

5.1. Conclusions

Over the last twenty years, the discovery of a link between specific human papillomavirus types and the aetiology of certain human carcinomas has led to a rapid expansion in the study of human papillomaviruses. The advent of molecular cloning has provided an intimate insight into the mechanisms employed by the virus to deregulate cellular growth controls and promote viral replication. The HPV16 E6 and E7 proteins have been established as key viral oncoproteins whose multiple activities are necessary, but not sufficient, for the transformation of primary human keratinocytes (Halbert *et al.*, 1991). The HPV16 E7 protein has been shown to deregulate normal cellular growth controls at a number of levels. The most important and well characterised is the ability to disrupt the retinoblastoma tumour suppressor pathway through the binding and proteasomal degradation of hypophosphorylated pocket proteins (Dyson *et al.*, 1989; Jones & Munger, 1997). Recent work has elucidated a central role for previously unknown pRb-independent E7 activities. Specifically, the HPV16 E7 protein has been shown to bind to and functionally inactivate the cdki p21^{WAF1/CIP1} (Funk *et al.*, 1997; Jones *et al.*, 1997). The efficient inactivation of both pRb and p21^{WAF1/CIP1} is required to overcome G₁ arrest and uncouple cellular differentiation and proliferation in infected keratinocytes (Helt *et al.*, 2002) and pocket protein inactivation is not sufficient to induce cervical dysplasia in a transgenic mouse model (Balsitis *et al.*, 2006).

Despite growing interest, HPV research has primarily focused on the medically significant high risk HPV type 16 and the low risk HPV type 6. In contrast to the high risk HPV16 E7 protein, low risk and cutaneous HPV E7 types vary in their affinity for the pocket proteins (Ciccolini *et al.*, 1994) and do not induce their degradation (Alunni-Fabbroni *et al.*, 2000; Gonzelez *et al.*, 2001). An inability to inactivate the pocket

proteins was thought to explain why these HPV types do not efficiently override cellular growth controls functioning at the G₁/S checkpoint and very rarely dispose a cell to malignant progression (Ciccolini *et al.*, 1994; Halbert *et al.*, 1992). In spite of this, each HPV type must deregulate cellular growth controls to a limited extent to ensure viral replication within cells that would normally have exited the cell cycle (Ciccolini *et al.*, 1994). Only that recently, p130 and p107 protein have been found to be constituents of a transcriptionally repressive complex termed DREAM (or LINC). In this complex p130 associated with E2F4 to bind to the promoters of genes that regulate entry into S phase (Litovchick *et al.*, 2007; Pilkinton *et al.*, 2007; Schmit *et al.*, 2007; Knight *et al.*, 2007). The complex was then dissociate from p130 and form an alternative complex with B-myb, a transcription factor, to regulate genes for S/G₂ and mitosis (Litovchick *et al.*, 2007; Pilkinton *et al.*, 2007; Schmit *et al.*, 2007; Knight *et al.*, 2007).

In this study, we investigated whether expression of HPV16 E6/E7 in cervical cancer cells results in disruption of the p130/DREAM and p107/DREAM complexes and conversely favours the formation of B-myb/DREAM. In addition, we have studied whether depletion of E6/E7 by RNA interference leads to reformation of p130/DREAM and whether this is required for the G₁ arrest precipitated by E6/E7 depletion. Our results show that disruption of the p130/DREAM complex by HPV16 E6/E7 in CaSki cervical cancer cells is critical in order to promote the cell cycle from G₁ to S phase.

This study addressed whether repressive DREAM complexes are a significant target for HPV16 oncoproteins in cervical cancer cells. Our work showed that the repressive p130/DREAM was virtually abolished in CaSki cells, which express high levels of 16E6/E7, and was also much depleted in SiHa cells, which express lower levels of 16E6/E7. Depleting E6/E7 expression by RNA interference caused cell cycle arrest in both CaSki and SiHa cells, as noted in some previous studies (Tang *et al.*,

2006), and it was evident that cell cycle arrest in CaSki cells depended on reformation of a repressive p130/DREAM complex. It can therefore be inferred that the continued proliferation of these HPV16 transformed cells is dependent upon disruption of p130/DREAM. Although both E6 and E7 are depleted by targeting the bicistronic mRNA with the shRNAs used, it is likely that the major effect on DREAM complexes, and thus the cell cycle, results from E7 depletion. HPV16 E7 is well known to bind to all three members of the Rb pocket protein family, preventing formation of transcriptionally repressive complexes by physically precluding interaction with E2F4/5 and additionally through degradation of pocket proteins. It cannot be excluded, however, that re-expression of p53 following E6 depletion could also impact on DREAM complexes. Under these conditions, p21Cip1 could inhibit cyclinE/A-cdk2 complexes, which would preclude both activating phosphorylation of B-myb (Saville *et al.*, 1998) and inactivating phosphorylation of p130 (Cheng *et al.*, 2000).

It is very well established that the association of different HPV types with cervical cancer correlates closely with the propensity of E7 to target inactivation and degradation of cellular Rb. More recently, it has been recognised that E7 proteins from both high- and low-risk HPV types share an ability to target p130 for degradation (Zhang *et al.*, 2006; Genovese *et al.*, 2008). Degradation of p130 by E7 appears to be important for driving quiescent cells into S phase and also decreases the expression of differentiated epithelial cell markers (Zhang *et al.*, 2006; Genovese *et al.*, 2008). It can therefore be argued that p130 rather than Rb is the more significant cellular target of E7 during the normal replication cycle to establish conditions in suprabasal cells favouring HPV genome replication. This argument has been propounded in previous studies (Zhang *et al.*, 2006; Genovese *et al.*, 2008), however, it is notable in one of these publications that p130 levels in submerged cultures of CaSki and SiHa cells were found

to be rather similar to the control primary human keratinocytes, suggesting that E7 had little impact on p130 stability in these cervical cancer cells (Genovese *et al.*, 2008).

We also detected p130 by western blotting in SiHa and CaSki cells in our study, albeit at lower levels than the control T98G and C33A cells. Importantly, our work showed a profound p130/DREAM deficit in the cervical cancer cells compared to the controls (Fig. 2). This was particularly evident with CaSki cells, and suggests the p130 that was detected in this system is not part of the DREAM complex. The residual p130 protein may be unable to bind to E7, presumably reflecting the complex phosphorylation events that regulate its activity (reviewed in Cobrinik, 2005), and is thus protected against degradation in CaSki cells. It is notable that a transcriptionally inactive p130-E2F4-cyclinE/A-cdk2 complex was observed in HeLa cells (Popov *et al.*, 2005), however, this complex was unable to revert to a repressive p130-E2F4 complex in this HPV18-transformed cervical cancer cell. It is therefore plausible that E7 specifically targets p130 in the DREAM complex, thereby removing the barrier to entry into S phase.

HPV16 E6 and E7 proteins are known to interact with and regulate the activity of multiple cellular proteins implicated in cell proliferation, apoptosis and senescence (reviewed in Yugawa *et al.*, 2009). Other studies have found that targeting E6/E7 expression by RNA interference induces apoptosis and/or senescence (Jiang & Milner, 2002; Hall & Alexander, 2003; Sima *et al.*, 2008; Johung *et al.*, 2007; Yamato *et al.*, 2008), in addition to effects on the cell cycle. We observed no obvious apoptosis in CaSki or SiHa cells during the course of our experiments, and there is no implication from our study that DREAM complexes regulate cell survival. Recent studies do suggest, however, that p130/DREAM is important for establishment of senescence, at least in the context of the RAS oncogene (Tschop *et al.*, 2011). It will be interesting to determine whether the induction of senescence mediated by E7 repression, which has

been reported to be initiated by a transcriptional cascade driven by the Rb family (Johung *et al.*, 2007), also depends upon reformation of p130/DREAM.

Developments in HPV research have continued to uncover a plethora of mechanisms exploited by the viruses to overcome cellular growth controls and ensure productive infection. It is this accumulation of small differences in viral protein function that has led to the diversity of HPV types that can be seen today. As yet, the complex process of cervical carcinogenesis is incompletely understood. It would seem that the functional amalgamation of different E7-encoded activities within a dynamic cellular background paves the way for the gradual process of cervical carcinogenesis. The results obtained in this study stress the importance of studying a variety of HPV types for a more complete understanding of this process. It is feasible that the E7 proteins may not only induce quiescent cells to divide by releasing pocket protein complexes from the promoters of genes required for G₁/S transition, but can also promote the transcription of genes required for G₂/M progression. Further investigation could identify another layer of viral function, while shedding more light onto a novel area of transcriptional control.