitro, were also detected in cervical carcinomas, both squamous cell carcinomas (SCC) and adenocarcinomas (AdCA). The promoter of MGMT was most frequently methylated in both histotypes (92%). Methylation of genes DAPK1 and CADM1 was significantly higher in SCC, while APC, CDKN2B, RASSF1A, TIMP3 and TP73 were more frequently methylated in AdCA. However, analyzing a different location within the promoter region of MGMT using Methylation Specific PCR (MSP) indicated a lower frequency of methylation (31%). The apparent discrepancy may in part be explained by heterogeneity of MGMT promoter methylation in cervical carcinomas. For other genes, such as DAPK1, ESR1, RAR β and CADM1 there was more concordance between MS-MLPA and MSP results, although complete concordance was never reached.

In **Chapter 3** three regions within the tumor suppressor gene CADM1 promoter were analyzed for DNA methylation in HPV-immortalized keratinocytes as well as cervical cancer cell lines and cervical tissue specimens. Dense methylation of the CADM1 promoter (i.e. methylation at two or more of the three regions analyzed) was found to contribute to gene silencing of CADM1 and was associated with anchorage independent growth in the cell lines. Methylation analysis of the three CADM1 promoter regions in cervical squamous lesions revealed that the frequency of methylation as well as the density of methylation increased with the severity of disease. Significantly more dense methylation was detected in CIN3 (30%) and SCC (83%) than in normal cervical epithelial samples and low grade CIN lesions (5%) ($p=0.005$). However, dense CADM1 promoter methylation was less frequent in AdCA (23%) Immunohistochemical analysis revealed that dense methylation was also significantly associated with decreased CADM1 protein expression. These data indicate that CADM1 silencing by DNA methylation has functional consequences and may have potential to stratify hrHPV positive women for risk of high-grade squamous premalignant lesions. Nevertheless, the fact that CADM1 promoter methylation was not increased in cervical AdCa warranted further studies to identify biomarkers with a broader coverage of cervical (pre)malignant disease.

By transcriptional profiling of cervical carcinomas MAL has been identified as the most significantly downregulated gene (2). In **Chapter 4** the mechanism underlying MAL silencing, its functional role in cervical carcinogenesis and the presence of DNA methylation in cell lines as well as cervical tissue specimens

and scrapings were examined. MAL messenger RNA expression was nearly undetectable in HPV immortalized cell lines as well as cervical cancer cell lines, but could be upregulated upon treatment with a demethylating agent. The anticipated epigenetic regulation of MAL expression was confirmed by methylation analysis of two promoter regions, showing methylation at both sites in all HPV-containing cell lines, but not in primary keratinocytes. Ectopic expression of MAL in the cervical cancer cell line SiHa suppressed oncogenic traits as proliferation, migration and anchorage independent growth, indicating the tumor suppressive character of MAL. In cervical tissue specimens analyzed, DNA methylation increased with the severity of the lesion, from 0% in normal tissue, to 9% in CIN1 lesions, 53% in CIN3 lesions, 90% in cervical SCC and 93% in AdCa. Also in cervical scrapings, detection of MAL DNA methylation appeared predictive for underlying high-grade lesions. In both tissue specimens and scrapings, MAL promoter methylation was significantly correlated to reduced mRNA expression. Hence, MAL promoter methylation and/or mRNA expression analysis on cervical scrapings may contribute to risk stratification of hrHPV positive women, not only for (precursors of) SCC, but also AdCa.

Several lines of evidence indicate the involvement of PI3-kinase signaling in HPV-mediated transformation (3-6). Chromosomal analysis has revealed that a gain of chromosome 3q is the most common event in the development of cervical SCC (5, 7, 8). Candidate oncogenes on chromosome 3q include the gene encoding p110 α , the active subunit phosphatidylinositol 3-kinase catalytic alpha (PIK3CA) of class I PI3-kinase. In **Chapter 5** we assessed the potential functional role of PIK3CA and the PI3-kinase pathway in HPV-induced transformation *in vitro*. Expression of the catalytic subunit PIK3CA as well as activation of downstream effector PKB/AKT increased with consecutive stages of transformation. Inhibition of PI3-kinase signaling in HPV16-transfected keratinocytes by chemical interference or siRNA-mediated silencing of PIK3CA resulted in a decreased phosphorylation of PKB/AKT. Moreover, blockage of PI3-kinase resulted in reduced cellular viability, migration, and anchorage independent growth. These properties were accompanied with a downregulation of HPV16E7 and hTERT mRNA expression. 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, there was a severe impairment in epithelial tissue development as well as a dramatic reduction in

p-PKB/AKT and CK10. Taken together, the present data suggest that the activation of the PI3-kinase/PKB/AKT pathway is of importance in HPV-induced carcinogenesis. Hence, PIK3CA and/or the PI3-kinase/PKB/AKT pathway may provide suitable targets for therapeutic intervention in patients with HPV-induced carcinomas.

Notch signaling is another pathway implicated in cervical cancer development. The actual role of altered Notch signaling in HPV-mediated transformation has been a long standing debate, as both tumor suppressive and oncogenic properties have been attributed to Notch signaling (9). In previous work, we demonstrated a fluctuation of Notch mRNA expression, i.e. increasing in early stages of transformation and decreasing in tumorigenic cells following a bell-shaped curve (10). This points to a strict regulation of Notch expression during HPV-induced transformation, with functional effects potentially being dose-dependent. Amongst the downstream effectors of Notch, the AP-1 transcription factor complex displayed a change as well at the transition from non-tumorigenic to a tumorigenic phenotype. Whereas the AP-1 complex mainly existed of cJun/Fra1 heterodimers in non-tumorigenic, immortalized cells, AP-1 in tumorigenic cervical cancer cells primarily consists of cJun/cFos dimers (10). In **Chapter 6** the dosage dependent functional effects of Notch in HPV-containing cells were examined, as well as the relation to AP-1. Two expression levels of Notch were created in the cervical cancer cell line SiHa, leading to two activation states of the signaling pathway. While moderate Notch activation contributed to increased transforming properties in terms of cell viability and anchorage independent growth, high level Notch activation exhibited tumor suppressive traits e.g. reduced colony formation. Moderate Notch expression led to an increased AP-1 transcriptional activity and DNA binding activity, but did not affect complex composition. High levels of Notch also increased AP-1 transcriptional activity, but additionally led to a change in AP-1 complex composition, in favour of cJun/Fra1 dimers, which is exemplary for non-tumorigenic HPV-immortalized cell lines. To find support for a mechanistical involvement of altered AP-1 complex composition in transformation, Fra1 expression was silenced in non-tumorigenic HPV16-immortalized keratinocytes (FK16A). Fra-1 silencing resulted in a (partial) shift in AP-1, from cJun/Fra-1 dimers to cJun/cFos dimers, which was accompanied with an increased colony formation. In conclusion, the functional role of Notch in HPV-mediated transformation is dosage dependent and correlated to a change in AP-1.

Discussion and future perspectives

Taken together, the results described in this thesis have enhanced our current understanding of HPV-induced transformation. A summary of the novel insights obtained is shown in Figure 1. A number of candidate tumor suppressor genes that during the course of HPV-induced transformation become silenced by promoter methylation has been defined. These include in order of onset TP73, ESR1, RAR β , DAPK1, MGMT, CADM1, CDH13 and CHFR. Some methylation events are apparently subtle, as is the case for MGMT, whereas others, like those of CADM1, are more extensive and therefore easily detectable by MSP assays. A further comprehensive analysis of two of the latter genes, i.e. CADM1 and MAL, showed that their silencing by DNA methylation can be functionally involved in cervical carcinogenesis. Moreover, data from this thesis indicate that DNA methylation of CADM1 and MAL may provide promising molecular markers that can aid in the triage of hrHPV-positive women at risk of \geq CIN3. Next to the methylation events two signaling pathways were found to be critical in later stages of HPV-induced transformation. PI3-kinase and Notch signaling became progressively activated during HPV-mediated transformation up to anchorage independent growth, while expression of Notch dropped in tumorigenic cervical cancer cells. Our recent unpublished findings indicate that also PI3-kinase activity is reduced in SiHa cervical cancer cells. Whereas PIK3CA appeared a critical regulator of PI3-kinase signaling in HPV-transformed cells, AP-1 was identified as a potentially important mediator in Notch signaling.

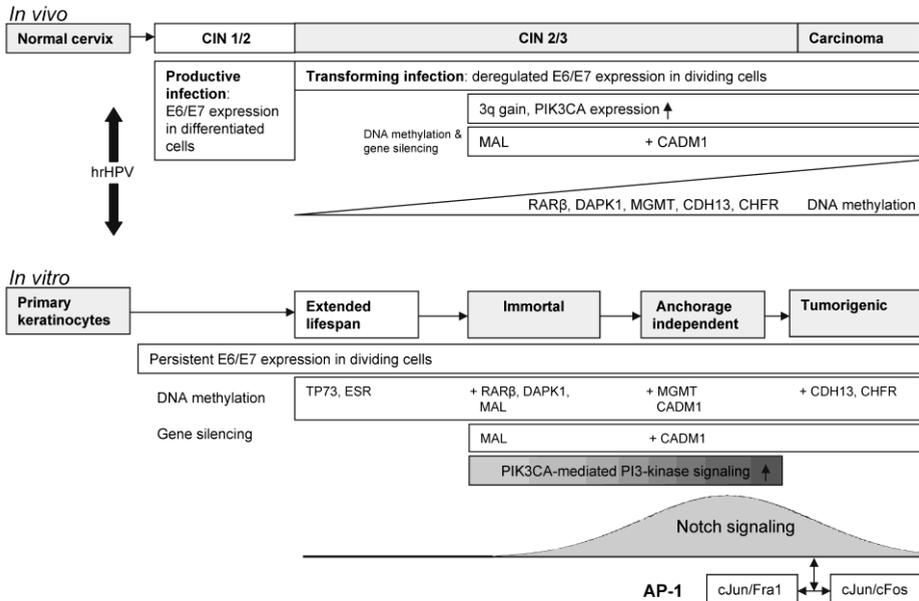


Figure 1. Schematic presentation of the novel insights obtained in this thesis related to both cervical carcinogenesis *in vivo* (upper part) and HPV-mediated transformation *in vitro* (lower part). A gain of chromosome 3q is found in high grade lesions and carcinomas, which results in an increased expression of PIK3CA. DNA methylation of the MAL and CADM1 promoter was increasingly detected in high grade lesions and carcinomas, as well as other genes identified by MS-MLPA and confirmed by others. In the *in vitro* model system, the accumulation of DNA methylation alterations occurs in the following order: TP73 and ESR in cells in their extended lifespan, complemented with RARβ, DAPK1 and MAL in immortal cells, MGMT and CADM1 in anchorage independent cells and CDH13 and CHFR in tumorigenic cells. MAL and CADM1 gene expression is silenced, which reduced tumor suppressive functioning of these two genes. Other functionally important events include activation of PI3-kinase-PKB/AKT signaling (in part) resulting from PIK3CA overexpression, and altered Notch signaling influencing AP-1 composition.

Following the results from this thesis there are some issues that require further investigation.

In Chapter 2 the identity and sequential order of 8 methylation events during HPV-mediated transformation were determined. To the best of our knowledge, of these 8 genes only CADM1 has yet been shown to be functionally involved in cervical carcinogenesis, see also Chapter 3 (11). Since our current data on CADM1 and also MAL indicate that genes with functional relevance in HPV-induced transformation (Chapter 3 and 4), provide promising molecular markers for triage purposes (see “Clinical implications” & (12), further functional analyses on the remaining 7 candidates identified in Chapter 2 are warranted.

Moreover, since MS-MLPA examines methylation of a single CpG dinucleotide within the promoter of the designated gene, more extensive methylation data are needed to determine the extent of promoter methylation. Recently, more extensive studies have revealed that three of the 8 genes we studied by MS-MLPA (i.e. DAPK1, CADM1 and RAR β) are consistently methylated in cervical cancer (13). Another issue is whether methylation of the promoter regions as assessed by MSP reflects altered gene expression. Although this was shown for CADM1 and MAL (see Chapter 3 and 4), no studies have addressed altered expression in relation to methylation for the other 7 candidates identified in Chapter 2.

The finding that PI3-kinase activation exceeded the increase in PIK3CA expression during transformation (Chapter 5) warrants further studies on potential additive mechanisms activating PI3-kinase activity during cervical carcinogenesis. It also remains to be examined whether the growth inhibitory effects seen upon interference with PI3-kinase signalling were a direct consequence of reduced viral oncogene expression or whether reduced viral expression is simply the result of growth inhibition. Towards this goal it may for instance be examined whether ectopic expression of E6 and/or E7 may overcome the growth inhibiting effects seen upon PI3-kinase interference.

The role of Notch signaling in cervical carcinogenesis has been controversial and present literature remains inconclusive. The observations presented in this thesis indicate that the dual outcomes in literature may at least in part be explained by varying Notch expression levels. However, the correlation between Notch expression and changes in AP-1 composition, as described in Chapter 6, is not completely resolved. High level Notch expression can drive a change in AP-1 complex composition from cJun/cFos to cJun/Fra1, which may, at least in part, explain the observed tumor suppressive effect. However, although moderate Notch resulted in increased cJun expression, its growth promoting effect could so far not be attributed to a change in the AP-1 complex involving Fra-1 and cFos. It would be of interest to find out whether changes in complex formation of other members of the Jun or Fos family could result from moderate Notch activity. Although the various signaling pathways linked to cancer are mostly analysed individually, these can naturally not be segregated from each other. Crosstalk between signaling pathways has been described for PI3-kinase and Notch signaling pathways (14-16). Jagged ligand interaction generated resistance to anoikis and induced phosphorylation of PKB/AKT in HaCaT cells (15). This cooperative action is thought to contribute to epithelial-

to-mesenchymal transition (EMT), which is supposed to be important for migration and invasion. Conversely, PI3-kinase signaling was also shown to contribute to Notch-induced HPV mediated-transformation by providing survival signals during transformation (17).

The *in vitro* model described in this thesis provides a means to further investigate the potential link between the different pathways as well as the regulation of HPV oncogene expression. The fact that both expression of p-PKB/AKT and Notch increase with transformation, though are reduced in the tumorigenic cell line SiHa, may imply a correlation between the two. It has previously been suggested that reduced PI3-kinase activity releases the inhibitory function on Notch signaling (18, 19). A potential effect of PI3-kinase activity on Notch signaling also suggests that PI3-kinase may affect AP-1 activity and/or complex composition, an interaction which has been shown in literature to occur via GSK3 β (20, 21).

Clinical implications

Molecular diagnostics

High risk-HPV testing is considered a more attractive and alternative primary screening tool compared to the current screening based on assessment of cytomorphological alterations in cervical scrapings (22). Although primary hrHPV testing has convincingly demonstrated to improve protection against CIN3 lesions and cervical cancer, a drawback of hrHPV testing is the lower specificity compared to cytology. As most hrHPV positive women will not develop clinically relevant lesions, risk stratification based on additional molecular markers is required to avoid over-diagnosis and -treatment. A recent study in our laboratory showed that the combined testing of hrHPV and CADM1 and MAL promoter methylation was equally discriminatory for \geq CIN3 as current cytology or cytology with HPV16/18 genotyping (12). In this light, it remains to be resolved whether the genes found to be methylated in Chapter 2 have any additional value over these two recently evaluated methylation markers. Methylation markers are especially appealing as they are also applicable to self-sampled cervico-vaginal material that is not suitable for cytology (Brink et al., (23) , Hesselink et al. in preparation)

Therapeutic options

To further investigate the possible future applications of for instance PI3-kinase pathway inhibitors in treatment of cervical cancer, this pathway needs to be dissected further both in *in vitro* studies and by analysis of clinical samples representative of the status of the cervix. As there exists a plethora of inhibitors, targeting specific branches of PI3-kinase signaling (e.g. mTOR), it should be determined which (downstream) elements in the pathway actively contribute to cervical carcinogenesis. Currently, isoform specific inhibitors of PI3-kinase are most promising, as these types of inhibitors show minimal side effects. However, due to the complexity of PI3-kinase signaling, including pathway cross talk and negative feed-back loops, the future application of these inhibitors is still unsure. Additionally, based on recent findings indicating involvement of PI3-kinase signaling in epigenetic silencing it may be speculated that a combination of specific PI3-kinase inhibitors and epigenetic drugs may provide a promising treatment option for cervical cancer patients. (24).

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