Functional interactions between the human papillomavirus type 16 E7 oncoprotein and the tumor suppressor p53

Vom Fachbereich Chemie
der Universität Hannover
zur Erlangung des Grades
Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation
von

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geboren am 17. Februar 1972 in Prüm

21. Juni 2002

Die vorliegende Dissertation wurde an der Universität Hannover, Fachbereich Chemie, Hannover, Deutschland unter der Leitung von Prof. Dr. Dieter Paul und an der Harvard Medical School, Department of Pathology, Boston, MA, USA unter der Leitung von Prof. Dr. Karl Münger angefertigt.

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Tag der Promotion: 21. Juni 2002

Zusammenfassung

Die Expression von „high-risk“ HPV-16 E7 in einer Zelle ohne HPV-16 E6 führt zur Degradation von pRB, aber auch zur Stabilisierung von p53. Dieser Anstieg wird nicht durch verstärkte Transkription, sondern durch eine verlängerte Halbwertszeit des p53 Proteins bewirkt (Jones and Munger, 1997). Die Ergebnisse dieser Arbeit verdeutlichen, dass das stabilisierte p53 in HPV-16 E7-expremierenden Zellen weder in der Präsenz, noch beim Fehlen von Wachstumsfaktoren als Transkriptionsfaktor aktiv ist, obwohl p53 eine Rolle in der HPV-16 E7 vermittelten Apoptose durch Wachstumsfaktorentzug zu spielen scheint. Außerdem kann das stabilisierte p53 in HPV-16 E7 expremierenden Zellen mdm2 binden, befindet sich in einer Wild-Typ Konformation und ist im normalen Zellkompartment lokalisiert. Weder der nukleäre Export von p53, noch seine Degradation durch das Proteasom wird von HPV-16 E7 verhindert. Es bleibt zu untersuchen, ob HPV-16 E7 das Zusammensetzen des p53 Transkriptions-Initiations-Komplexes oder die Ubiquitinierung von p53 inhibiert und p53 dadurch stabilisiert und inaktiviert

Vorhergehende Studien zeigten, dass Zellen, die Oncogene, wie z.B. HPV-16 E7 expremieren, unter suboptimalen Wachstumsbedingungen prädestiniert Apoptose zu begehen. Untersuchungen der molekularen Mechanismen dieser HPV-16 E7 abhängigen Apoptose durch Wachstumsfaktorentzug zeigten die Involvierung des Mitochondriums anhand von Messungen der Cytochrom C Freisetzung und der Caspase 3 Aktivierung,. Der Apoptose-Induzierende Faktor (AIF) verlässt das Mitochondrium nicht und scheint deshalb nicht in die HPV-16 E7 vermittelte Apoptose verwickelt zu sein. Es wurde eine verstärkte Expression von IGFBP-2 und IGFBP-5 in HPV-16 E7-expremierenden proliferierenden Zellen beobachtet. Die Expression dieser beiden Proteine steigt nach Wachstumsfaktorentzug weiter an. Es wurde zuvor berichtet, dass diese Proteine den Wachstumsfaktor IGF-I binden können und seine Verfügbarkeit für die Zelle regulieren. Bislang ist unklar, ob die Expression dieser beiden Proteine HPV-16 E7-expremierende Zellen für Apoptose prädestiniert oder ob dies eine antiapoptotische Antwort der Zelle auf den Wachstumsfaktorentzug darstellt

Die Ergebnisse dieser Arbeit tragen zum besseren Verständnis bei, wie Wirtszellen auf die, durch virale Oncoproteine induzierte Deregulation des Zellzykluses und die nachfolgende, unkontrollierte Proliferation reagieren. Detailliertes Wissen über die Effekte von HPV-16 E7 sowohl auf proliferierende als auch auf unter Wachstumsfaktorentzug kultivierten Zellen könnte zur Entwicklung neuartiger Behandlungsmethode von HPV-positiven Krebsarten führen, indem man die Zellen anfällig für HPV-16 E7 vermittelte Apoptose macht.

Schlagworte: Tumor Suppressor p53, Human Papillomavirus Typ 16 E7 Oncoprotein, Apoptose

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1. Repeatedly used abbreviations

AIF	apoptosis inducing factor
CKI	cyclin-dependent kinase inhibitor
E6AP	E6 associating protein
HFK	human foreskin keratinocyte
HPV	human papillomavirus
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IκB	inhibitor of NFκB
NFκB	nuclear factor kappa B
ORF	open reading frame
p53DD	carboxyl terminal truncation mutant of p53
pRB	retinoblastoma tumor suppressor protein
TAF	TBP-associated factor
TBP	TATA-binding protein
TF	transcription factor
TGF-β	transforming growth factor beta
TNFR	TNF receptor
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand

2. Introduction

2.1 Papillomaviruses

Papillomaviruses are widespread and have been isolated from different vertebrate species including cattle, birds, deer, mice and humans. Although papillomaviruses isolated from these various species are quite similar, they show a high degree of species specificity, which means that a papillomavirus isolated from one species does not cause a productive infection in a second species. In addition, papillomaviruses exhibit a specific tropism for squamous epithelial cells. Papillomavirus infections cause lesions at either cutaneous or mucosal sites of the genital tract, oral pharynx or esophagus. The productive infection of cells by papillomaviruses can be divided into early and late phases, which are linked to the differentiation state of the infected epithelial cell (Fig. 1). Because the basal cell is

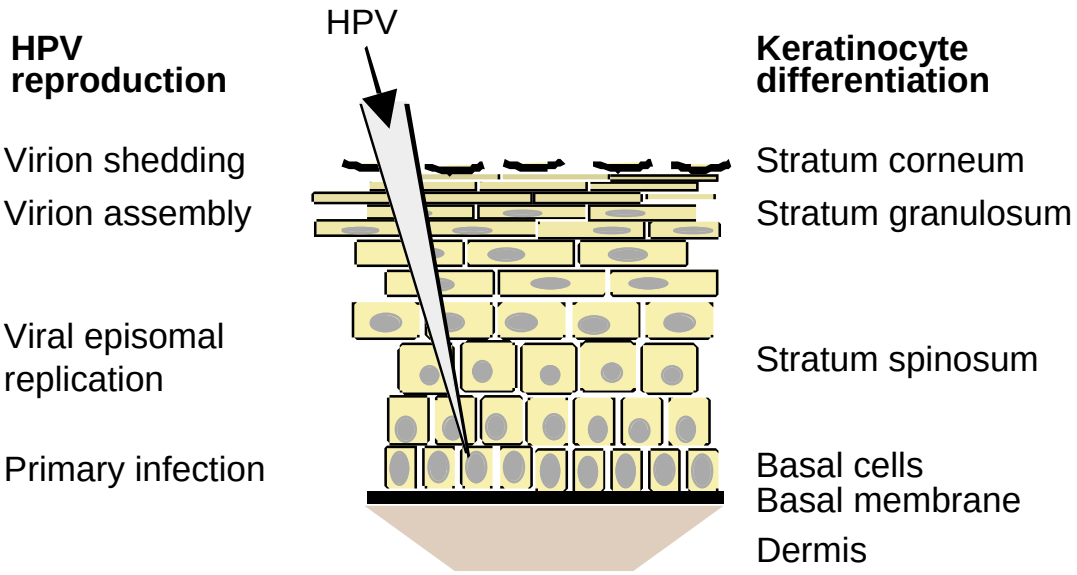

Figure 1: The differentiation state of the host cell and the viral life cycle are tightly linked. To induce a persistent infection, HPV infects the basal cell. Viral DNA replication, late gene expression and virion assembly occurs in the differentiating cells.

the only cell in the squamous epithelium that is capable of dividing, the virus must infect the basal cell to induce a lesion that can persist. Late gene expression, synthesis of capsid

proteins, viral DNA synthesis, and assembly of virions occur only in terminally differentiating squamous epithelial cells (Fig. 1). A persistent papillomavirus infection usually results in epithelial hyperplasias, also referred to as warts.

Papillomaviruses are non-enveloped viruses with double-stranded circular DNA genomes of about 8000 base pairs. The approximately 10 designated open reading frames (ORFs) of the virus genome are all encoded on one DNA strand. These ORFs are classified into early or late ORFs. The numbers of the early (E1-8) and late (L1 and L2) ORFs reflect their sizes (Fig. 2). The terms “late” and “early” ORFs reflect the time of their expression during the viral life cycle. The virus genome also contains a non-coding long control region (LCR), which contains the origin of replication and the constitutive transcriptional enhancer sequence as well as the viral E2 responsive enhancer (Fig. 2).

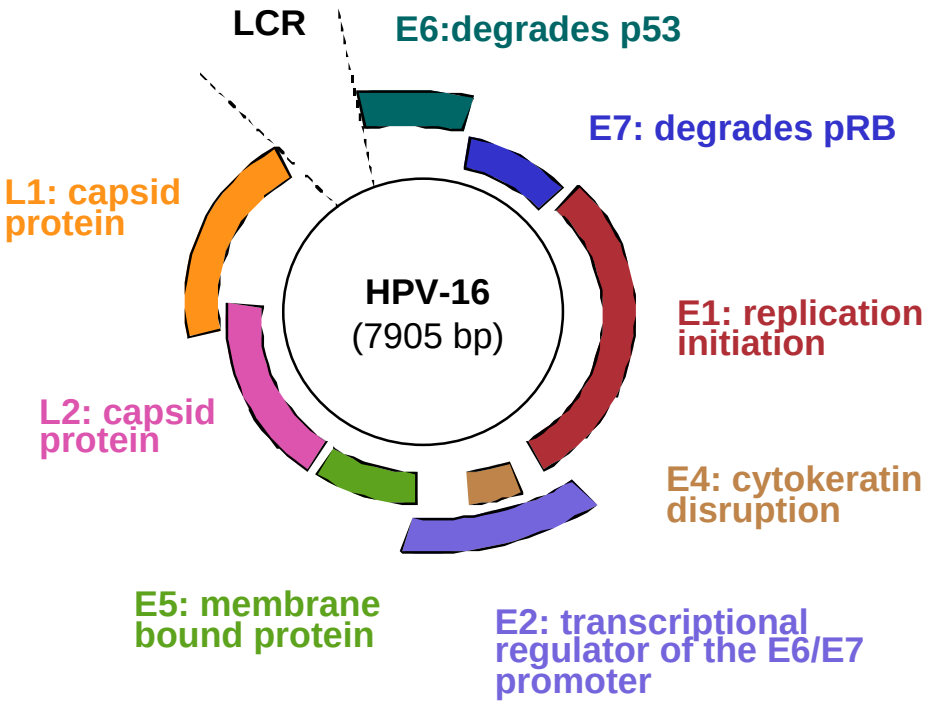

Figure 2: HPV genome structure. The HPV-16 genome consists of a double stranded DNA genome of 7905 basepairs and encodes early and late genes. The early genes (E) encode proteins that are necessary for viral replication during the early viral life cycle, while late genes (L) encode viral capsid proteins that are expressed later in viral life cycle. The long control region (LCR) is a non-coding DNA sequence that controls viral replication and transcription.

2.1.1 Human Papillomaviruses

The continually growing list of human papillomaviruses (HPVs) now comprises more than 100 distinct types. Like all papillomaviruses, HPVs possess a pronounced tropism for squamous epithelial cells. Despite their similar overall genomic make-up, different HPVs infect epithelia at specific anatomic locations. Approximately 30 HPV types preferentially infect the anogenital tract mucosa. These HPVs can also be detected in the oral mucosa, and are further classified into 'high-risk' and 'low-risk' types based on the clinical prognosis of the lesions that they cause. Low-risk HPVs are associated with benign warts, whereas high-risk HPVs cause lesions that have a propensity for carcinogenic progression. Even though the rate of carcinogenic progression is relatively low, infections with high-risk HPVs account for almost all human cervical cancers, a leading cause of cancer in younger women worldwide (reviewed in zur Hausen, 1996)). Moreover, approximately 20% of oral cancers, particularly oropharyngeal carcinomas in patients that lack risk factors such as alcohol and tobacco abuse, are also associated with high-risk HPVs (Gillison *et al.*, 2000).

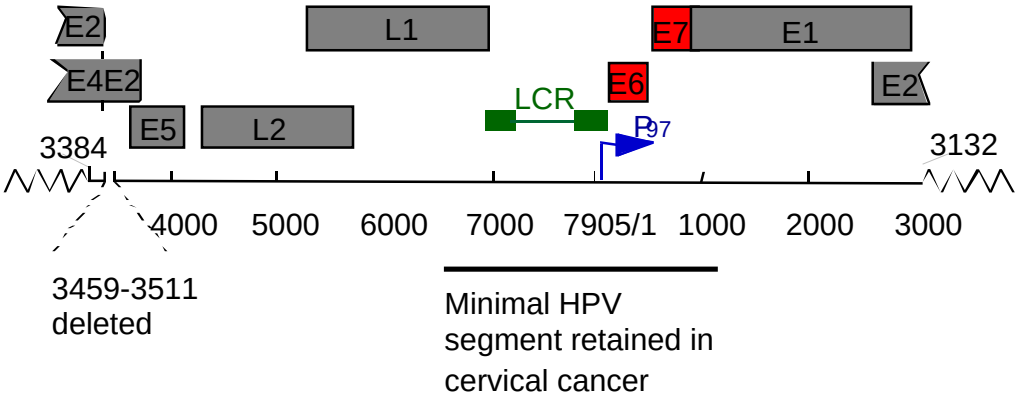

Figure 3: HPV genome integration. Integration of the HPV genome into a host chromosome often results in the disruption of the E2 gene. E2 is a negative regulator of E6 and E7 expression, and the loss of E2 results in the constitutive expression of the two viral oncoproteins. Constitutive E6/E7 expression contributes to the carcinogenic progression of a cell. E6 and E7 are transcribed from a single promoter (P_{97}). The minimal HPV segment retained in cervical cancer, consisting of the LCR and the E6 and E7 genes, is indicated by a black bar.

During carcinogenic progression of a high-risk HPV infected cell, the viral genome frequently integrates into the host chromosome. This event is quite random with respect to the host chromosome but follows a specific pattern with respect to the viral genome. As a consequence of integration, expression of two early genes (E6 and E7) becomes dysregulated due to the frequent disruption of the gene that encodes the viral transcription factor E2 (Fig. 3), a negative regulator of the E6 and E7 promoter. The E6 and E7 proteins of the high-risk HPVs have oncogenic activities. E7 functionally inactivates the retinoblastoma tumor suppressor protein (pRB) and the related "pocket proteins" p107 and p130 by destabilization (Boyer *et al.*, 1996; Dyson, 1998; Gonzalez *et al.*, 2001; Jones and Münger, 1997), while E6 protein binds to (Werness *et al.*, 1990) and promotes the degradation of the tumor suppressor p53 (Scheffner *et al.*, 1990). Expression of high-risk HPV E6 and E7 results in the extension of the life span and immortalization of normal human genital epithelial cells, the normal host cell type of these viruses (Hawley-Nelson *et al.*, 1989; Hudson *et al.*, 1990; Münger *et al.*, 1989). Moreover, continuous expression of E6 and E7 in cervical cancer cell lines is required for the maintenance of the transformed state (Goodwin and DiMaio, 2000; Wells *et al.*, 2000).

2.1.2 The HPV E7 oncogene

HPV E7 was the first oncogene of high-risk HPVs to be discovered. HPV E7 induces transformation of rodent fibroblast cell lines (Bedell *et al.*, 1989; Phelps *et al.*, 1988; Vousden *et al.*, 1988; Yasumoto *et al.*, 1986) and can cooperate with the ras oncogene to transform primary rodent fibroblasts (Matlashewski *et al.*, 1987; Phelps *et al.*, 1988). HPV E7 proteins are phosphoproteins (Smotkin and Wettstein, 1987) composed of approximately 100 amino acid residues that contain two casein kinase II consensus phosphorylation sites (Barbosa *et al.*, 1990) as well as another site for an unknown protein kinase (Massimi and Banks, 2000). They contain a LxCxE motif at the

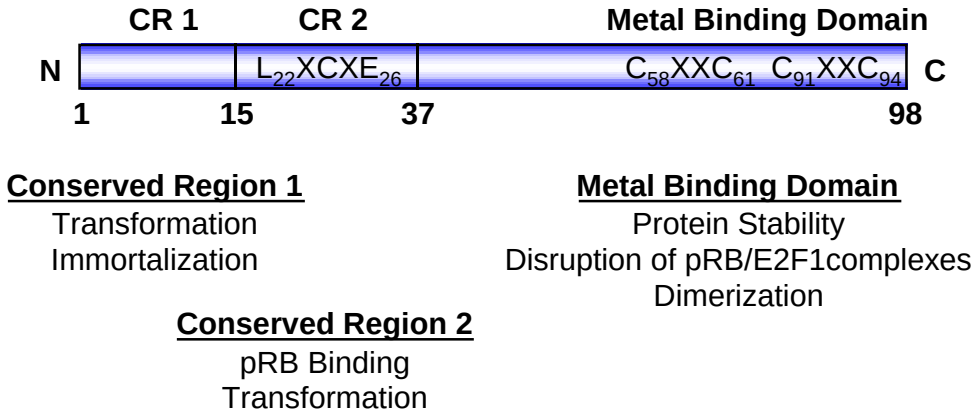

Figure 4: Schematic structure of the HPV-16 E7 oncoprotein. The two conserved regions (CR1 and CR2) at the amino-terminus are involved in transformation. CR2 also contains a LxCxE motif, which represents the core binding site for pRB and the related pocket proteins p130 and p107. A metal binding domain at the carboxyl-terminus consists of two copies of a Cys-X-X-Cys sequence motif can act as a dimerization or multimerization domain and influences protein stability and disruption of pRB/E2F1complexes.

amino terminus, which represents the core binding site for pRB and the related pocket proteins p130 and p107 (Fig. 4). A zinc-binding domain consisting of two copies of a Cys-X-X-Cys sequence motif separated by 29 amino acid residues in the carboxyl terminus (Barbosa *et al.*, 1989; McIntyre *et al.*, 1993) can act as a dimerization/multimerization domain (Clemens *et al.*, 1995; Clements *et al.*, 2000; McIntyre *et al.*, 1993) (Fig. 4). Immunofluorescence studies have shown that HPV E7 proteins are predominantly nuclear (Greenfield *et al.*, 1991; Sato *et al.*, 1989). A number of non-nuclear cellular target proteins of E7 have been described in recent years (reviewed in Zwerschke and Jansen-Durr, 2000) and hence it is possible that at least under some conditions, a certain fraction of E7 may reside in the cytoplasm (Smotkin and Wettstein, 1987). E7 has also been detected at nucleolar structures in some cell types (Zatsepina *et al.*, 1997). It has a relatively short half-life, which results in low cellular steady-state levels (Smotkin and Wettstein, 1987). HPV-16 E7 is a target of ubiquitin-dependent proteolysis through a rather uncommon mechanism that involves conjugation of ubiquitin mojeties to the amino terminal methionine residue (Reinstein *et al.*, 2000).

HPV E7 can uncouple differentiation and proliferation and hence, retain differentiating keratinocytes in a DNA replication competent state (Cheng *et al.*, 1995; Woodworth *et al.*, 1992). Several studies in mouse and human keratinocytes have shown that like in other cell types (Guo *et al.*, 1995; Steinman *et al.*, 1994), $p21^{CIP1}$ plays an important role in coupling cell cycle arrest and differentiation in keratinocytes (Alani *et al.*, 1998; Di Cunto *et al.*, 1998; Missero *et al.*, 1996). The steady state levels of $p21^{CIP1}$ increase during cellular differentiation resulting in inactivation of cdk2 activity and growth arrest. The inhibitory activities of cyclin dependent kinase inhibitors (CKIs) such as $p21^{CIP1}$ and $p27^{KIP1}$ can be abrogated by HPV E7 (Funk *et al.*, 1997; Jones *et al.*, 1997a; Zerfass-Thome *et al.*, 1996). The retinoblastoma tumor suppressor and the related 'pocket proteins' p107 and p130 are major cellular targets of high-risk HPV E7 proteins. High-risk HPV E7 proteins induce the proteolytic elimination of the pRB family members (Boyer *et al.*, 1996; Gonzalez *et al.*, 2001). This ability to inhibit CKIs and to destabilize the pocket proteins and the subsequent activation of E2F results in enhanced expression of cellular proteins that are rate-limiting for cellular DNA synthesis and contribute to the function of E7 as a viral replication competence factor. Consistent with this notion, it was shown that expression of E7 and its ability to subvert cellular targets such as pRB are indispensable for HPV replication and the full viral life cycle (Flores *et al.*, 2000; Thomas *et al.*, 1999).

High-risk HPV E7 interferes with additional regulatory pathways of a normal cell to guarantee the continued proliferation of the infected cell. It can abrogate the cytostatic activities of cytokines such as the transforming growth factor beta (TGF-β) (Pietenpol *et al.*, 1990) and tumor necrosis factor alpha (TNF-α) (Basile *et al.*, 2001), and severely compromise cellular signaling by interferon (IFN) (Barnard and McMillan, 1999; Barnard *et al.*, 2000; Park *et al.*, 2000; Perea *et al.*, 2000). It has also been reported that HPV E7 interacts with the 110 kDa TBP-associated factor (TAF110) which may contribute to the ability of E7 to modulate transcription (Mazzarelli *et al.*, 1995). In addition, E7 was reported to interact with the insulin-like growth factor binding protein-3 (IGFBP-3), a protein that is involved in negatively regulating the cellular availability of the important cellular survival factors, insulin-like growth factors (IGFs) (Mannhardt *et al.*, 2000). IGFBP-3 is a proapoptotic protein (Rajah *et al.*, 1997) that is regulated by p53

(Buckbinder *et al.*, 1995) and thereby involved in p53 dependent apoptosis (Hollowood *et al.*, 2000).

Furthermore, cells expressing high-risk HPV E7 have an altered cellular metabolism including decreased glycolytic flux (Mazurek *et al.*, 2001; Zwerschke *et al.*, 1999) and lowered intracellular glycogen stores (Zwerschke *et al.*, 2000). These effects may drive cellular hyperproliferation or represent a consequence of the altered energy requirements of rapidly growing transformed cells. An additional physiological hallmark of cells transformed by HPV E7 is an increase of the intracellular pH by stimulation of Na^+/H^+ exchanger activity. Intracellular alkalinization is thought to represent an early and important step that contributes to cellular transformation (Reshkin *et al.*, 2000).

HPV E7 plays a role in carcinogenic progression by inducing genomic instability (Kessis *et al.*, 1996; Thomas and Laimins, 1998; White *et al.*, 1994). HPV E7 expressing cells were found to be polyploid or aneuploid (Duensing *et al.*, 2000; Hashida and Yasumoto, 1991) (Southern *et al.*, 2001). Aneuploidy is generated by the gain or loss of chromosomes as a consequence of non-symmetrical chromosome segregation during cell division and is frequently observed in tumor cells. Recent studies have shown that HPV-16 E7 can interfere with the ordered synthesis of centrosomes (Duensing *et al.*, 2001; Duensing *et al.*, 2000), the major microtubule organizing centers during mitosis (reviewed in Murray, 2001).

2.2 The effects of viral oncogenes on the tumor suppressors p53 and pRB

2.2.1 The tumor suppressor p53

The p53 gene encodes a protein composed of 393 amino acids that functions as a transcription factor. It contains five highly conserved regions, termed domains I-V that are crucial for p53 functions (Soussi *et al.*, 1990; Soussi *et al.*, 1987; Soussi and May, 1996) and represent "hot spot" areas for mutations. Structure function analyses have identified four major functional domains: An amino terminal transcriptional activation domain (amino acids 1-42), a central sequence-specific DNA-binding domain (amino acids 102-292), an oligomerization domain (amino acids 323-356) and a regulatory

domain (amino acids 360-393) in the carboxyl terminal portion (reviewed in Levine, 1997) (Fig. 5). The amino terminal transcriptional activation domain of p53 is acidic and allows p53 to recruit the basal transcriptional machinery, including the TATA-box binding protein (TBP) and the TBP-associated factors (TAFs) 40 and 60, which are components of TFIIH (Lu and Levine, 1995; Thut *et al.*, 1995). The central DNA binding region of p53 contains the four conserved domains II-V, where 80-90 % of the mutations associated with human tumors are clustered. This part of the protein recognizes and binds a consensus DNA sequence which consists of two copies of the 10 base-pair motif 5'-PuPuPu-C(A/T)(T/A)G-PyPyPy-3' separated by 0-13 nucleotides (el-Deiry *et al.*, 1992). The C-terminal region contains a tetramerization domain and a basic region. The tetramerization of p53 (Kraiss *et al.*, 1988) via its oligomerization domain (Clore *et al.*, 1994) (Jeffrey *et al.*, 1995) is required for efficient transactivation *in vivo* and for p53-mediated growth suppression (Pietenpol *et al.*, 1994). The basic region acts as a negative regulator of sequence specific DNA binding (Wang *et al.*, 1996; Wang and Prives, 1995) (Fig. 5).

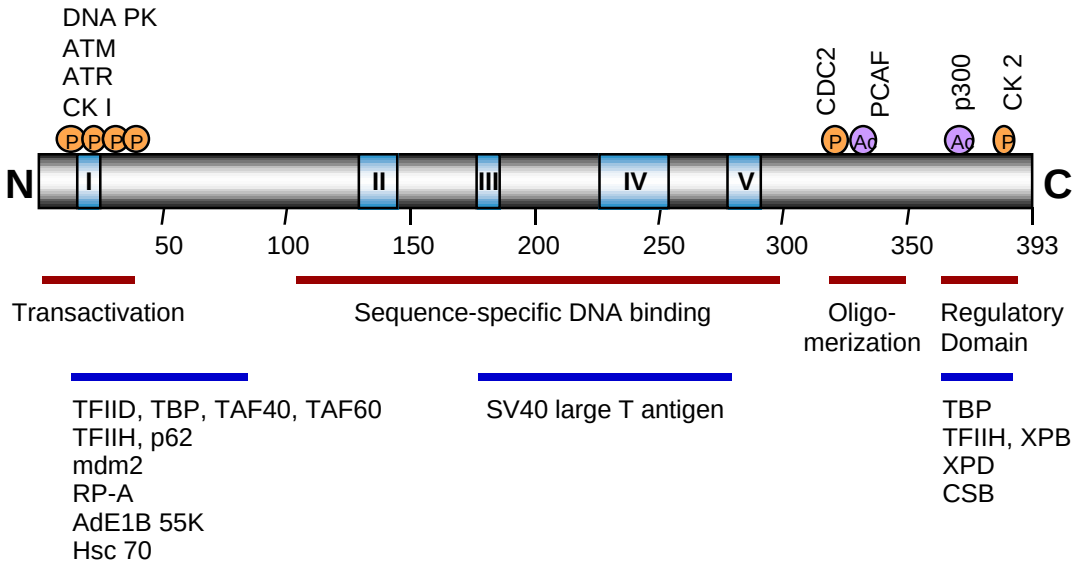

Figure 5: Schematic p53 structure. Roman numerals represent the five regions of p53 that are conserved within p53 from all vertebrates and are mutational "hot spots". Known phosphorylation (P) and acetylation (Ac) sites are indicated as well as their known modifiers. Shown below and indicated by red horizontal bars are the 4 functional domains while blue bars indicate various domains important for biological activities and p53-protein complex formation. See text for details.

In normal cells, p53 is present at extremely low levels because the protein is rapidly degraded following synthesis (Kubbutat and Vousden, 1998). Degradation of p53 is mediated by a large multiprotease complex, the 26S proteasome and is dependent on ubiquitination. The E3 ubiquitin ligase marking p53 for degradation is mdm2 (Honda *et al.*, 1997), a transcriptional target of p53 (Barak *et al.*, 1993). The mdm2 protein interacts with the amino terminal transcriptional activation domain of p53 and inhibits its transcriptional function (Oliner *et al.*, 1993). The importance of mdm2 as a regulator of p53 became apparent when mdm2-deficient mice showed very early embryonic lethality and the simultaneous deletion of p53 completely rescued this phenotype (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

The p53 protein plays an important role in the suppression of tumorigenesis. The p53 gene is deleted or contains point mutations in approximately 60% of all human cancers (Baker *et al.*, 1989; Baker *et al.*, 1990; Caron de Fromentel and Soussi, 1992; Hollstein *et al.*, 1994; Hollstein *et al.*, 1996; Marshall, 1991; Nigro *et al.*, 1989). Wild type p53 is believed to guard the integrity of the cellular genome (Lane, 1992) by inhibiting cell cycle progression or inducing apoptosis in response to aberrant proliferation signals, DNA damage or cellular stress. It acts as a key regulator of a complex circuitry involving mdm2, $p14^{ARF}$ and other cellular proteins (Freedman and Levine, 1999; Sherr, 1998), that link the pRB pathway to the p53 pathway. Upon the hyperphosphorylation or the loss of pRB, E2F is released. $p14^{ARF}$ is a transcriptional target of E2F and the upregulation of $p14^{ARF}$ levels results in the inhibition of mdm2 (Stott *et al.*, 1998) and subsequent stabilization and activation of p53 (Chin *et al.*, 1998), which induces either growth arrest or apoptosis in response to deregulation of pRB function (reviewed in May and May, 1999).

Rapid degradation of p53 in normal cells is critical to tightly regulate p53 activity. Cellular stress, such as DNA damage, heat shock, hypoxia, oncogene activation, metabolic changes and pH alterations can all result in the stabilization and activation of p53. This is due to posttranslational modifications such as phosphorylation and acetylation in the amino terminal and carboxyl terminal regions of p53. The protein kinases ATM and ATR become activated upon DNA damage and phosphorylate p53 at serine 15 and serine 37 in its amino terminus (Canman *et al.*, 1998; Chehab *et al.*, 1999;

Tibbetts *et al.*, 1999), which disrupts the interaction of mdm2 with p53. This causes p53 stabilization and enhances its DNA binding activity. The stabilized p53 becomes activated as a transcription factor by acetylation of the lysine residues 320 and 382 at its carboxyl terminus (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998). This leads to the induction of target genes that mediate either growth arrest or apoptosis (reviewed in Meek, 1999).

2.2.2 The retinoblastoma tumor suppressor (pRB)

The retinoblastoma tumor suppressor protein (pRB) is a nuclear phosphoprotein that contains two conserved domains A and B, also called the A/B pocket. This pocket domain is involved in protein-protein interactions. The transforming proteins of different DNA tumor viruses, namely Ad E1A, SV40 TAg and HPV-16 E7 all bind this domain and thereby inactivate pRB. This domain is also involved in E2F binding. pRB as well as the other members of the "pocket protein" family, p107 and p130, act as important regulatory subunits of E2F transcription factor complexes. E2F transcription factors are heterodimers consisting of an E2F subunit bound to a member of the "dimerization partner" family (DP) (Bandara *et al.*, 1993; Helin *et al.*, 1993). E2F/DP complexes are critical regulators of many genes that are rate limiting for entry into S phase. Binding of hypophosphorylated pocket proteins to the E2F/DP complexes masks their transcriptional activation domain and since encodes a transcriptional repressor domain, pRB binding converts E2F/DP complexes into transcriptional repressors that actively prevent S phase entry (Luo *et al.*, 1998; Weintraub *et al.*, 1995; Weintraub *et al.*, 1992). Upon cdk-dependent phosphorylation of pRB in late G1-phase, these complexes dissociate and E2F complexes are converted into transcriptional activators that stimulate S phase entry. The G1/S phase transition is tightly regulated by pRB through this mechanism.

2.2.3 Viral oncoproteins and the tumor suppressor p53 and pRB

Several DNA tumor viruses such as Adenoviruses and SV40 encode oncoproteins that target both p53 and pRB. The binding of viral oncoproteins to the cellular tumor suppressor proteins p53 and pRB is fundamental to the induction of S-phase in host cells by the small DNA tumor viruses to ensure viral replication (Nevins, 2001). The induction

of S-phase in differentiating cells is crucial to viral replication since epithelial cells withdraw from the cell cycle during differentiation and stop expressing enzymes that are necessary for DNA replication. Viruses had to evolve mechanisms to guarantee expression of S-phase genes of the host cell to ensure the availability of these rate-limiting proteins for their own replication. In a cell infected with high-risk HPV, the E7 oncoprotein inactivates pRB and the related proteins p107 and p130 by inducing their destabilization by ubiquitin mediated degradation (reviewed in Munger *et al.*, 2001) which causes the release of active E2F transcription factors. The free transcription factor E2F induces the transcription of a set of S-phase genes like DNA polymerase-α, dihydrofolate reductase (DHFR), thymidine kinase (TK), that are rate limiting for DNA synthesis. Activation of E2F allows the virus to establish a replication competent cellular milieu in differentiated keratinocytes that are normally terminally withdrawn from the cell cycle.

High levels of E2F can also result in the inhibition of mdm2 through $p14^{ARF}$. The $p14^{ARF}$ protein binds to mdm2 and inhibits the transfer of ubiquitin to p53. This results in subsequent stabilization and activation of p53. To prevent p53 from inducing growth arrest or apoptosis of an HPV infected cell, the HPV E6 protein binds to and promotes the degradation of the tumor suppressor p53 (Scheffner *et al.*, 1990; Werness *et al.*, 1990). E6 functions together with the cellular protein E6AP as a ubiquitin ligase and marks p53 for ubiquitin mediated degradation through the 26S proteasome (Rolfe *et al.*, 1995). The inactivation of p53 allows cells inappropriately stimulated by free E2F to circumvent these p53 checkpoints, enter S-phase and replicate viral DNA (Fig. 6). This inactivation of the two tumor suppressors pRB and p53 in a high-risk HPV-infected cell results in the uncontrolled proliferation of the cells and may contribute to subsequent development of malignant tumors.

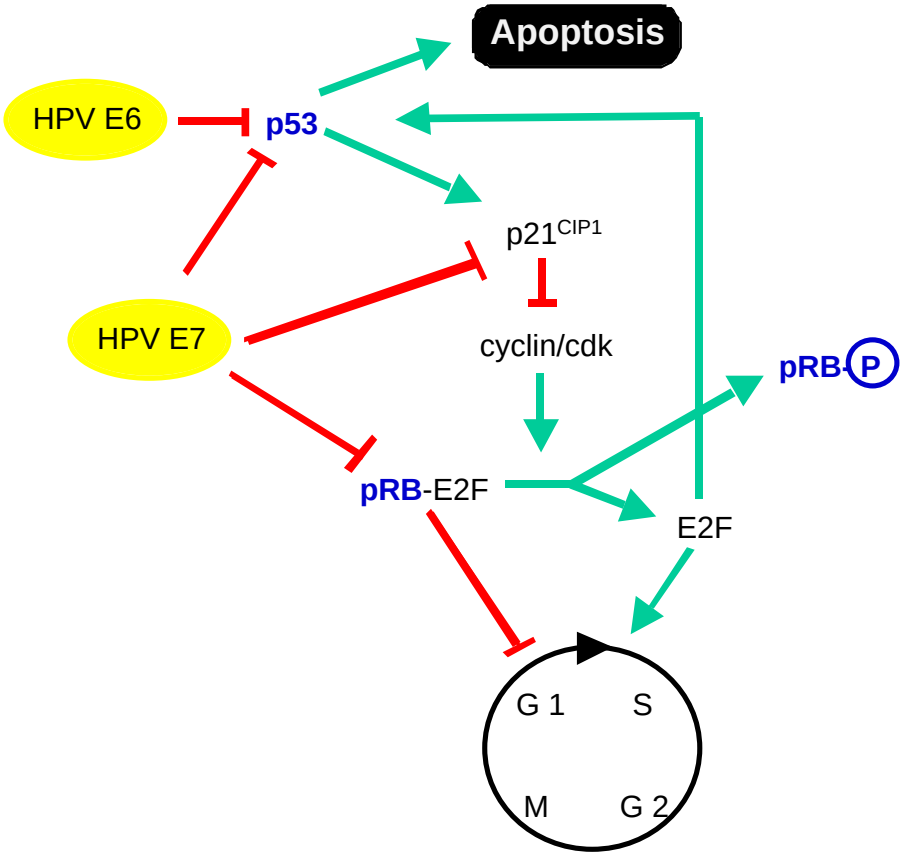

Figure 6: Effects of HPV E6 and HPV E7 on the cell cycle. Positive (green arrows) and negative (red bars) effects are shown. The degradation of p53 by HPV E6 and the destabilization of pRB and inactivation of $p21^{CIP1}$ by HPV E7 result in uncontrolled transition from G1- to S-phase and progression through the cell cycle. The negative impact of HPV E7 on p53 function is the subject of this thesis.

2.2.4 The HPV E7 oncogene and the tumor suppressor p53

HPV E7 can interfere with the ability of p53 to induce G1 growth arrest in response to cellular insults (Demers *et al.*, 1994a; Hickman *et al.*, 1994; Slebos *et al.*, 1994; Vousden *et al.*, 1993). Multiple mechanisms likely contribute to this activity of E7, including E2F-mediated aberrant expression of cyclins E and A (Hickman *et al.*, 1997) and cdc25A (Katich *et al.*, 2001), inactivation of the p53-responsive CKI $p21^{CIP1}$ (Funk *et al.*, 1997; Jones *et al.*, 1997a) and the decreased cellular steady-state levels of pRB (Jones and Münger, 1997). It was shown previously that HPV E7 expressing cells contain increased steady-state levels of p53 (Demers *et al.*, 1994b) due to an extended half-life of

p53, suggesting that E7 may perturb p53 degradation (Jones and Münger, 1997). It was reported that the p53-specific ubiquitin ligase mdm2 is not as efficiently bound to p53 in E7 expressing cells as in normal cells (Seavey *et al.*, 1999). Given the ability of E7 to activate E2F-responsive promoters (Phelps *et al.*, 1991), it has been postulated that the increased p53 levels in E7 expressing cells may be due to E2F-mediated transcriptional induction of $p14^{ARF}$ (Bates *et al.*, 1998), an inhibitor of mdm2-dependent p53 degradation (Stott *et al.*, 1998; Zhang *et al.*, 1998). Interestingly, however, expression of E7 does not cause a rapid increase of $p14^{ARF}$, and stabilization of p53 by E7 is also observed in mouse embryo fibroblasts that lack $p19^{ARF}$, the murine homolog of the human $p14^{ARF}$ (Seavey *et al.*, 1999). These results suggest that alternative mechanisms contribute to the ability of E7 to stabilize p53. Nevertheless, it has been demonstrated that the normal physiological pathway of mdm2-mediated p53 turnover is not active in high-risk HPV E6/E7 expressing cervical carcinoma cell lines and the rapid turnover of p53 in these cell lines is solely a function of the E6/E6AP p53-specific ubiquitin ligase complex (Hengstermann *et al.*, 2001)

The steady-state levels of the CKI $p21^{CIP1}$, a transcriptional target of p53, are increased in E7 expressing cells just as p53 levels are (Demers *et al.*, 1994a; Thomas and Laimins, 1998). This increase, however, is mostly a consequence of protein stabilization and is not due to transcriptional induction by p53 (Jones *et al.*, 1999; Noya *et al.*, 2001). In fact, it appears that despite its increased levels, the transcriptional activity of p53 is disturbed in E7 expressing cells. Consistent with this finding, it was shown that E7 could interfere with the activation of p53-responsive reporter constructs in transient transfection assays (Massimi and Banks, 1997). This has been linked to the ability of E7 to interact with TBP (Massimi and Banks, 1997; Phillips and Vousden, 1997).

Previous studies indicate that in E7 expressing cells, p53 is activated upon DNA damage to a comparable extent as in control cells (Jones *et al.*, 1999). This indicates that the p53 in E7 expressing cells retains the ability to be functionally activated upon specific stimuli.

2.3 Viral oncoproteins predispose cells to apoptosis upon cellular stress

2.3.1 Programmed cell death or apoptosis

Apoptosis, or programmed cell death, is a process required for development and maintenance of metazoan organisms. In normal cells apoptotic cell death is tightly linked to cell proliferation and leads to the regulated turnover of cells, which is crucial to the preservation and function of organisms. Defects in cell death pathways, or uncoupling of cell death and proliferation, can lead to developmental abnormalities and importantly contribute to the pathogenesis of degenerative and neoplastic diseases (reviewed in Zornig *et al.*, 2001). Apoptosis also eliminates aberrant cells created by cellular stress such as DNA damage, hypoxia or viral infection. Cells undergoing apoptosis show highly characteristic morphological changes including chromatin condensation, DNA fragmentation, membrane blebbing, cell shrinkage, and compartmentalization of the dead cells into membrane-enclosed vesicles or apoptotic bodies (Kerr *et al.*, 1972; Oberhammer *et al.*, 1993; Wyllie, 1980). Survival factors prevent normal cells from undergoing apoptosis unless they receive specific genotoxic signals like DNA damage or viral infection, which can lead to the initiation of death pathways.

Apoptosis is mediated by the sequential activation of caspases, a family of cysteine proteases with specificity for aspartate residues in their target substrates. Caspases are constitutively produced in most cells as inactive monomeric proenzymes and are activated by specific proteolytic cleavage and assembly into tetramers (Green and Kroemer, 1998). Caspases have the capacity to activate each other and co-operate in an intracellular cascade leading to the activation of downstream ‘effector’ caspases, such as caspase-3, -6 and –7. Engagement of effector caspases is a key event that commits the cell to the cleavage of substrates which results in the typical morphological changes of apoptosis and the degradation of chromosomal DNA (Enari *et al.*, 1998). At least two pathways converge on the activation of caspase-3. These pathways were previously thought to be independent, but are now known to interact in a cell-specific manner (Li *et al.*, 1998). The first pathway is initiated by internal cellular stress such as DNA damage and is mostly mediated by mitochondria, which play a central role in regulating cell death

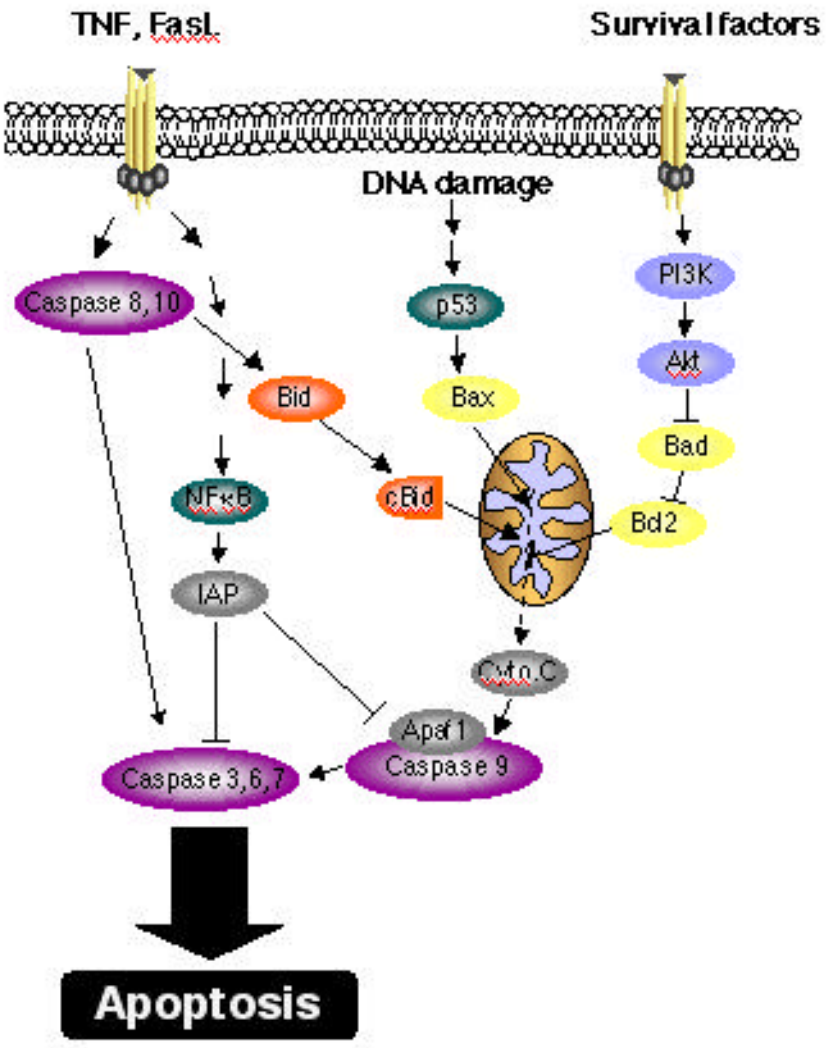

Figure 7: Overview of apoptotic signaling pathways. Pro-apoptotic stimuli include the activation of cell death receptors by their ligands (FasL, TNF) and DNA damage. The binding of Fas or TNF to their receptor activates the initiator caspases 8 and 10, while DNA damage leads to the activation of p53 and the subsequent activation of the initiator caspase 9. Once activated, these caspases cleave and activate downstream effector caspases (including caspase 3, 6 and 7) which in turn induce apoptosis. Cytochrome c released from mitochondria is coupled to the activation of caspase 9, a key initiator caspase. Survival factors including growth factors that activate Akt thereby inhibit Bad and prevent cytochrome c release.

and is mostly mediated by mitochondria, which play a central role in regulating cell death (Green and Reed, 1998; Kroemer and Reed, 2000). In response to apoptotic stimuli cytochrome c is released from the mitochondria, forms a complex with Apaf-1 and procaspase 9 and this complex, the apoptosome, converts procaspase 9 into its active form (Li *et al.*, 1997; Zou *et al.*, 1999). Activated caspase 9 cleaves downstream caspases

including caspase 3. The second pathway is induced by the activation of the initiator caspases, caspase-8 and –10 upon ligation of cytotoxic cytokines such as TNF-α, Fas ligand or TRAIL to death domain receptors at the cell surface. Activated caspase-8 then acts directly on caspase-3 to initiate apoptosis (Kroemer and Reed, 2000) (Fig. 7). There is some crosstalk between these two pathways. One molecule that connects the two pathways is Bid. Bid is a cytoplasmic protein that is cleaved by activated caspase 8 or 10. The cleaved Bid translocates to the mitochondria where it induces cytochrome c release and triggers an apoptotic response (Gross *et al.*, 1999).

The tumor suppressor p53 can induce apoptosis in response to cellular stress such as DNA damage. Under such conditions p53 is stabilized and activated as a transcription factor. This results in the transcriptional induction of apoptosis mediating target genes including bax or IGFBP-3 (reviewed in Meek, 1999). Bax is a proapoptotic member of the Bcl-2 family of mitochondrial apoptosis regulators. It can counteract the death suppressive activity of the antiapoptotic family members Bcl-2 or Bcl-x_L (reviewed in Korsmeyer, 1999). IGFBP-3 is regulated by p53 as well (Buckbinder *et al.*, 1995) and was shown to play a role in p53 dependent apoptosis (Rajah *et al.*, 1997). IGFBP-3 was reported to suppress proliferation and induce apoptosis (reviewed in Oh *et al.*, 1995) by negatively regulating the availability of the growth factor IGF-I to its receptor (Binoux, 1996). It was also reported to be involved in senescence (Goldstein *et al.*, 1991; Grigoriev *et al.*, 1995). Other p53 target genes like PERP, Noxa and p53AIP1 are involved in p53 mediated apoptosis as well (Attardi *et al.*, 2000; Oda *et al.*, 2000a; Oda *et al.*, 2000b). Various other genes induced during p53 dependent apoptosis were identified by serial analysis of gene expression (SAGE). These p53-inducible genes (PIGs) were predicted to encode proteins involved in the cellular response to oxidative stress (Polyak *et al.*, 1997).

p53 induces apoptosis not only through transcription-dependent but also through transcription-independent mechanisms. The transcription-independent mechanisms of p53-mediated apoptosis are not very well studied. However it was reported to be dependent on caspase 8 activity (Ding *et al.*, 2000) as well as on protein phosphatase 1 and 2A activity (Yan *et al.*, 1997). Another report showed that p53 can induce relocalization of the cytokine receptor Fas from the Golgi to the cell surface, thereby

rendering a cell more prone to apoptosis (Bennett *et al.*, 1998). p53 may also contribute to apoptosis by directly signaling to the mitochondria when a small fraction of stress-induced p53 protein traffics to the mitochondria where it induces the release of cytochrome c and subsequent activation of caspase 3 (Marchenko *et al.*, 2000).

2.3.2 Viruses and apoptosis

In addition to its role in tissue homeostasis and development, apoptosis is an essential component of the cellular response to injury (Thompson, 1995). In particular, many cells will undergo programmed cell death following viral infection. Many viruses take advantage of host cell-mediated apoptosis while finishing the infectious cycle. Death of the host cell by apoptosis offers several advantages for the virus. During apoptosis the entire cellular content, including progeny virus, is packaged into membrane-bound apoptotic bodies that are rapidly taken up by surrounding cells. This limits the inflammatory response and allows the infection to spread undetected by the host organism. Virus particles enclosed within apoptotic vesicles are also protected from inactivation by host antibodies and proteases.

Viruses have also developed a range of strategies to prevent elimination of the infected host cell by the host immune response. These strategies include the inhibition of the interferon response as well as the abrogation of death receptor (TNFR and Fas) signaling by either blocking TNF and Fas signaling, mimicking the death receptors, interacting with signaling factors or mimicking death signaling factors (vFLIPS). Some viruses have also evolved mechanisms to inhibit cellular caspases due to the expression of viral inhibitors of apoptosis proteins (vIAP) or they express viral homologues of the antiapoptotic protein Bcl-2. In addition, some viruses can inhibit the transcriptional activity of p53 (reviewed in Roulston *et al.*, 1999).

2.3.3 Apoptosis as an anti-oncogenic defense mechanism

Cancer cells, products of deregulated proliferation, have to develop mechanisms to evade programmed cell death as well, or else be eliminated. It was shown previously that the predisposition of cells to apoptosis by oncogenes and transformation are linked. The regions of the HPV-16 E7 oncoprotein required for transformation (Barbosa *et al.*,

1990; Edmonds and Vousden, 1989; Phelps *et al.*, 1992) overlap with those required for sensitizing E7 expressing cells to apoptosis upon suboptimal growth conditions (Jones *et al.*, 1997b). In contrast to some rodent cell lines, primary human cells are not transformed by expression of a E7 alone. Normal human cells expressing the HPV-16 E7 oncogene are predisposed to apoptosis (Jones *et al.*, 1997b), and similar observations were made with cells expressing the c-myc oncogene (Evan *et al.*, 1992).

Cells expressing the c-myc oncoprotein have been used extensively to investigate the relationship between apoptosis and proliferation in cells depending on the quality of their growth conditions. It was shown that in addition to its well-documented growth-promoting activity, myc is a powerful inducer of apoptosis, especially under conditions of stress, genotoxic damage or depletion of survival factors (Askew *et al.*, 1991; Evan *et al.*, 1992). Myc induced apoptosis upon growth factor deprivation was shown to be independent of p53 by using a carboxyl terminal fragment of p53 that acts as a dominant negative mutant (Juin *et al.*, 1999). This indicates that p53 which is involved in most apoptotic responses upon DNA damage, also called the “damage sentinel”, is not involved in the “trophic sentinel” response of c-myc expressing cells upon deprivation of growth factors. The apoptotic potential of c-myc appears to counteract its oncogenic capacity (Evan and Littlewood, 1993; Evan *et al.*, 1992; Harrington *et al.*, 1994b). In addition, there is a clear parallel between the activation of cell cycle progression by the adenoviral oncoprotein E1A and the early products of other small DNA tumor viruses and the sensitization of cells to apoptosis by activated oncogenes as well. In the absence of well-coordinated growth signals, these viral oncogene-expressing cells are primed to undergo apoptosis unless this response is blocked by cellular survival factors such as Bcl-2 or by viral factors that inactivate p53 such as Ad E1B or HPV E6. A similar duality has been described for many known growth-promoting proteins, including E2F-1 (Almasan *et al.*, 1995; Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994).

2.3.4 The HPV E7 oncoprotein and apoptosis

Expression of HPV-16 E7 targeted to lens or retinal photoreceptor cells induces apoptosis in differentiating cells of transgenic mice (Howes *et al.*, 1994; Pan and Griep, 1994). Coexpression of HPV-16 E6, which targets p53 for degradation abrogates

apoptosis, resulting in tumorigenic progression (Pan and Griep, 1994). In this model, cells expressing an E7 mutant that is unable to bind pRB do not undergo apoptosis. By employing this model in mice with a p53 null background it became clear that the observed HPV-16 E7 mediated apoptosis in the lens is mediated through p53-dependent and p53-independent pathways (Pan and Griep, 1995). The fact that a pRB binding deficient E7 mutant does not induce apoptosis, supported the model that E2F-1, which is regulated by pRB may be involved in E7 mediated apoptosis. The investigation of the lens phenotype in mice that express E7 in an E2F-null background indicated that the ability of HPV-16 E7 to induce apoptosis is indeed partially dependent on E2F-1 (McCaffrey *et al.*, 1999).

There is conflicting evidence on whether HPV-16 E7 can predispose cells to apoptosis upon different stimuli in different cell systems. The p53 tumor suppressor functions as a “damage sentinel” in a DNA damage-induced cellular apoptosis response. HPV-16 E7 infected human foreskin keratinocytes (HFKs) are not more prone to p53-dependent apoptosis upon DNA damage induced by cisplatin, etoposide or mitomycin C (Liu *et al.*, 2000), whereas these cells are sensitized to apoptosis upon treatment with TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) or sulfur mustard (Basile *et al.*, 2001; Stoppler *et al.*, 1998). In contrast, expression of HPV-16 E7 inhibits the induction of TNF-α mediated apoptosis in normal human diploid fibroblasts (IMR90) (Thompson *et al.*, 2001). The expression of HPV-16 E7 in human uroepithelial cells predisposed these cells to apoptosis in response to γ-irradiation (Puthenveettil *et al.*, 1996).

The “trophic sentinel” is engaged in response to growth factor withdrawal and is dependent on cellular survival factors such as the Akt kinase. Its dependence on p53 is less well established. However, it has been shown that HPV-16 E7 can predispose human diploid lung fibroblasts to undergo apoptosis in response to growth factor deprivation (Jones *et al.*, 1997b). The finding that pRB remains hyperphosphorylated in HPV-16 E7 expressing cells upon growth factor withdrawal indicated that the ability of HPV-16 E7 to predispose cells to apoptosis is linked to its growth promoting activity, which triggers the apoptosis pathway in the absence of growth factors (Jones *et al.*, 1997b). Since HPV-16 E7 mediated apoptosis upon growth factor deprivation can be abrogated by the

coexpression of HPV-16 E6, it has been suggested that this cell death may be dependent on p53 (Jones *et al.*, 1997b).

2.4 Aims of the research

HPV-16 E7 expressing cells were reported to have higher steady state levels of p53, which is due to a prolonged half-life of the protein (Jones and Münger, 1997; Jones *et al.*, 1997b). The fact that HPV-16 E7 expressing cells proliferate rapidly in the presence of high p53 levels indicates that the stabilized p53 might not be fully active as a transcription factor. HPV-16 E7 expressing cells are predisposed to apoptosis upon suboptimal growth conditions (Jones *et al.*, 1997b). The finding that HPV-16 E7 mediated apoptosis is inhibited by the coexpression of HPV-16 E6, suggested that HPV-16 E7 triggered apoptosis in primary human cells may be at least in part p53 dependent.

Hence we studied the biochemical mechanisms and molecular consequences of HPV-16 E7 induced p53 stabilization. Moreover we determined whether the apoptotic response of HPV-16 E7 expressing cells to growth factor deprivation was p53 dependent and mediated by increased expression of known p53 responsive proapoptotic genes.

2.4.1 Aim 1: To investigate the biochemical mechanisms and biological consequences of p53 stabilization by the HPV-16 E7 oncoprotein in proliferating cells

As one part of the thesis the mechanisms of p53 stabilization and the consequences for the proliferating HPV-16 E7 expressing cells were studied. It was investigated whether p53 stabilization is due to an inability of mdm2 to bind to and degrade p53 in the presence of HPV-16 E7. Other mechanisms that might be involved in p53 stabilization by HPV-16 E7 including the possible interference of HPV-16 E7 with the nuclear export of p53 or the folding of p53 into a degradation resistant mutant conformation were investigated as well. Discovering the mechanism of E7 mediated stabilization may yield new insights into how viral oncoproteins inactivate cellular regulators to escape growth arrest or apoptosis.

2.4.2 Aim 2: To determine the molecular components of the "tropic sentinel" response of HPV-16 E7 expressing cells upon growth factor deprivation

To determine the mechanisms involved in HPV-16 E7 mediated apoptosis upon growth factor withdrawal, the transcriptional involvement of p53 in the "trophic sentinel" response in E7 expressing cells was investigated. To identify other mediators of HPV-16 E7 mediated apoptosis, gene expression profiling was performed and two genes were observed to be upregulated more than 2 fold in HPV-16 E7 expressing cells and their levels increased even further in these cells upon deprivation of growth factors. The two genes are insulin-like growth factor binding proteins (IGFBPs) 2 and 5, which are involved in regulating the availability and binding capacity of the survival factor Insulin-like growth factor I (IGF-I) to its receptor and thereby have a major impact on the pro- or anti-proliferative signals the cell receives. The possible roles of these two genes in HPV-16 E7 mediated apoptosis upon growth factor deprivation was investigated. Different apoptosis pathways were described previously and the possible involvement of these pathways in HPV-16 E7 mediated apoptosis like the involvement of the caspase cascade, the mitochondria and the apoptosis-inducing factor (AIF) was investigated as well.

A better understanding of the mechanisms of HPV-16 E7 mediated stabilization and inactivation of p53 as well as HPV-16 E7 mediated apoptosis upon growth factor withdrawal may lead to the possible development of novel treatments of HPV-associated cervical cancer. One possible treatment could eliminate HPV-positive cervical cancer cells by inactivating the HPV-16 E6 oncoprotein and subsequently induce HPV-16 E7 mediated apoptosis by suboptimal growth conditions.

3. Materials and Methods

3.1 Cell culture methods

3.1.1 Cell culture and cell lines

Low passage normal human diploid lung fibroblasts (IMR90), the human keratinocyte cell line HaCaT, the HPV negative cervical cancer cell line C33A, the breast cancer cell lines SK-Br-3 and MCF-7, the osteosarcoma cell line U2OS and the african green monkey kidney cells COS-7 were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin.

IMR90 cells immortalized by telomerase (hTERT) (pBABE vector with puromycin resistance) expressing IMR90 cells that co-express p53DD (CTF), HPV-16 E6, HPV-16 E7, HPV-16 E6 and HPV-16 E7 or the empty LXSN vector (LXSN vector with G418 resistance) were obtained from Sheila Steward (Weinberg laboratory, Whitehead Institute, MIT, Boston). These cells were infected with either empty pBABE or pBABE-E7 expressing retroviruses (pBABE vector with hygromycin resistance) to obtain the following stable hTERT expressing IMR90 cell lines: hT LX, hT E7, hT E6, hT p53DD, hT E6E7, and hT p53DDE7.

3.1.2 Retroviral infections

Recombinant retroviruses LXSN and LXSN-HPV-16 E7 were obtained from D. Galloway and used for retroviral transduction of IMR90 cells. Recombinant retroviruses pBABE and pBABE-HPV-16 E7 were obtained by transiently transfecting the retroviral packaging line 293T cells (Yang *et al.*, 1998) using the Calcium phosphate method. In brief, 293T cells were transfected with 6 µg of pMD.MLV gagpol (helper plasmid), 2 µg of pMD.G (VSVG pseudotype) and 8 µg of plasmid of interest. After 16 hours of incubation cells were washed twice, new medium was added and the virus containing supernatant was collected 48-72 hours post-transfection.

For the retroviral infection, the cells were incubated for 4 h with virus-containing supernatant in the presence of 4 µg/ml hexadimethrine bromide (polybrene) (Sigma).

Pooled stable cell populations were obtained by selection with 300 µg/ml G418 (Gemini Bio-Products) for 4 days or with 150 µg/ml hygromycin.

3.1.3 Transfections

Transient transfections of IMR90 cell populations were performed by incubating the cells in 6-well plates for 4 h at 37°C in 1 ml Opti MEM I (Gibco) containing 4 µg DNA and 10 µg/ml polybrene followed by incubation with 24 % DMSO (ICN Biomedicals Inc.) in Opti MEM I for 4 minutes at room temperature. The cells were washed three times with PBS, then supplemented with DMEM with 10 % FBS, and processed 24 hours post-transfection.

Transient transfection of U2OS cells was performed using lipofectamine according to the manufacturer's instructions.

3.1.4 Treatments

Geldanamycin (Sigma) was dissolved in DMSO at a concentration of 1.5 mM. IMR90 control and E7 expressing cells and SK-Br-3 cells were treated for 4 h with 1.5 µM geldanamycin or mock treated with an equal volume of DMSO. Leptomycin B (Sigma) was dissolved in ethanol at a concentration of 10 µg/ml. IMR90 control and E7 expressing cells were treated for 8 h with 40 ng/ml leptomycin B. Lactacystin (BIOMOL) was dissolved in water at a concentration of 40 mM. IMR90 control and E7 expressing cells were treated for 4 h with 40 µM lactacystin. Actinomycin D (Sigma) was dissolved in Ethanol at a concentration of 2.5 µM. IMR90 control and E7 expressing cells were treated for 24 h with 2.5 nM actinomycin D. Cisplatin (Sigma) was made up fresh before each use in DMSO at a concentration of 1 mg/ml and used at the indicated concentrations. TNF-α (R&D Systems) was dissolved in PBS + 0.2 % BSA to a stock concentration of 10 ng/ml and was used at the indicated concentrations. Cycloheximide (CHX) was dissolved in H_2O at a concentration of 3 mg/ml and was used at a concentration of 30 µg/ml. UV treatment was performed by exposing cells to 50 mJ/m^2 in a stratalinker (Stratagene) followed by incubation of 3 h in DMEM + 10 % FBS. Taxol

(paclitaxel) (Sigma) was dissolved in DMSO at a concentration of 50 mg/ml and was used at 100 nM for 24 h followed by an incubation with serum-free medium for 48 h.

3.2 DNA and RNA analysis methods

3.2.1 RNA isolation

Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturers recommendations, followed by treatment with 1 U/µl DNase-I (Roche) for 1 h at 37 °C. To test for DNA contamination, RNA samples were subjected to PCR analysis using GAPDH primers. mRNA was isolated from total RNA by using the OligoTex kit (Qiagen) according to the manufacturer's manual.

3.2.2 cDNA arrays

mRNA was used as a template to prepare a ^{32}P-labeled single stranded cDNA hybridization probe according to the manufacturers manual (Clontech) to use as a probe on ATLAS cDNA arrays. A Human Apoptosis ATLAS cDNA array (Clontech) was used. The signals were visualized with a STORM PhosphoImager and the results were quantitated using ATLAS image 1.5 software (Clontech).

3.2.3 Northern blotting

For Northern Blot analyses 10 µg total RNA was separated on a 1% formaldehyde/agarose gel, transferred to a NYTRAN SuPerCharge nylon membrane (Schleicher & Schuell) and crosslinked with a Stratalinker (Stratagene). The cDNA probes were ^{32}P-labeled using the STRIP-EZ DNA kit (Ambion) following the manufacturer's instructions and hybridized in ExpressHyb (Clontech) according to the manufacturer's manual. The signals were visualized with a STORM PhosphoImager and quantitated using ImageQuant software.

3.3 Protein analysis methods

3.3.1 Immunoblot analyses

Cells were lysed in 1% NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Nonidet P-40, 0.01% PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin), immediately scraped off the plates, incubated on ice for 20 minutes and centrifuged for 10 minutes at 4°C at 16,000 x g in a microcentrifuge. Protein concentrations of the lysates were determined by the Bradford method (Bio-Rad). Samples containing 100 µg of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Immobilion P, Millipore). Antibody complexes were detected by enhanced chemiluminescence (Renaissance Enhanced Luminol Reagent, NEN Life Science Products). Primary antibodies were used at the following dilutions: p53 (Ab-6, mouse monoclonal, 1:1000, Calbiochem), $p21^{CIP1/waf1}$ (Ab-1, mouse monoclonal, 1:1000, Calbiochem), bax (mouse monoclonal, 1:500, Pharmingen), ß-actin (mouse monoclonal, 1:10,000, CHEMICON), GAPDH (mouse monoclonal, 1:600, CHEMICON), E7 (mixture of 8C9, 1:100, Zymed and ED17, 1:1000, Santa Cruz, both mouse monoclonal), mdm2 (N-20, rabbit polyclonal, 1:300, Santa Cruz), DR5 (1:500, rabbit polyclonal, IMGENEX, San Diego, CA, USA), IκBα (1:200, rabbit polyclonal, Santa Cruz), cytochrome c (1:250, mouse monoclonal, Pharmingen), apoptosis inducing factor (AIF) (1:500, rabbit polyclonal, Alexis), cytochrome c oxidase subunit II (CoxII) (1:300, mouse monoclonal, Molecular Probes), IGFBP-1 (C-19, 1:100, goat polyclonal, Santa Cruz), IGFBP-2 (C-18, 1:100, goat polyclonal, Santa Cruz), IGFBP-3 (C-19, 1:100, goat polyclonal, Santa Cruz), IGFBP-4 (C-20, 1:100, goat polyclonal, Santa Cruz), IGFBP-5 (C-18, 1:100, goat polyclonal, Santa Cruz), IGFBP-6 (C-20, 1:100, goat polyclonal, Santa Cruz). The phospho-specific p53 antibodies used were Ser6, Ser9, Ser20, Ser37, Ser392 (1:1000, rabbit polyclonal, Cell Signaling Technology), Ser15 (1:2000, mouse monoclonal, Cell Signaling Technology) and Ser46 (1:1000, rabbit polyclonal, gracious gift from Y. Taya, Tokyo, Japan). Secondary HRP-conjugated antibodies were used at 1:10,000 dilutions (mouse, rabbit: Amersham, goat: Santa Cruz). ECL images were acquired using a Fluoro-S MultiImage MAX (Bio-Rad) with a supercooled digital camera (Nikon) and quantitated using

QuantityOne software (Bio-Rad). Equal loading was controlled using GAPDH or ß-actin immunoblots.

3.3.2 Immunoprecipitations

Cells were lysed and processed as described previously. Samples containing 0.5-1 mg total protein were incubated for 1.5 h at 4°C with 2 µg of specific antibody, followed by protein A-sepharose beads (Pharmacia) or protein G PLUS-agarose beads (Santa Cruz) for 1 h at 4°C at constant rotation. The p53 antibodies used were PAb1620 (Calbiochem) and PAb240 (Calbiochem), and for mdm2 Ab-1 (Calbiochem) was used. Immuncomplexes were washed 4 to 6 times in 1% NP 40 lysis buffer and subjected to SDS-PAGE and immunoblot analysis. An anti-mouse κ light chain specific secondary antibody (1:10,000, Southern Biotechnology Associates) was used to facilitate detection of immunoprecipitated p53.

3.3.3 Half life determinations

Control and E7 expressing IMR90 cells were treated with 30 µg/ml of the translation inhibitor cycloheximide (Sigma) for the indicated time periods. The cells were lysed and processed as described previously and the p53 steady state levels were determined by immunoblot analysis. Images were digitally acquired using a BioRad Fluor-S-Max Multi-imager and quantitated using the manufacturer's software. The levels of p53 were normalized for ß-actin expression.

3.3.4 Immunofluorescence analyses

Cells were transiently transfected as described above. After 36 h the cells were fixed in 2% paraformaldehyde for 30 min at 25°C, washed in PBS, permeabilized with 100% Methanol for 10 min at -20°C and washed with PBS. For the detection of AIF and Tid in untransfected IMR90 cells the cells were permeabilized in 4% paraformaldehyse with 0.2 % Triton X for 20 minutes at room temperature. After that the cells were incubated with 2.5% goat serum in PBS for 30 min at 37°C, washed with PBS, incubated

with primary antibody (p53, mouse monoclonal, Ab-6, Calbiochem, 1:100; AIF, rabbit polyclonal, Santa Cruz 1:25; Tid, mouse monoclonal, hybridoma cell supernatant, 1:5) for 1.5 h at 37°C, washed twice in PBS followed by an incubation with the FITC-coupled secondary goat α−mouse antibody (1:200) for p53, Cy3-coupled secondary goat α−mouse antibody (1:500) for Tid and Alexa Flour-coupled goat α−rabbit antibody (1:400) for AIF for 1 h at 37°C. The nuclei were stained with Hoechst 33258 (1:2000 in H_2O).

3.3.5 Luciferase assays

Cells were transfected in 6-well-plates with 500 ng of firefly luciferase reporter plasmid either under the control of an artificial p53 responsive promoter carrying 17 tandem repeats of the p53 consensus DNA binding sequence derived from the ribosomal gene cluster (Kern *et al.*, 1991) (pRGC-luc) or under the control of the p53 responsive human mdm2 promoter (pGL2-mdm2-luc) (Zauberman *et al.*, 1995) (kind gifts from M. Oren). 20 ng of the non-inducible renilla luciferase reporter plasmid (pRL-TK) was cotransfected as a transfection control. The cells were lysed after 24 hours in 30 µl lysis buffer (Dual-Luciferase Reporter Kit, Promega) per well, scraped off the plate and centrifuged for 10 min at 4°C at 16,000 x g. The supernatants were subjected to the dual luciferase assay and the firefly luciferase activity was normalized for renilla luciferase expression.

3.3.6 Subcellular fractionation

Stable control and E7 expressing IMR90 cells were washed in PBS and scraped in sucrose buffer (250 mM sucrose, 10 mM MOPS pH 7.2, 1 mM EDTA, 0.01% PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin). The cells were then homogenized by 30 strokes of a Teflon tissue homogenizer (Glas-Col, Terre Haute, IN). Nuclei were pelleted at 700 x g at 4°C for 10 min. The supernatant was centrifuged at 10,000 x g at 4°C for 10 min to pellet the mitochondria. The supernatant represents the cytoplasmic fraction. The nuclear pellet was resuspended in 1% NP 40 buffer and the nuclear lysate was cleared by centrifugation for 10 min at 4°C at 16,000 x g. The mitochondrial pellet was lysed in cell lysis buffer provided with the Quantikine human cytochrome c ELISA kit (R&D

systems) and cleared by centrifugation for 10 min at 4°C at 16,000 x g. Equal amounts of protein from the cytoplasmic and nuclear fraction were separated by SDS-PAGE and p53 levels were determined by immunoblotting. To determine cytochrome c release equal amounts of protein of the cytoplasmic and the mitochondrial fraction were used in the Quantikine human cytochrome c ELISA kit.

3.3.7 Two-dimensional gel electrophoresis

For two dimensional (2D) gel analyses of endogenous p53 (Stewart *et al.*, 2001), 1 mg of protein lysate from LXSN cells or 250 µg of protein lysate from E7 cells were prepared in a final volume of 600 µl of isoelectric focusing (IEF) sample buffer (9.5 M urea (Pharmacia), 2% NP-40, 2% ß-mercaptoethanol, 0.2% ampholytes pH 5-8 (Pharmacia), 0.001% bromophenol blue). A truncated form of recombinant human p53 (amino acids 1-353) was incubated in each sample as an internal standard to permit alignment of p53 phospho-forms; the truncated human p53 was prepared as previously described (Szak and Pietenpol, 1999). IEF was performed using the PROTEAN IEF system (BioRad) and 17 cm isoelectric strip gels pH 5-8 (BioRad). The isoelectric gels were passively rehydrated for 10 h in IEF sample buffer containing the protein lysate prior to focusing for 60,000 volt-hours. After IEF, gels were incubated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 130 mM DTT) and 15 min in equilibration buffer II [6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 135 mM iodoacetamide (Sigma)] prior to separation by SDS-PAGE. Detection of p53 was by immunoblot using PAb1801 (1 µg/ml; Calbiochem).

3.3.8 Electrophoretic Mobility Shift Assay (EMSA) for NFκB

The doublestranded H2K oligonucleotide with BamH1 overhangs (sense 5'-GATCCAGGGCTGGGGATTCCCCATCTCCACAGG-3'; antisense 5'-GATCCCTGTGGAGATGGGGAATCCCCAGCCCTG-3') was radioactively labeled in a 25 µl reaction using 3.5 µl ^{32}P α-dATP (6000 µCI/µl), 250 ng H2K oligonucleotide, 2.5 µl 10x Klenow buffer, 1 µl Klenow and 1.8 µl 5 mM dNTP mix without dATP. The mixture was incubated at 30°C for 30 minutes followed by a purification using the QIAquick

Nucleotide Removal kit (Qiagen). The efficiency of the labeling was determined by scintillation counter.

Cells were washed in PBS and lysed in EBL buffer (20 mM HEPES/NaOH, pH 7.9, 350 mM NaCl, 20 % glycerin, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 % NP-40, 0.1 mM NaO_3VO_4, 1 mM DTT, 0.57 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM NaF, 10 mM β-glycerophosphate) for 30 minutes at 4˚C. Lysates were cleared at 14,000 rpm for 10 minutes at 4˚C and the DNA binding assay was performed as follows: 5 µg of protein lysate was incubated with 20,000 cpm labeled H2K oligonucleotide in DNA binding buffer (40 mM HEPES/NaOH, pH 7.9, 120 mM KCl, 8 % Ficoll, 0.5 mM DTT, 0.1 mg/ml BSA, 0.1 µg/µl poly dIdC, 0.1 % NP40) in a total reaction volume of 20 µl for 30 minutes at 30°C. Supershifts were performed by preincubating the protein lysate with α-p50 (Rockland) or α-p65 antibodies (Santa Cruz) for 10 minutes on ice. Protein lysates form U2OS cell transfected with p50 and p65 expression plasmids (gracious gifts of M. Hinz, MDC Berlin, Germany) were used as positive controls. The complexes were separated on a 4 % acrylamide gel and the vacuum-dried gel was exposed to X-ray film.

3.3.9 Electrophoretic Mobility Shift Assay (EMSA) for p53

The oligonucleotide provided in the p53 NUSHIFT kit (GENEKA) was radioactively labeled according to the manufacturer's instructions. The efficiency of the labeling was determined by scintillation counter.

Cells were washed in PBS and lysed in p53 EMSA lysis buffer (100 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 % NP-40, 1 mM EDTA, 0.57 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin) for 30 minutes at 4˚C. Lysates were cleared at 14,000 rpm for 5 minutes at 4˚C and the DNA binding assay was performed using the p53 NUSHIFT kit (GENEKA) according to the manufacturers' instructions. 20 µg of protein lysate was used per reaction. The complexes were separated on a 4% acrylamide gel and the vacuum-dried gel was exposed to X-ray film.

3.4 Apoptosis analysis methods

3.4.1 Caspase Activity Assay

Caspase activity assays were carried out according to manufacturers instructions (Clontech, ApoAlert Caspase 3 and 8 Fluorometric Assay Kit). $1x10^6$ cells from different stable IMR90 cell lines that were subject to different treatments were lysed and incubated with caspase 3 or caspase 8 specific substrates for 1 hour at 37°C. The addition of caspase specific inhibitors served as a control to assure the measurement of specific caspase activity. The resulting enzymatic activity was determined by measuring the emitted fluorescence of the caspase cleaved substrate in a fluorometer.

3.4.2 Hoechst Staining

Cells treated to induce apoptosis by different stimuli and their control treated counterparts were fixed with 100 % methanol vapors for 10 minutes and stained with 1 µg/ml of bisbenzimide (Sigma, Hoechst 33258) in dH_2O for 8 minutes. Apoptotic nuclei were visualized by fluorescence microscopy as described previously (Jones *et al.*, 1997b). Cells were scored as apoptotic based upon the condensed nuclear morphology and expressed as a percentage of total cells.

3.4.3 DNA fragmentation assay

DNA fragmentation was measured by using the Cell Death Detection ELISA plus system (Roche) according to the manufacturers manual. $1x10^4$ cells were used for each assay and experiments were performed in triplicate.

3.4.4 Cytochrome c release assay

Cells treated to induce apoptosis by different stimuli and their control treated counterparts were subjected to subcellular fragmentation. 2µg of the cytoplasmic and mitochondrial fraction were used to determine the cytochrome c content using the Quantikine human cytochrome c ELISA assay (R&D systems) according to the manufacturers manual.

4. Results

4.1 Stabilization and functional impairment of the tumor suppressor p53 by the human papillomavirus type 16 E7 oncoprotein

4.1.1 The functional impairment of the tumor suppressor p53 by the HPV-16 E7 oncoprotein

4.1.1.1 The stabilized p53 in HPV-16 E7 expressing cells is transcriptionally incompetent.

It was reported previously that overexpression of the HPV-16 E7 oncogene results in accumulation (Demers *et al.*, 1996) and increased half-life of the p53 protein (Jones and Münger, 1997; Jones *et al.*, 1997b). These results are surprising as increased p53 activity is associated with cell cycle arrest or apoptosis. However, HPV-16 E7 expressing cells grow rapidly. Therefore it was critical to analyze p53 activity in proliferating HPV-16 E7 expressing cells.

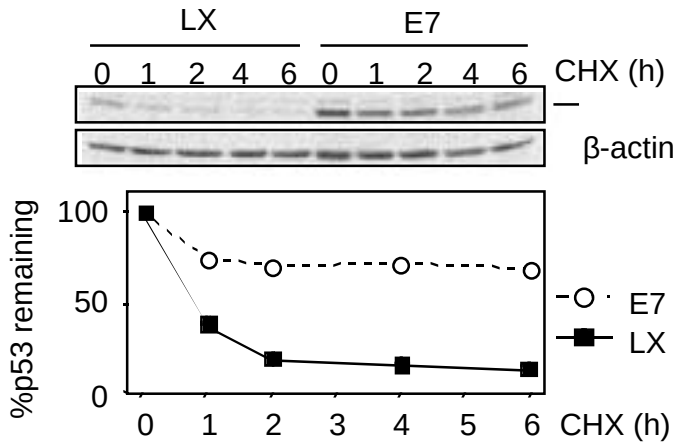

Figure 8: Estimation of p53 half-life. Cultures were treated with cycloheximide (CHX) for the indicated periods of time and 100 µg aliquots of protein lysates analyzed by SDS-PAGE and immunoblotting. Quantification was performed after normalization for ß-actin expression and is shown underneath.

We generated stable cell lines by infecting IMR90 human diploid lung fibroblasts with empty retroviral vector LXSN (LX) or LXSN vector expressing HPV-16 E7 (E7). In agreement with previous studies (Jones and Münger, 1997) the p53 steady state levels

were consistently 3 to 4 times higher in HPV-16 E7 expressing IMR90 cells compared to control cells and the half-life of p53 was extended in stable HPV-16 E7 expressing cells (Fig. 8). Previous studies showed that p53 mRNA levels are not increased in HPV-16 E7 expressing cells indicating that the increased levels of p53 protein are mostly due to increased protein stability (Jones *et al.*, 1997b). Note that the decrease of p53 levels in both control (LX) and HPV-16 E7 expressing cells is greater between the 0 and 1 hour time points than at subsequent times suggesting that these cells may contain two p53 populations with different half lives.

There is conflicting evidence concerning the transcriptional activity of the stabilized p53 in HPV-16 E7 expressing cells. A previous study demonstrated that transcription of $p21^{CIP1}$, a major transcriptional target of p53, was not significantly altered in HPV-16 E7 expressing cells (Jones *et al.*, 1999). Consistent with this finding it was also reported that HPV-16 E7 can dampen expression of p53-responsive reporter plasmids in transient assays (Massimi and Banks, 1997). In contrast, another study showed that stable HPV-16 E7 expressing fibroblasts contained elevated mdm2 mRNA levels derived from the p53-responsive P2 promoter (Seavey *et al.*, 1999) suggesting that p53 may be transcriptionally active in HPV-16 E7 expressing cells. To determine whether the elevated p53 levels correlate with an increase in transcriptional activity of p53, transient reporter assays were performed. Control and HPV-16 E7 expressing IMR90 cells were transfected with a constant amount of firefly luciferase reporter plasmid under the control of an artificial p53-responsive promoter consisting of 17 p53 binding sites (pRGC-luc). Activity was determined and normalized against the expression of the non-p53-responsive renilla luciferase reporter pRL-TK that was cotransfected. Expression of the p53 responsive reporter construct was decreased in HPV-16 E7 expressing cells to a level of approximately 40% of control cells (Fig. 9A) even though this HPV-16 E7 expressing cell population contains 3.6 fold higher p53 levels than control cells (Fig. 10). Activity of the renilla luciferase reporter was not affected by HPV-16 E7 expression (data not shown).

Next the expression levels of known p53 target genes in control and HPV-16 E7 expressing IMR90 cells was investigated performing expression profiling using a cDNA array. The array contained a total of 12 known p53 responsive genes. The basal level of

expression of the p53 responsive genes was variable, but none of these genes was expressed at a significantly higher level in HPV-16 E7 expressing cells (Fig. 9B). As expected from earlier studies (Cheng *et al.*, 1995), expression of PCNA, a marker of proliferation, was higher in E7 expressing cells than in control cells (Fig. 9B).

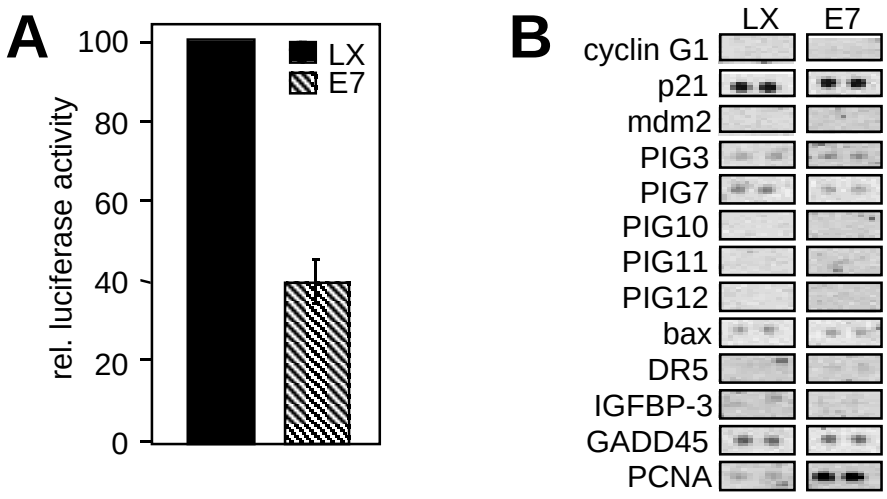

Figure 9: Decreased transcriptional activity of the stabilized p53 in E7-expressing IMR90 cells. **(A)** Cells were transfected with the artificial p53-responsive firefly luciferase reporter plasmid pRGC-luc. Activity values were normalized for expression of a non-responsive renilla luciferase reporter. Values represent averages and standard deviations of an experiment performed in triplicate. **(B)** Expression analysis of a set of p53 responsive genes by transcriptional profiling. A "Human Apoptosis ATLAS" cDNA nylon array (Clontech) was analyzed by sequential hybridization with ^{32}P-labeled single stranded cDNA probes. PCNA is a known E7-responsive gene and was used as a positive control.

The protein levels of some p53 target genes on the cDNA array were determined by immunoblot analysis as well (Fig. 10). Steady state levels of the pro-apoptotic p53 target genes bax (Miyashita and Reed, 1995) and DR5 (alias TRAIL-R2/Apo2/Killer) (Wu *et al.*, 1997) were only slightly increased or were unchanged, respectively (Fig. 10). Increased levels of $p21^{CIP1}$ have been observed in HPV-16 E7 expressing cells and are a consequence of increased protein stability (Jones *et al.*, 1997b) and there is no comparable increase in mRNA levels (Fig. 9B).

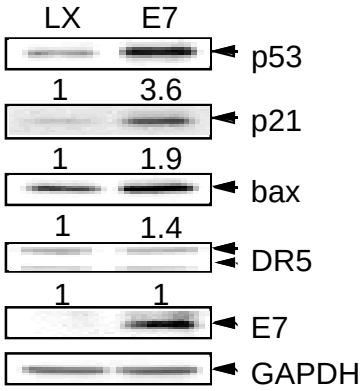

Figure 10: Immunoblot analysis of p53, and the p53 transcriptional targets $p21^{CIP1}$ (p21), bax and DR5 (alias TRAIL-R2/Apo2/Killer). Expression of HPV-16 E7 is also shown. Relative expression levels of each protein were determined after correction for GAPDH and is indicated underneath the panels in arbitrary units.

4.1.1.2 Exogenous p53 is transcriptionally active in HPV-16 E7 expressing cells.

To investigate whether HPV-16 E7 can interfere with the transcriptional activity of exogenous p53 we transiently transfected control and HPV-16 E7 expressing IMR90

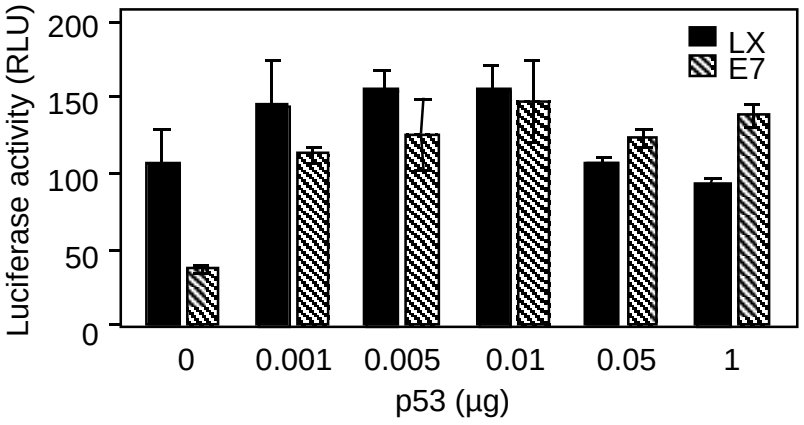

Figure 11: Transcriptional activity of exogenous p53 in control (LX) and HPV-16 E7-expressing (E7) IMR90 populations. Cells were cotransfected with 500 ng of the p53-responsive firefly luciferase reporter pRGC-luc and the indicated amounts of p53. Activity values were normalized for expression of a non-responsive renilla luciferase reporter. Values represent averages and standard deviations of an experiment performed in triplicate.

cells with increasing amounts of p53 and the p53-responsive luciferase reporter plasmid pRGC-luc and determined its transcriptional activity by luciferase assays. In agreement with our previous result (Fig. 9A) transcriptional activity of endogenous p53 is diminished by approximately 60% in HPV-16 E7 expressing cells compared to the control population. Exogenous p53, however, was able to efficiently activate reporter gene expression in HPV-16 E7 expressing cells to a similar level as in control cells (Fig. 11). This indicates that in IMR90 cells, HPV-16 E7 does not impair the transcriptional activity of exogenous p53.

4.1.2 The susceptibility of p53 to mdm2 mediated degradation in HPV-16 E7 expressing cells

4.1.2.1 p53 is bound to mdm2 in HPV-16 E7 expressing cells.

The major regulator of p53 stability in cells is mdm2, which is a transcriptional target of p53 as well. Mdm2 binds to p53 and marks it for degradation. Upon cellular stress, such as DNA damage, p53 becomes modified and is no longer susceptible to mdm2 binding. This increase in p53 activity results in elevated mdm2 levels and this feedback loop response terminates the p53 response. Due to the importance of mdm2 for the regulation of p53 activity it was critical to determine mdm2 activity in HPV-16 E7 expressing cells containing stabilized p53.

It has been reported previously that HPV-16 E7 expressing cells contain higher mdm2 levels (Seavey *et al.*, 1999; Thomas and Laimins, 1998). In our cells steady state levels of mdm2 mRNA and protein in control and HPV-16 E7 expressing cells were too low to detect by transcriptional profiling (Fig. 9B) or direct immunoblot analysis (data not shown), respectively. Hence, we performed coupled immunoprecipitation/immunoblot analyses and found that mdm2 levels were increased approximately 1.8 fold in HPV-16 E7 expressing cells compared to control cells (Fig. 12A, upper panel). This is a less dramatic increase than previously reported in other cell systems (Seavey *et al.*, 1999; Thomas and Laimins, 1998). Since a previous study reported that HPV-16 E7 could interfere with the interaction of mdm2 and p53 (Seavey *et al.*, 1999), the amount of p53 bound to mdm2 was determined by coimmunoprecipitation

experiments. Approximately 2.5 fold more p53 was coimmunoprecipitated with mdm2 in HPV-16 E7 expressing cells, which parallels the increase of mdm2 (1.8 fold) in these cells (Fig. 12A). These results suggest that mdm2 is effectively bound to p53 in HPV-16 E7 expressing IMR90 cells indicating that HPV-16 E7 does not interfere with the association of p53 and mdm2.

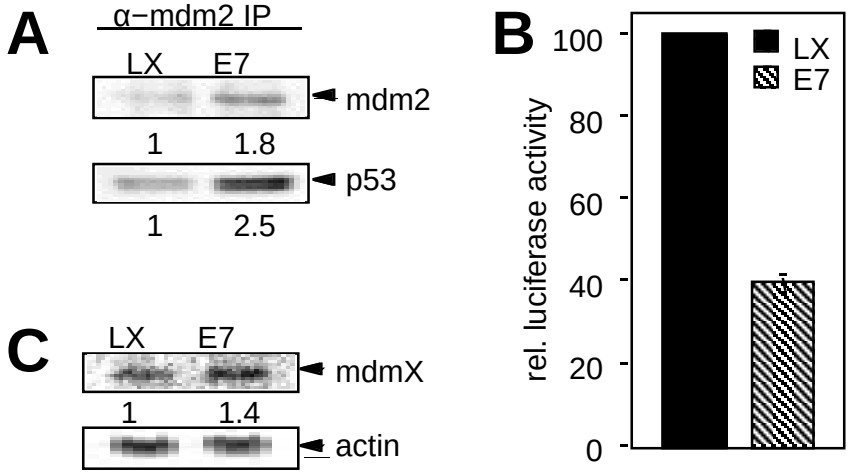

Figure 12: Expression of mdm2 and the mdm2 homologue mdmX in control (LX) and HPV-16 E7 expressing (E7) IMR90 cells. **(A)** p53 is in complex with mdm2 in E7-expressing cells. Equal amounts of protein lysates were immunoprecipitated with mdm2 specific antibodies followed by immunoblot analysis for mdm2 (upper panel) and co-precipitated p53 (lower panel). Quantitation of the signals is shown underneath. **(B)** Activity of the p53-responsive P2 promoter of human mdm2. Cells were transfected with the corresponding firefly luciferase reporter construct. Activity values were normalized for expression of a non-responsive renilla luciferase reporter. Values represent averages and standard deviations of an experiment performed in triplicate. **(C)** Northern blot analysis of mdmX RNA expression. Relative levels of mdmX mRNA are shown underneath and have been corrected for equal loading as determined by actin mRNA.

Since the mdm2 promoter contains p53 responsive elements, it was determined whether the transcriptional activity of the mdm2 promoter is increased in HPV-16 E7 expressing cells. A luciferase reporter assay was performed by transfecting a reporter plasmid under the control of the p53 responsive element of the human mdm2 P2 promoter into control and HPV-16 E7 expressing IMR90 cells. Similar to the synthetic p53 responsive reporter construct (Fig. 9A), the expression of the mdm2 reporter plasmid was decreased in HPV-16 E7 expressing cells to a level of approximately 40% compared

to control cells (Fig. 12B). These experiments further support the notion that HPV-16 E7 mediated p53 stabilization does not lead to a concomitant increase of transcriptional activity.

The recently described mdm2 homologue, mdmX, was reported to bind and stabilize p53 by protecting it from mdm2-mediated degradation (Jackson and Berberich, 2000). Interestingly, mdmX-bound p53 is transcriptionally inert (Shvarts *et al.*, 1997). Furthermore, mdmX can bind and stabilize mdm2 and inhibit its ability to degrade p53 (Sharp *et al.*, 1999). Hence mdmX appeared an attractive candidate to mediate HPV-16 E7 effects on p53. Analysis of mdmX protein levels in HPV-16 E7 expressing cells has not been conclusive due to limitations of the existing antibodies. Northern blot analyses revealed a slight, 1.4 fold increase of mdmX mRNA in HPV-16 E7 expressing cells (Fig. 12C). Hence it is possible that mdmX may be involved in p53 stabilization in HPV-16 E7 expressing cells.

4.1.2.2 Stabilized p53 in HPV-16 E7 expressing cells is susceptible to degradation by exogenous mdm2.

To determine whether HPV-16 E7 can protect p53 from degradation by exogenous mdm2, mdm2 was transiently expressed in stable HPV-16 E7 expressing IMR90 cells and changes in p53 levels were determined by immunofluorescence. Transfection of HPV-16 E6, which induces E6AP-mediated p53 degradation, was used as a positive control. The DS-red plasmid encodes a fluorescent protein, and was used as transfection marker for these experiments. Like HPV-16 E6, expression of mdm2 decreased p53 levels in stable HPV-16 E7 expressing cells (Fig. 13). This indicates that HPV-16 E7 stabilized p53 is still susceptible to degradation by exogenous mdm2.

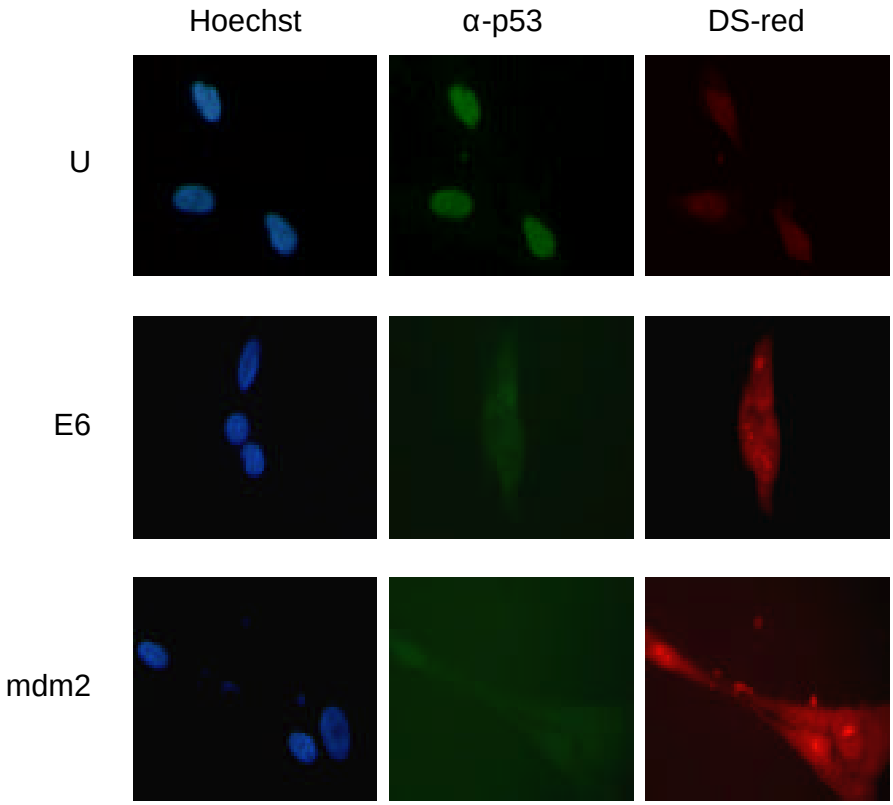

Figure 13: Stabilized p53 in HPV-16 E7-expressing cells remains susceptible to degradation by exogenous mdm2. HPV-16 E7 expressing IMR90 cells were transiently transfected with mdm2 or HPV-16 E6 as a positive control. Untransfected E7-expressing IMR90 cells (U) are shown as controls. The autofluorescent protein DS-red was cotransfected to allow specific identification of the transfected cells. p53 was detected by immunofluorescence. Nuclei were visualized by Hoechst staining.

4.1.3 The stabilization of the tumor suppressor p53 by the HPV-16 E7 oncoprotein

Several other factors may also contribute to p53 stability and activity. Various forms of cellular stress result in stabilization and accumulation of the p53 protein as a consequence of posttranslational modifications. Changes in p53 phosphorylation status have been implicated in both stabilization and activation of the protein (reviewed in Stewart and Pietenpol, 2001). In addition, changes in conformation, subcellular localization or the inhibition in degradation can result in stabilization and activation of the p53 protein (Bosari *et al.*, 1995; Cho *et al.*, 1994; Elmore *et al.*, 1997; Moll *et al.*, 1995; Moll *et al.*, 1992; Pavletich *et al.*, 1993; Schlamp *et al.*, 1997; Ueda *et al.*, 1995; van den Heuvel *et al.*, 1993).

4.1.3.1 No significant changes in the number of p53 phospho-forms in HPV-16 E7 expressing cells.

To determine if increased p53 levels in HPV-16 E7 cells were associated with changes in p53 phosphorylation we performed two-dimensional gel electrophoresis in collaboration with the laboratory of J. Pietenpol at Vanderbilt University in Nashville, TN. To adjust for the different p53 levels in the two cell lines, 1 mg of protein lysate from control cells and 250 µg protein from HPV-16 E7 expressing IMR90 cells were analyzed (Fig. 14). A recombinant carboxyl terminal truncated human p53 protein (amino acids 1-353) was added to the IEF sample buffer to serve as internal standard for alignment of the indicated phospho-forms (Stewart *et al.*, 2001). Although several phosphorylated p53 species were detected, with the exception of the most highly phosphorylated isoform, a significant difference in p53 phosphorylation was not observed between the control and HPV-16 E7 expressing cells.

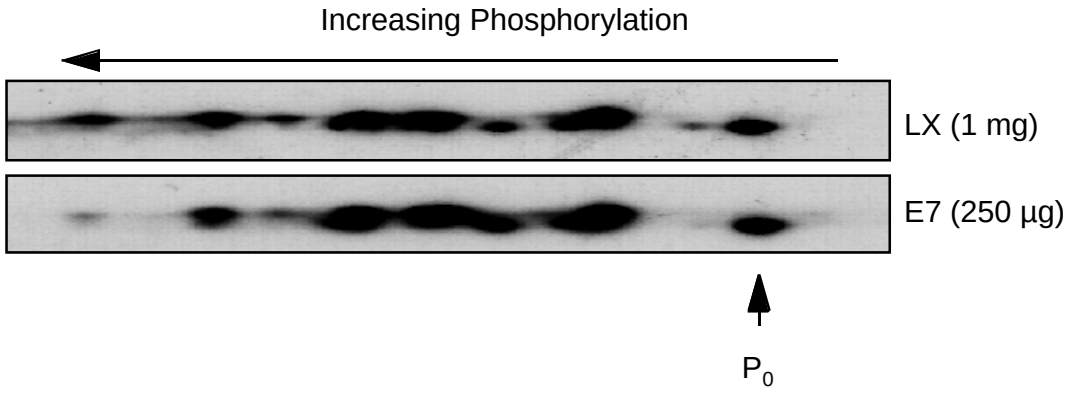

Figure 14: No changes in the number if phospho-forms in the stabilized p53 in HPV-16 E7 expressing cells. Two dimensional SDS-PAGE analysis of p53 was performed to analyze aliquots containing similar amounts of p53 (1 mg LX; 250 µg E7). P_0 denotes unphosphorylated p53.

4.1.3.2 Wild type conformation of p53 in HPV-16 E7 expressing IMR90 cells.

The tumor suppressor p53 is inactivated in many cancers by being folded in a mutant conformation (Finlay *et al.*, 1988; Milner and Medcalf, 1991; Milner *et al.*, 1991; Whitesell *et al.*, 1997). This misfolding results in the stabilization of the inactive protein. To investigate whether the stabilized p53 in HPV-16 E7 expressing cells is in a mutant

conformation we performed immunoprecipitation experiments with conformation-specific antibodies. The antibody PAb1620 preferentially recognizes wild type conformation p53 and the antibody PAb240 preferentially detects p53 with a mutant conformation (Gannon *et al.*, 1990). HaCaT cells, which contain a p53 with a mutant conformation (Lehman *et al.*, 1993), and C33A cells, which contain a p53 with a point mutation at codon 273 (Scheffner *et al.*, 1991) that has a wild type conformation (Medcalf and Milner, 1993), were used as controls. Since these are mutant forms of p53

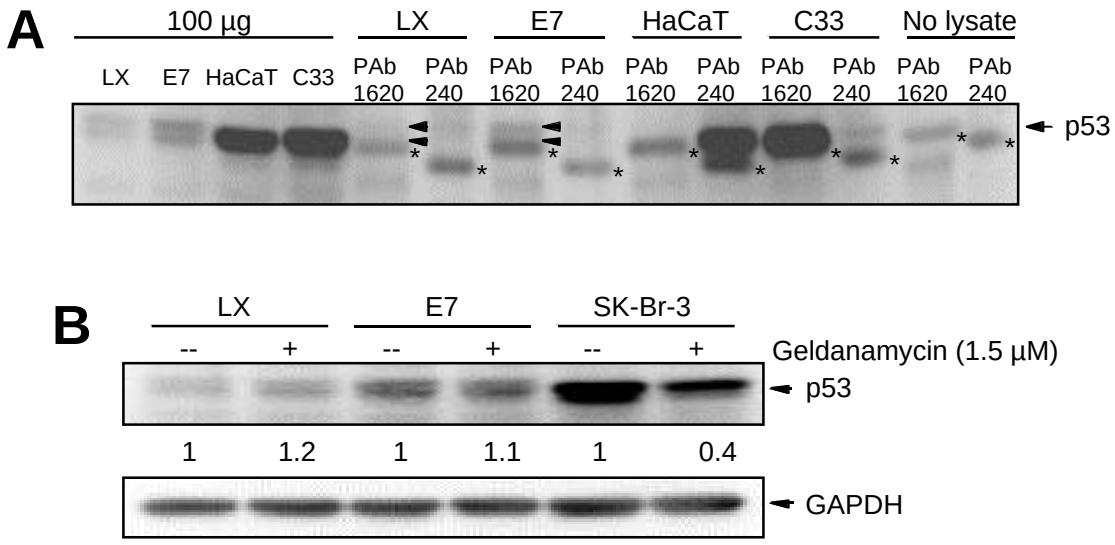

Figure 15: The stabilized p53 in HPV-16 E7 expressing cells is in a wild type conformation. **(A)** Immunoprecipitations with p53 antibodies PAb1620 and PAb240 were performed with lysates of control (LX) and HPV-16 E7-expressing (E7) IMR90 cells (1 mg), HaCaT and C33A (C33) cells (500 µg) followed by p53 immunoblot analysis. PAb1620 preferentially recognizes wild type conformation p53, whereas PAb240 preferentially precipitates mutant conformation p53. Samples containing 100 µg total protein were used as controls for direct Western Blots. Background bands specific to each of the antibodies used were detected in the "No lysate" control lanes and are indicated by a (*), immunoprecipitated p53 is indicated by arrows **(B)** Control (LX) and HPV-16 E7 expressing (E7) IMR90 were treated with geldanamycin, a compound that disrupts complexes of mutant p53 and hsp90 and renders p53 susceptible for proteasomal degradation. SK-Br-3 cells contain hsp90-bound mutant p53 and were used as a positive control. Levels of p53 were determined by immunoblot analysis normalized for GAPDH levels and are shown underneath.

they do not exactly comigrate with the wild type p53 in IMR90 cells. Precipitation with the wild type conformation specific PAb1620 but not with mutant conformation specific PAb240 yielded a detectable signal in HPV-16 E7 expressing cells (Fig. 15A). These

results suggest that the majority of p53 in HPV-16 E7 expressing cells is retained in a wild-type conformation, which correlates with p53 being able to bind to its consensus sequence and induce transcription as well as it being in a conformation susceptible to mdm2 binding.

To corroborate this result we next determined whether the stabilized p53 in HPV-16 E7 expressing cells is bound to hsp90. Hsp90 can bind to p53 that is in a mutant conformation and stabilize it by preventing proteasome-mediated degradation (Blagosklonny *et al.*, 1996). Geldanamycin is a drug that disrupts the interaction of mutant conformation p53 with hsp90 and renders it susceptible for proteasomal degradation (Blagosklonny *et al.*, 1996; Nagata *et al.*, 1999; Whitesell *et al.*, 1997). Control and HPV-16 E7 expressing cells were treated with geldanamycin and p53 steady state levels were determined by immunoblotting. The breast cancer cell line SK-Br-3 contains hsp90-bound p53 and was used as a positive control (Whitesell *et al.*, 1997). The p53 levels remained unchanged in control and HPV-16 E7 expressing cells upon Geldanamycin treatment, while the p53 level in the control cells SK-Br-3 cells decreased (Fig. 15B). These results suggest that the stabilized p53 in HPV-16 E7 expressing cells is not complexed to hsp90. Taken together these results indicate that the stabilized p53 in HPV-16 E7 expressing cells is in a wild type conformation.

4.1.3.3 The stabilized p53 in HPV-16 E7 expressing cells is localized to the nucleus.

p53 signaling can be inhibited by mechanisms other than mutations or conformational alterations. One such mechanism involves aberrant subcellular localization. We determined whether expression of HPV-16 E7 alters the subcellular localization of p53. Nuclear and cytoplasmic fractions were prepared from control and HPV-16 E7 expressing cells, subjected to SDS-PAGE and p53 immunoblot analysis. IκBα is an exclusively cytoplasmic protein and was used as a marker. Some p53 was detected in the cytoplasmic fractions in both control and HPV-16 E7 expressing cells, but the ratio between cytoplasmic and nuclear p53 was similar in both cell populations (Fig. 16). Hence, the subcellular localization of p53 is not altered in HPV-16 E7 expressing cells.

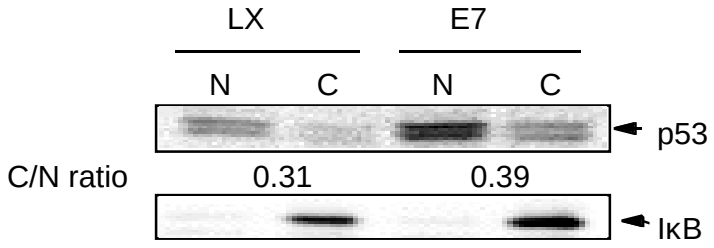

Figure 16: p53 is nuclear in control (LX) and HPV-16 E7-expressing (E7) IMR90 cells. Nuclear (N) and cytoplasmic (C) fractions were subjected to p53 immunoblot analysis. IκB was used as a cytoplasmic marker. The ratio of cytoplasmic to nuclear p53 (C/N ratio) is indicated.

4.1.3.4 Expression of HPV-16 E7 does not block nuclear export or proteasomal degradation of p53 per se.

Since the stabilized p53 is mainly nuclear in HPV-16 E7 expressing cells it was investigated whether HPV-16 E7 might block nuclear export of p53, which might result in its stabilization. Control and HPV-16 E7 expressing IMR90 cells were treated with the

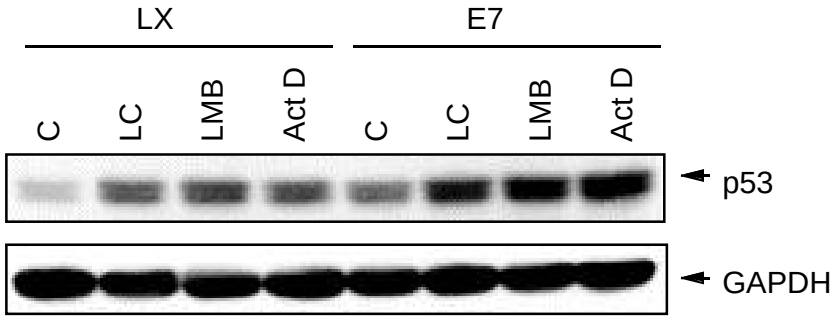

Figure 17: The nuclear export or proteasomal degradation of the stabilized p53 inHPV-16 E7 expressing cells is not generally impaired. Cells were treated for 4 h with 40 µM lactacystin (LC - a proteasome inhibitor), or for 8 h with 40 ng/ml leptomycin B (LMB - a nuclear export inhibitor) and p53 levels were determined by immunoblot analyses. Treatment with a radiomimetic dose (2.5 nM) Actinomycin (Act D) for 24 hours was used as a positive control. GAPDH is shown to document equal loading.

nuclear export inhibitor leptomycin B (LMB) and p53 steady state levels were determined by immunoblotting. p53 steady state levels increased to a similar extent in control and HPV-16 E7 expressing cells (Fig. 17). This result indicates that HPV-16 E7 expression does not block the nuclear export of p53. Since it has been reported that HPV-16 E7 can interact with the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi,

1997) we determined whether HPV-16 E7 might stabilize p53 by generally interfering with the proteasomal degradation machinery. Control and HPV-16 E7 expressing IMR90 cells were treated with the proteasome inhibitor lactacystin. Similar increases in p53 steady state levels were observed in control and HPV-16 E7 expressing cells, indicating that HPV-16 E7 does not generally interfere with the proteasomal degradation machinery (Fig. 17). Treatment with the DNA damaging agent actinomycin D, which results in p53 stabilization in both control and HPV-16 E7 expressing cells (Demers *et al.*, 1994a; Slebos *et al.*, 1994), was used as a control in these experiments (Fig. 17).

4.2 The "trophic sentinel" response of HPV-16 E7 expressing cells upon growth factor withdrawal

4.2.1 The involvement of the tumor suppressor p53 in the "tropic sentinel" response of HPV-16 E7 expressing cells

Oncogenes promote apoptosis under several conditions. Previous studies have shown that the expression of HPV-16 E7 predisposes cells to apoptosis upon growth factor withdrawal (Jones *et al.*, 1997b). It is not clear, however, whether this "trophic sentinel" response is mediated by p53. The involvement of p53 in most other apoptotic responses, such as upon DNA damage, is well established, but the "trophic sentinel" response upon limitation of growth factors is not as well studied as the "damage sentinel" response.

4.2.1.1 The stabilized p53 in HPV-16 E7 expressing cells is not phosphorylated at known serine residues upon growth factor deprivation

Changes in the p53 phosphorylation status have been implicated in both stabilization and activation of the protein (reviewed in Stewart and Pietenpol, 2001). To determine whether the stabilized p53 in HPV-16 E7 expressing cells is differentially phosphorylated upon growth factor withdrawal, the phosphorylation status of known serine residues was probed using phosphospecific antibodies in immunoblot analyses. No increase in phosphorylation of p53 at serine residues 6, 9, 15, 20, 37, 46 or 392 was observed in apoptotic HPV-16 E7 expressing IMR90 cells (Fig. 18). UV treated COS7 cells were used as positive controls for the phosphospecific antibodies (Fig. 18). These

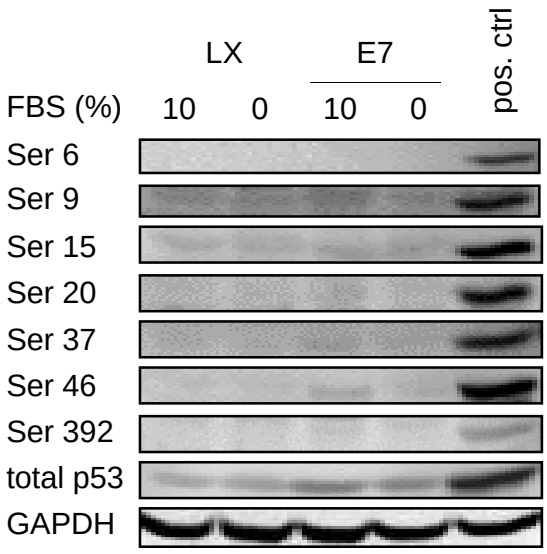

Figure 18: p53 is not phosphorylated in HPV-16 E7 expressing cells upon growth factor deprivation. The phosphorylation status of p53 in control and HPV-16 E7 expressing IMR90 cells in the presence and absence of growth factors was determined by immunoblot analyses using antibodies specific for known serine phosphorylation sites. UV treated COS7 cells served as positive controls for the phospho-specific antibodies. The total amount of p53 protein in control (LX) and HPV-16 E7 (E7) expressing IMR90 cells was determined as well. The protein expression levels of GAPDH were used as a loading control.

findings are consistent with the 2 dimensional gel electrophoresis studies that showed no changes in the number of phospho-forms in the stabilized p53 in proliferating HPV-16 E7 expressing cells compared to control cells (Fig. 14). These data indicate that the stabilized p53 is not activated as a transcription factor in HPV-16 E7 expressing cells by phosphorylation upon growth factor withdrawal.

4.2.1.2 The DNA binding activity of p53 in HPV-16 E7 expressing cells is not enhanced upon growth factor withdrawal.

P53 has to bind to its binding sequence in the promoter region of the target gene to activate transcription (el-Deiry *et al.*, 1992; El-Deiry *et al.*, 1993). Since the stabilized p53 in growth factor deprived HPV-16 E7 expressing cells is not phosphorylated at known serine residues and is thus likely not transcriptionally active it was investigated whether it is capable of binding to its DNA consensus sequence at all. A p53 EMSA was performed using whole cell lysates from control (LX) and HPV-16 E7 (E7) expressing cells grown in the presence and absence of growth factors. The EMSA showed that the

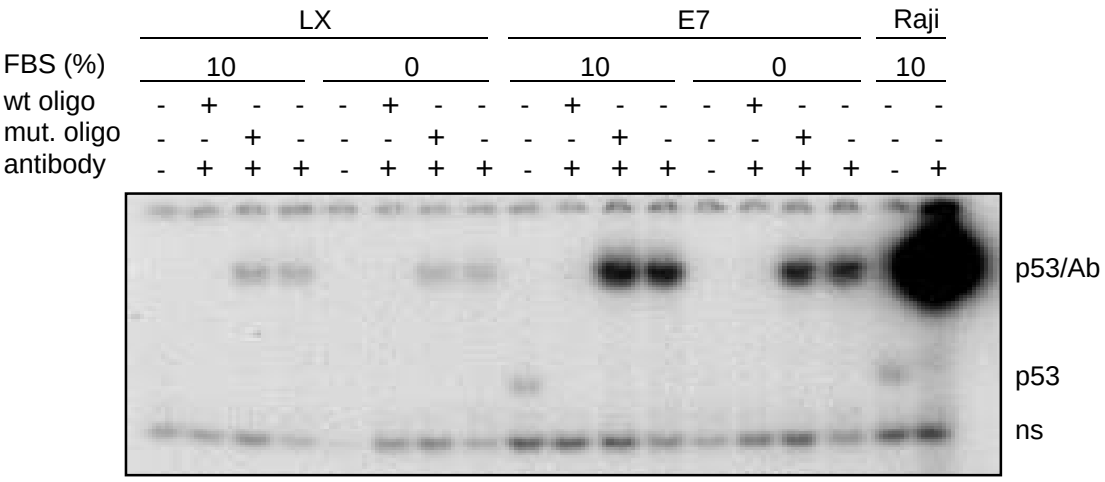

Figure 19: The DNA binding activity of p53 in control and HPV-16 E7 expressing IMR90 cells does not change upon growth factor withdrawal. The DNA binding capacity of p53 in control and HPV-16 E7 expressing IMR90 cells upon growth factor deprivation was determined by electrophoretic mobility shift assay (EMSA). Raji cell lysates served as positive control. The p53/oligonucleotide complexes (p53) and the supershifted p53/oligonucleotide/antibody complexes (p53/Ab) are indicated, as well as a nonspecific band (ns). Non-radioactive labeled oligonucleotides containing either wild-type (wt) or mutant (mut) p53 DNA binding consensus sequences were used for competition studies. The free probe was run off the gel to guarantee adequate separation of the complexes.

stabilized p53 in proliferating HPV-16 E7 expressing cells can bind to its DNA consensus sequence. Furthermore no increase in the DNA binding activity of p53 in growth factor deprived control and HPV-16 E7 expressing IMR90 cells was observed (Fig. 19). This indicates that the p53 in proliferating and apoptotic HPV-16 E7 expressing cells is capable of binding to its consensus sequence at a similar efficiency. Taken together these results indicate that p53 is not phosphorylated at known serine residues and that its ability to bind DNA is neither impaired nor enhanced in HPV-16 E7 expressing IMR90 cells in the presence or absence of growth factors.

4.2.1.3 The stabilized p53 is not active as a transcription factor during HPV-16 E7 mediated apoptosis.

The transcription factor activity of p53 is elevated in most apoptotic settings (Bennett, 1999; Zornig *et al.*, 2001), and several p53 responsive genes directly contribute to the apoptotic response. Since it was shown previously that the stabilized p53 in HPV-

16 E7 expressing cells can be activated as a transcription factor upon DNA damage, it was critical to investigate whether the observed apoptosis in HPV-16 E7 expressing cells upon growth factor withdrawal involves the transcriptional activation of the otherwise inactive p53.

A cDNA array containing a total of 12 known p53 target genes was analyzed using probes derived from mRNAs isolated from control and HPV-16 E7 expressing cells in the presence and absence of growth factors. None of these 12 genes was expressed at a significantly higher level in growth factor deprived HPV-16 E7 expressing cells, including the two proapoptotic genes bax and DR5, although the basal level of expression of these genes varied (Fig. 20). As expected PCNA, a marker of proliferation, was upregulated by HPV E7 expressing cells (Cheng *et al.*, 1995).

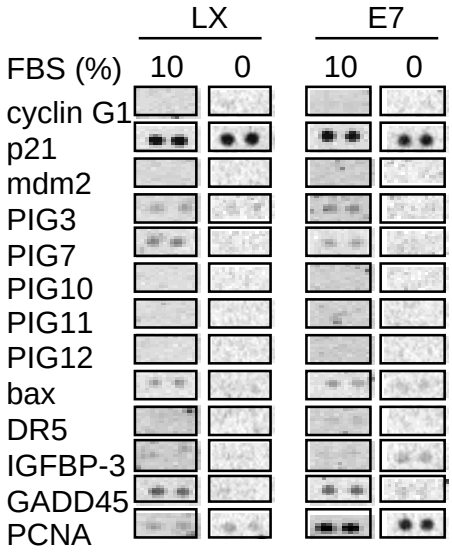

Figure 20: Expression analysis of a set of p53 responsive genes by transcriptional profiling. A "Human Apoptosis ATLAS" cDNA nylon array (Clontech) containing 12 p53 responsive genes was analyzed by sequential hybridization with ^{32}P-labeled single stranded cDNA probes. PCNA is a known HPV-16 E7-responsive gene and was used as a positive control.

Taken together these findings indicate that p53 is not activated as a transcription factor during HPV-16 E7 mediated apoptosis upon growth factor withdrawal.

4.2.1.4 The “trophic sentinel” response upon growth factor withdrawal depends on p53.

It was shown previously that the coexpression of HPV-16 E6 with HPV-16 E7 abolishes E7 mediated apoptosis upon growth factor withdrawal. Since expression of HPV-16 E6 results in the degradation of p53 it was concluded that HPV-16 E7 mediated apoptosis is p53 dependent. The observation that neither the DNA binding activity of p53 nor its phosphorylation status is changed and no p53 responsive genes are expressed at higher levels upon growth factor withdrawal does not support this model. This lead us to investigate whether HPV-16 E7 mediated apoptosis is independent of p53 as a transcription factor or whether transcription-independent mechanisms may be involved. Stable HPV-16 E7 expressing IMR90 cell lines were generated, coexpressing either a carboxyl terminal truncation-mutant of p53 (p53DD) or HPV-16 E6. p53DD which lacks the transactivation domain interacts with wild type p53 and thereby results in the formation of transcriptionally inert tetramers. Hence p53DD acts as a dominant negative mutant at least with respect to transcription but may not affect other, non-transcriptional functions of p53. The expression of HPV-16 E6 results in the degradation of p53 and abolishes p53-mediated transcriptional and non-transcriptional functions. The stable IMR90 cell lines (LX, E6, E7, p53DD, E6E7 and p53DDE7) were deprived of growth factors and p53, $p21^{CIP1}$ and HPV-16 E7 protein levels were determined by immunoblot. The expression of HPV-16 E6 (E6) results in a decrease of p53 steady state levels, while the expression of p53DD increases the steady state levels. The expression of either proteins result in an inhibition of the transcriptional activity of p53, as was determined by investigating the steady state levels of the p53 target gene $p21^{CIP1}$. The expression levels of HPV-16 E7 are shown as well and seems to be lower in cells co-expressing HPV-16 E6 (E6E7) or p53DD (p53DDE7). Steady state levels of p53 or $p21^{CIP1}$ are not elevated in any of these cell lines upon growth factor deprivation (Fig. 21A).

Next, the different IMR90 cell populations were grown in the absence of growth factors and apoptosis was determined by two independent methods. Staining of nuclear DNA with the dye Hoechst 33258 visualizes nuclear changes like chromatin condensation while the DNA fragmentation assay quantitates the fragmentation of the

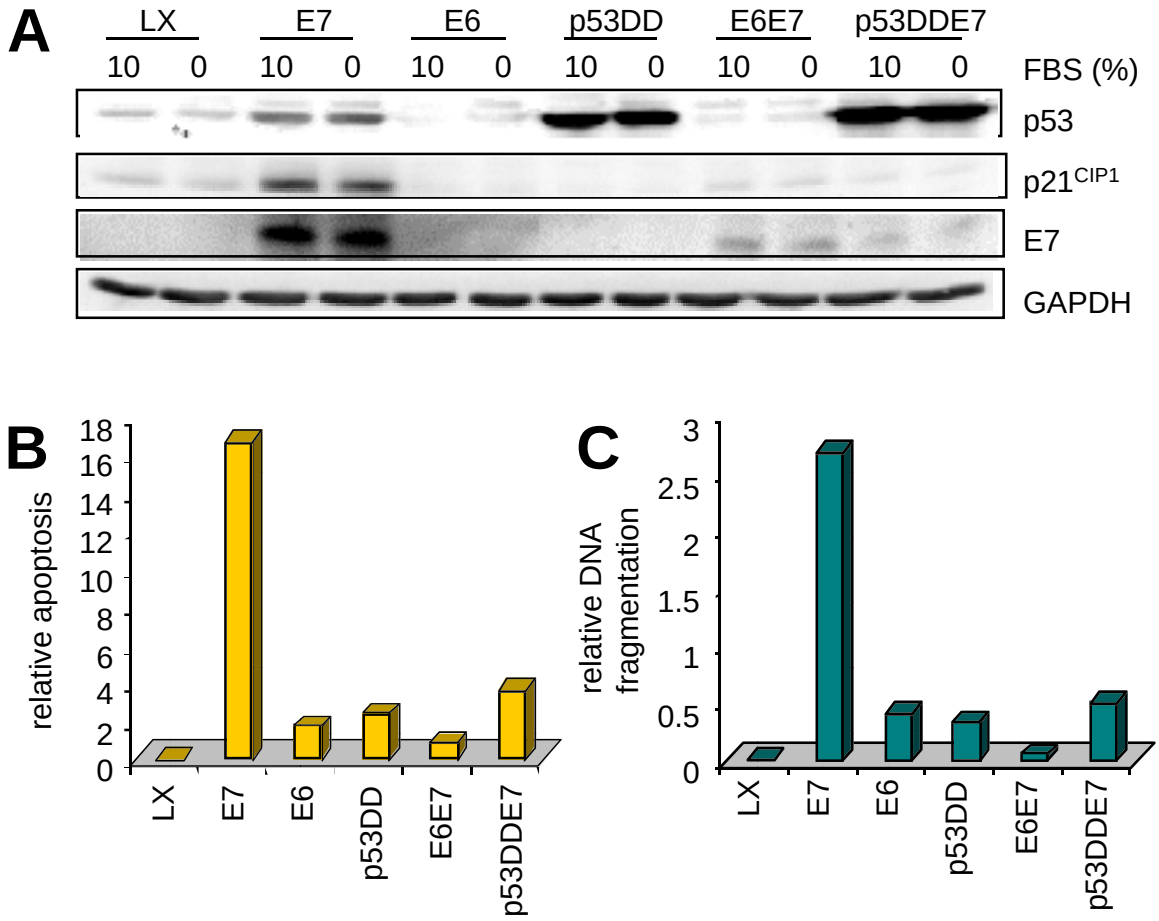

Figure 21: HPV-16 E7 mediated apoptosis is inhibited by coexpression of HPV-16 E6 or a dominant negative truncation mutant of p53 (p53DD). **(A)** IMR90 cells stably expressing empty vector (LX), HPV-16 E6 (E6), HPV-16 E7 (E7), a dominant negative C-terminal p53 fragment (p53DD) or HPV-16 E7 together with either HPV-16 E6 (E6E7) or p53DD (p53DDE7) were generated. These cell lines were either maintained in normal medium (10%) or deprived of growth factors (0%) and the protein levels of p53, $p21^{CIP1}$ and HPV-16 E7 levels were determined by immunoblot analysis. The protein expression levels of GAPDH were used as a loading control. **(B)** The different IMR90 cell lines were deprived of growth factors and apoptosis rates were determined by Hoechst staining. The background apoptosis rate in the presence of growth factors was between 0 and 0.5% in all cell lines. The relative apoptosis was determined by setting the amount of apoptosis observed in control cells upon growth factor withdrawal as the 0 baseline and comparing the apoptosis rates upon growth factor withdrawal of the other cell lines to that baseline. The shown results represent the mean of 3 independent experiments. **(C)** The apoptosis rates of the different IMR90 cell lines upon growth factor deprivation were determined by using a quantitative DNA fragmentation assay. The fold increase in DNA fragmentation of each cell line was determined by comparison of the amount DNA fragmentation in response to growth factor withdrawal to the amount of background DNA fragmentation in proliferating cells (0-0.2 %). The relative increase in DNA fragmentation was determined by setting the DNA fragmentation observed in control cells upon growth factor withdrawal as the 0 baseline and graphing the DNA fragmentation of the other cell lines relative to this baseline. The data represent the mean of 4 independent experiments.

chromosomal DNA to nucleosome-sized pieces. Both, nuclear condensation and nucleosomal DNA fragmentation are hallmarks of apoptotic cells (Kerr *et al.*, 1972; Oberhammer *et al.*, 1993; Wyllie, 1980). As expected HPV-16 E7 expressing cells show elevated levels of apoptosis. Cells expressing HPV-16 E6 or p53DD alone have similar apoptosis and DNA fragmentation levels as growth factor deprived control cells (Fig. 21B,C). Coexpression of either HPV-16 E6 or p53DD can each abrogate the HPV-16 E7 mediated apoptotic response (Fig. 21B,C). This indicates that, although p53 is not active as a transcription factor, as determined by gene expression profiling (Fig. 20), it is necessary for HPV-16 E7 mediated apoptosis upon growth factor withdrawal.

4.2.2 Changes in gene expression of HPV-16 E7 expressing cells undergoing apoptosis.

Gene expression profiling was performed using a cDNA array membrane (Human Apoptosis ATLAS cDNA array, Clontech) containing 205 different apoptosis related genes including tumor necrosis factor alpha (TNF-α), TNF related apoptosis inducing ligand (TRAIL), TNF-α receptor 1 (TNFR1), caspases and Bcl-2 family members. Only two of the 205 monitored genes were upregulated more that 2 fold in HPV-16 E7 expressing genes compared to control cells. Neither of these are known p53 responsive genes. The finding that the expression levels of both genes were upregulated even further in HPV-16 E7 expressing cells upon growth factor deprivation indicated their possible involvement in HPV-16 E7 mediated apoptosis. These two genes are insulin-like growth factor binding proteins (IGFBPs) 2 and 5. They are secreted proteins that regulate the availability of the survival factor Insulin-like growth factor (IGF-I) (reviewed in Binoux, 1996) to its receptor and thereby may have an impact on the "trophic sentinel" response.

4.2.2.1 Insulin-like growth factor binding proteins (IGFBPs) -2 and -5 are upregulated during HPV-16 E7 mediated apoptosis upon growth factor withdrawal.

Upregulation of IGFBP-2 and –5 gene expression was observed in proliferating HPV-16 E7 expressing cells compared to control cells. IGFBP-3, a p53 responsive protein that has been implicated in negatively regulating the ability of IGF-I to its receptor and thereby inhibiting its growth promoting properties (Rajah *et al.*, 1997) was

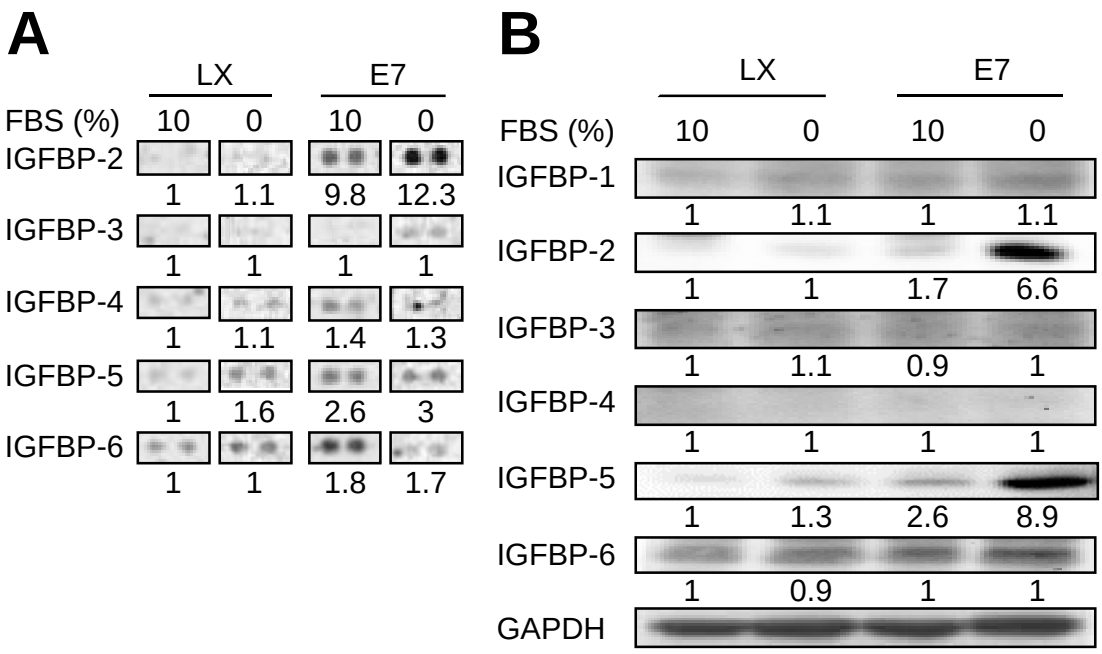

Figure 22: Gene expression and protein levels of Insulin-like growth factor binding proteins (IGFBPs) in control (LX) and HPV-16 E7 (E7) expressing IMR90 cells upon growth factor withdrawal. **(A)** Expression analysis of five IGFBP genes by transcriptional profiling. A "Human Apoptosis ATLAS" cDNA nylon array (Clontech) was analyzed by sequential hybridization with ^{32}P-labeled single stranded cDNA probes. **(B)** Immunoblot analyses of IGFBP 1-6 in control (LX) and HPV-16 E7 expressing IMR90 cells upon growth factor deprivation. The protein expression levels of GAPDH were used as a loading control.

reported to be bound and inhibited by HPV-E7 (Mannhardt *et al.*, 2000). However IGFBP-3 was not found to be upregulated in proliferating HPV-16 E7 expressing cells compared to control cells and its expression level upon growth factor deprivation did not change whereas the gene expression levels of IGFBP-2 and –5 increase even further in HPV-16 E7 expressing cells upon growth factor withdrawal (Fig. 22A). Immunoblot analyses were consistent with the gene expression profiling results showing that IGFBP-2 and –5 are upregulated in HPV-16 E7 expressing cells and that their expression is even further elevated upon growth factor deprivation (Fig. 22B). The gene expression levels as well as the protein levels of other known IGFBPs were investigated as well, but only IGFBP-2 and –5 were observed to be upregulated upon growth factor deprivation in HPV-16 E7 expressing cells (Fig. 22A,B).

4.2.2.2 The upregulation of IGFBP-2 and IGFBP-5 are dependent of p53

To investigate whether this upregulation of IGFBP-2 and-5 is dependent on p53, the HPV-16 E7 expressing IMR90 cell lines that co-express either HPV-16 E6 or p53DD were deprived of growth factors and the protein levels of IGFBP-2 and –5 were determined by immunoblot. The expression of IGFBP-2 and –5 is upregulated to a comparable extent in all cell lines expressing HPV-16 E7 independent of the coexpression of HPV-16 E6 or p53DD. Interestingly, the expression levels of IGFBP-2 and –5 are only further elevated in apoptotic HPV-16 E7 expressing cells while the protein levels remain almost unchanged in all other cell lines upon growth factor withdrawal (Fig. 23). This indicates that the upregulation of IGFBP-2 and –5 may be dependent on p53 to some extent, although it remains to be elucidated whether increased expression of these proteins mechanistically contributes to the induction of apoptosis. The fact that HPV-16 E7 expression in the E6 or p53DD coexpressing cells is lower correlates with the decreased expression of IGFBP-2 and –5 in these cells.

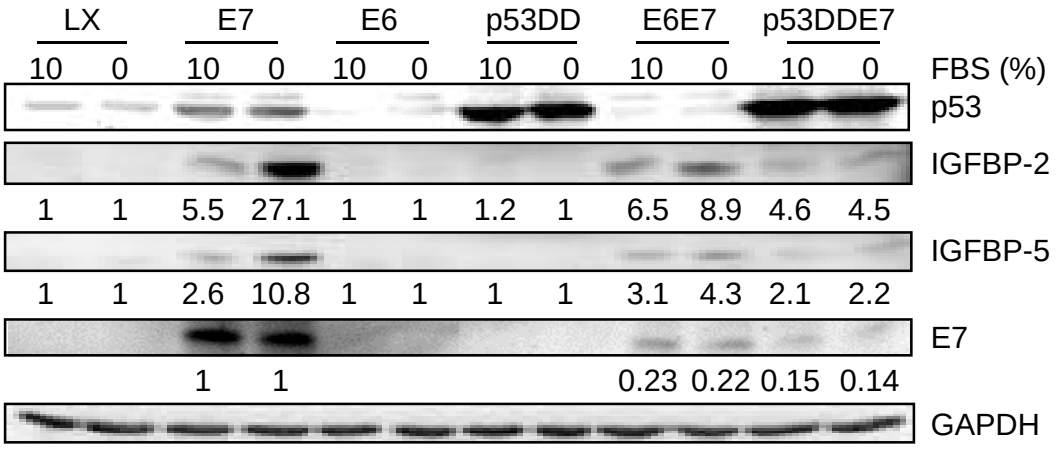

Figure 23: IGFBP-2 and IGFBP-5 protein levels are upregulated in apoptotic HPV-16 E7 expressing IMR90 cells. IMR90 cells stably expressing empty vector (LX), HPV-16 E6 (E6), HPV-16 E7 (E7), a dominant negative C-terminal fragment of p53 (p53DD) or HPV-16 E7 together with either HPV-16 E6 (E6E7) or p53DD (p53DDE7) were deprived of growth factors. IGFBP-2 and IGFBP-5 protein levels were determined by immunoblot analysis. The co-expression of HPV-16 E6 or a dominant negative mutant of p53 (p53DD) with HPV-16 E7 abolished the upregulation of IGFBP-2 and -5 protein levels upon growth factor deprivation. The protein expression levels of GAPDH were used as a loading control.

4.2.2.3 NFκB is activated in HPV-16 E7 expressing cells upon growth factor withdrawal.

NFκB is a transcription factor that can exhibit pro- as well as anti-apoptotic activities. It was reported that the IGFBP-2 gene has a NFκB binding site in its promoter (Cazals *et al.*, 1999). Since elevation of IGFBP-2 is observed, we investigated whether the transcription factor NFκB is activated upon the induction of apoptosis in HPV-16 E7 expressing IMR90 cells upon growth factor withdrawal. An EMSA was performed and it was observed that the DNA binding activity of NFκB is increased in apoptotic HPV-16 E7 expressing IMR90 cells (Fig. 24). This indicates that the activation of NFκB correlates with the induction of IGFBP-2 in these cells. Since NFκB can exhibit pro- as well as anti-apoptotic functions, it has to be further elucidated whether NFκB activation enhances or delays the observed apoptosis.

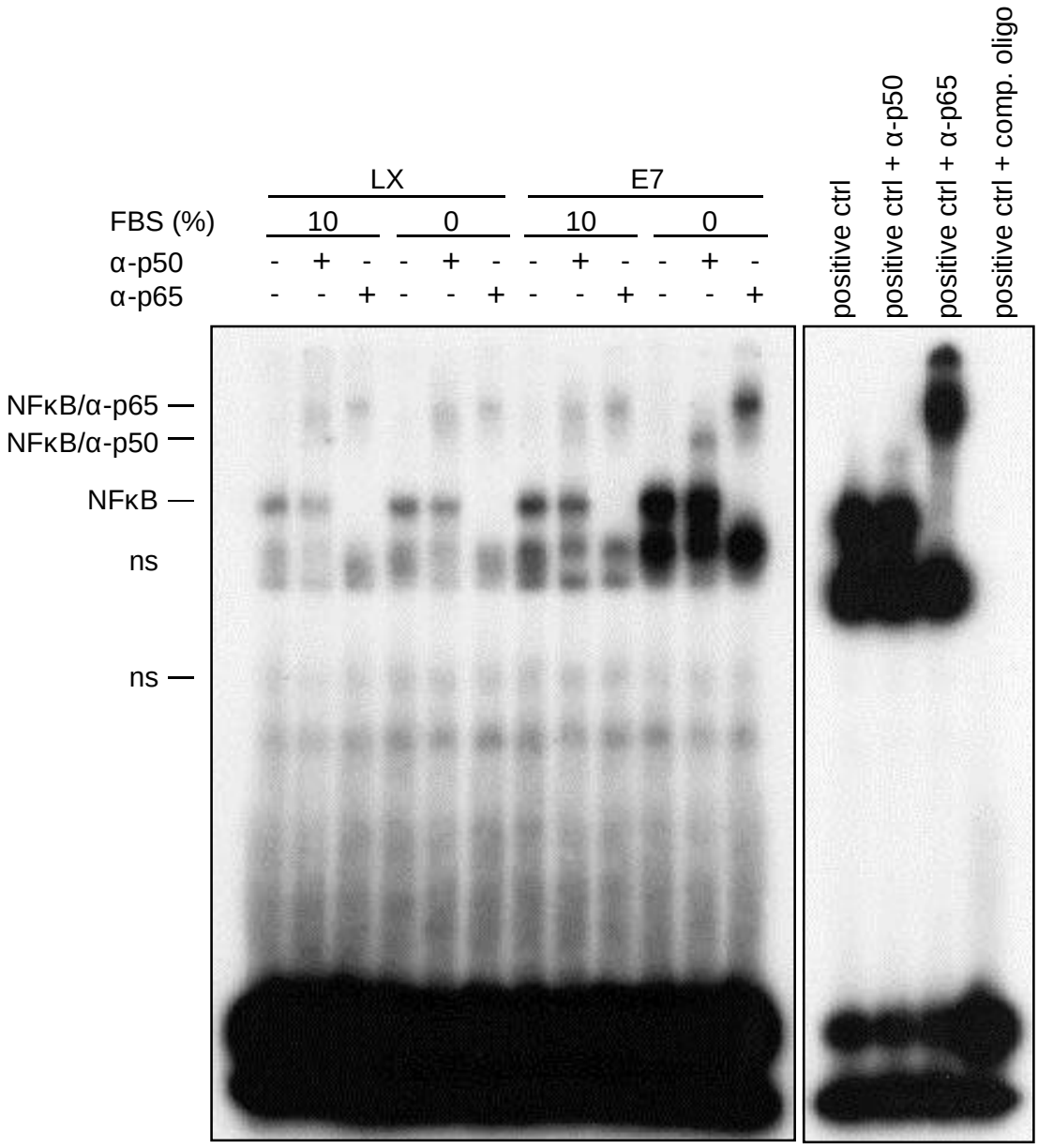

Figure 24: The activity of NFκB is elevated in HPV-16 E7 expressing cells upon growth factor deprivation. Control and HPV-16 E7 expressing IMR90 cells were deprived of growth factors. The DNA binding activity of NFκB was determined using Electrophoretic Mobility Shift Assay (EMSA). U2OS cells transfected with the NFκB subunits p50 and p65 were used as positive controls. The NFκB/oligonucleotide (NFκB) complexes were identified by supershift analysis using α-p50 and α−p65 antibodies.

4.2.3 The involvement of known apoptotic pathways in HPV-16 E7 mediated apoptosis

1.1.1.1 The involvement of the caspase cascade in HPV-16 E7 mediated apoptosis upon growth factor withdrawal.

Caspases are mediators of apoptosis (Enari *et al.*, 1998; Green and Kroemer, 1998). The activity of different caspases (caspase 3 and 8) were therefore determined in HPV-16 E7 expressing cells upon growth factor withdrawal. ApoAlert Fluorometric caspase activity assay kits (Clontech), which measure the specific activity of caspase 3 and caspase 8, were used. It was observed that the specific activity of caspase 8 remained

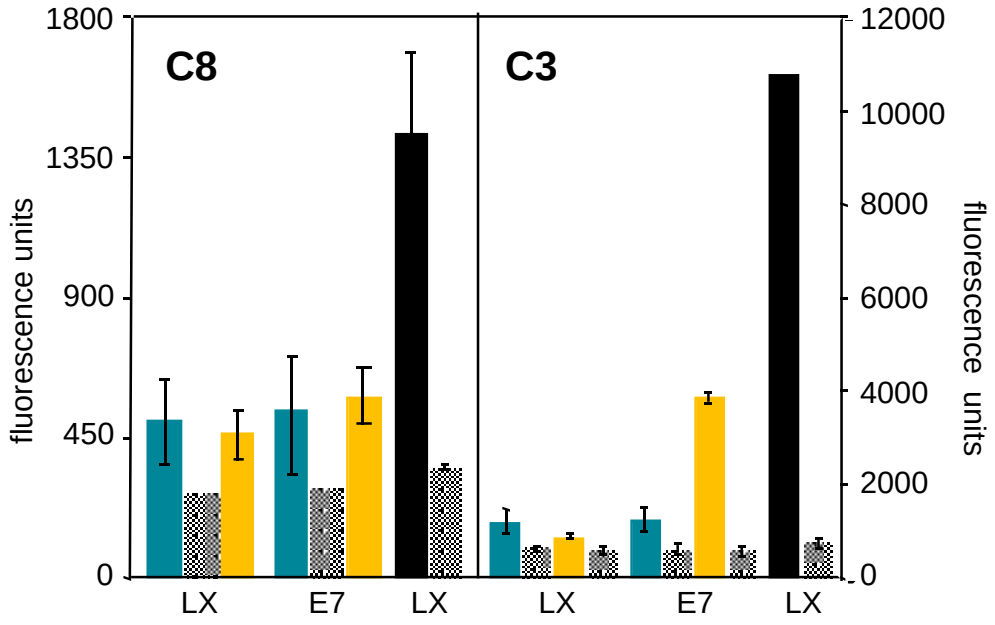

Figure 25: Activity levels of caspase 8 (C8) and caspase 3 (C3) in control (LX) and HPV-16 E7 expressing cells upon growth factor withdrawal. Control (LX) and HPV-16 E7 expressing IMR90 cells were deprived of growth factors and caspase 8 activity was determined using the ApoAlert Caspase 8 Fluorescence Assay kit. The addition of the specific caspase 8 inhibitor IETD-fmk was used to prove the specificity of the reaction. Control (LX) cells treated with TNF-α were used as a positive control. Caspase 3 activity of the same cells was determined using the ApoAlert Caspase 3 Fluorescence Assay kit. The addition of the specific caspase 3 inhibitor DEVD-CHO was used to guarantee the specificity of the reaction. Control (LX) cells treated with TNF-α and cycloheximide were used as a positive control.

unchanged during HPV-16 E7 mediated apoptosis upon growth factor withdrawal, while the specific activity of caspase 3 was increased (Fig. 25). This indicates that caspase 3 is activated through caspase 8 independent mechanisms. Caspase 8 activity is mostly regulated by death receptors such as the TNF receptor (TNFR), while caspase 3, a downstream effector caspase, can be activated by caspase 8 and 9. Control IMR90 cells that were treated with TNF-α served as a positive control for caspase 8 and caspase 3 activity.

4.2.3.2 Cytochrome c release in HPV-16 E7 mediated apoptosis upon growth factor withdrawal.

The finding that caspase 3 was activated while the activity of caspase 8 remained unchanged indicated that the mitochondria, which signal upstream of caspase 3 and do not necessarily receive their signals through caspase 8, might be involved. To investigate the possible role of the mitochondria in HPV-16 E7 mediated apoptosis upon growth factor withdrawal, HPV-16 E7 expressing cells and control cells were deprived of growth factors and subjected to subcellular fractionation. Untreated and taxol-treated MCF-7 cells were used as a positive control (Kottke *et al.*, 2002). The release of cytochrome c from the mitochondria was determined by measuring the cytochrome c contents of the mitochondrial and the cytoplasmic fraction using a human cytochrome c detection ELISA kit (Quantikine, R&D systems). The ratio of cytochrome c content of mitochondrial to cytoplasmic fraction of proliferating and growth factor deprived control and E7 expressing cells was determined. After setting the cytochrome c release of proliferating control and HPV-16 E7 expressing cells and untreated MCF-7 cells as zero-baseline, the differences in cytochrome c distribution were graphed. An increase in cytochrome c in the cytoplasmic fraction was observed in growth factor deprived HPV-16 E7 expressing cells (Fig. 26). These results correlate with the activation of caspase 3 (Fig. 25).

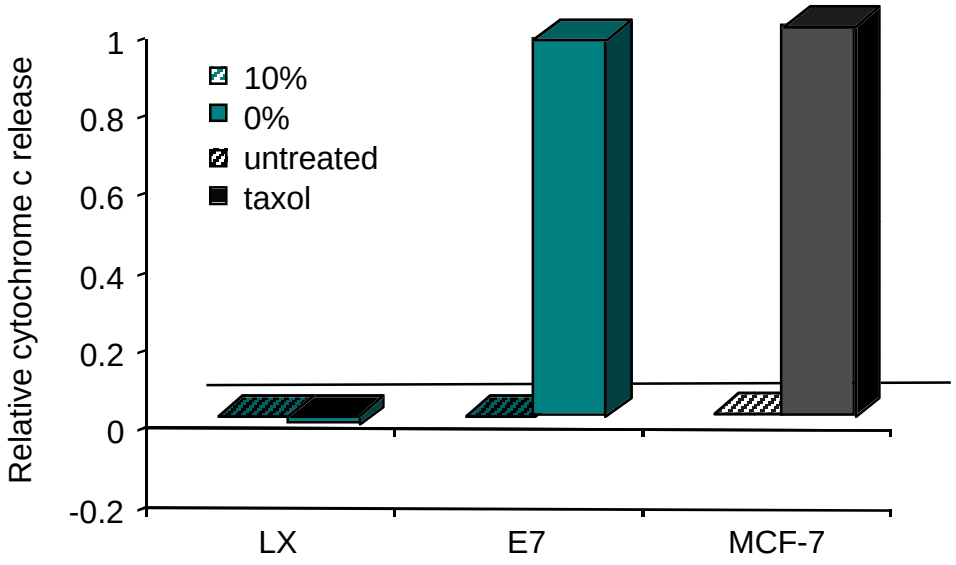

Figure 26: Cytochrome c release from the mitochondria in HPV-16 E7 expressing IMR90 cells upon growth factor deprivation. Control (LX) and HPV-16 E7 expressing cells (E7) were deprived of growth factors and subsequently subjected to subcellular fractionation. Untreated and taxol-treated MCF-7 cells were used as controls. The cytochrome c content of the mitochondrial and the cytoplasmic fraction was determined using the Quantikine human cytochrome c ELISA kit (R&D systems). The cytochrome c content of the cytoplasmic fraction was determined by normalizing it to the cytochrome c content of the mitochondrial fraction. Subsequently the cytochrome c contents in the cytoplasmic fraction of proliferating control and HPV-16 E7 expressing cells as well as untreated MCF-7 cells were set as zero baseline and the relative cytochrome c release upon growth factor deprivation or taxol-treatment was determined according to this baseline and graphed.

4.2.3.3 Translocation of AIF from the mitochondria to the nucleus in apoptotic HPV-16 E7 expressing cells

It was previously shown that the apoptosis inducing factor (AIF) is released from the mitochondria and translocates to the nucleus where it exhibits nuclease activity and results in DNA fragmentation (Susin *et al.*, 1999). To investigate whether AIF is involved in HPV-16 E7 mediated apoptosis upon growth factor deprivation immunofluorescence analyses were performed. The human Tid-1 gene encodes two alternatively spliced mitochondrial proteins (Syken *et al.*, 1999) that are not released from the mitochondria during apoptosis (unpublished data) and were used as controls for mitochondrial localization. AIF colocalizes with hTid-1 proteins in proliferating control and HPV-16 E7

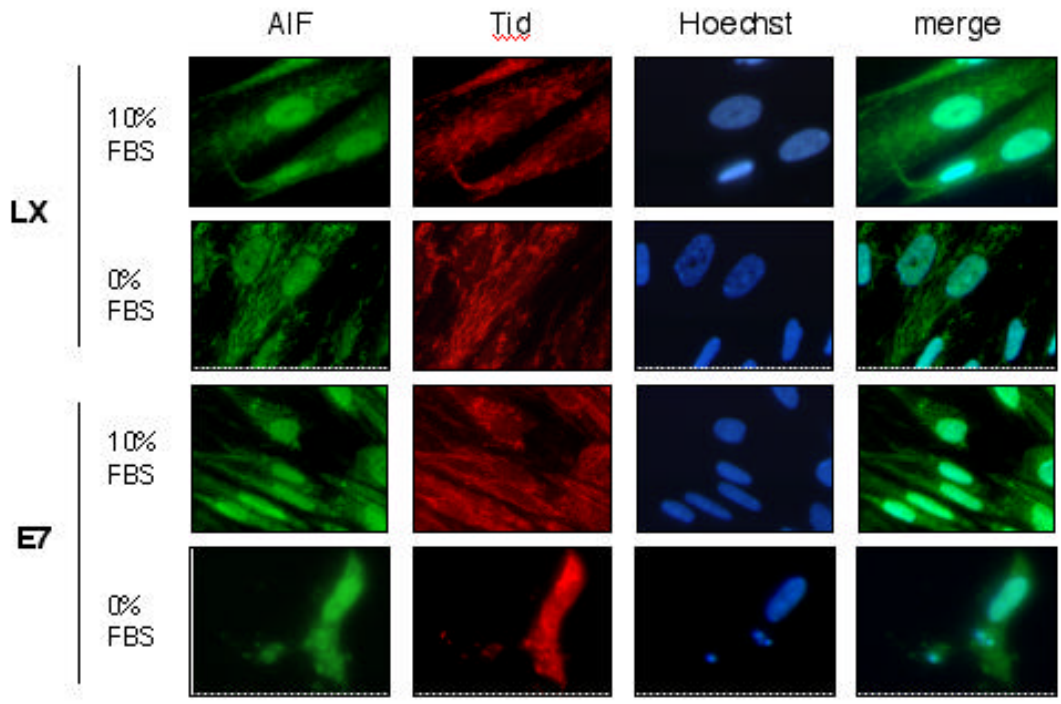

Figure 27: Localization of the apoptosis-inducing factor (AIF) in proliferating and growth factor deprived control (LX) and HPV-16 E7 expressing cells. Control and HPV-16 E7 expressing cells were deprived of growth factors and localization of AIF and the mitochondrial hTid-1 proteins (Tid) were determined by immunofluorescence. Nuclei were visualized by Hoechst 33258 staining.

expressing cells (Fig. 27). This colocalization of AIF with hTid-1 was also observed in growth factor deprived control and HPV-16 E7 expressing cells. This finding shows that AIF does not translocate to the nucleus in HPV-16 E7 expressing cells upon growth factor deprivation (Fig. 27) and indicates that it is not involved in HPV-16 E7 mediated apoptosis.

5. Discussion

5.1 Stabilization and functional impairment of the tumor suppressor p53 by the human papillomavirus type 16 E7 oncoprotein

5.1.1 Functional impairment of the tumor suppressor p53 by the HPV-16 E7 oncoprotein

In normal cells, stabilization of p53 is generally observed in response to genotoxic insults. Under these conditions, p53 stabilization leads to a corresponding increase of its transcriptional activation potential and transcriptional induction of downstream target genes (reviewed in Meek, 1999). Many of these genes encode growth suppressive or cytotoxic proteins, and consequently, activation of p53 generally leads to growth arrest or apoptosis (reviewed in Levine, 1997). In contrast, IMR90 cells stably expressing HPV-16 E7 grow rapidly in the presence of elevated p53 levels. The rate of spontaneous apoptosis in these cells is similar to control cells. Previous work has indicated that expression of HPV-16 E7, while it mimics a p53-dependent DNA damage response, does not fully activate p53 (Jones *et al.*, 1999). Most dramatically, $p21^{CIP1}$ protein but not mRNA levels are increased in E7 expressing cells (see Fig. 10, 9B) consistent with an effect on protein stabilization rather than p53-dependent transcriptional induction (Jones *et al.*, 1999). Hence we analyzed in more detail whether the stabilized p53 in HPV-16 E7 expressing cells is activated as a transcription factor. Analysis of the transcriptional activity of an artificial p53-responsive reporter construct in a matched pair of control and HPV-16 E7 expressing IMR90 cells suggested that, despite the higher steady state levels (Fig. 8,10) the transcriptional activity of p53 was decreased in HPV-16 E7 expressing cells (Fig. 9A). Consistent with these findings, transcriptional profiling of matched control and HPV-16 E7 expressing cells showed no marked increases in the mRNA levels of any of the known p53-responsive genes present on this array (Fig. 9B). Similarly, immunoblot analyses showed that levels of the pro-apoptotic p53 targets bax (Miyashita and Reed, 1995) and DR5 (alias TRAIL-R2/Apo2/Killer) (Wu *et al.*, 1997) were not increased in rapidly growing HPV-16 E7 expressing IMR90 cells (Fig. 10). The decreased activity of the p53 responsive luciferase reporter (Fig. 9A) appears to be at variance with the unchanged levels of p53 target genes as determined by transcriptional profiling (Fig. 9B)

and may suggest that other transcriptional elements are also involved in regulating the expression of these genes. Regardless, our results demonstrate that expression of HPV-16 E7 results in stabilization of the protein but not activation of p53.

The mechanisms that lead to p53 stabilization in response to HPV-16 E7 expression appear different from those involved in DNA damage. Our findings suggest that posttranslational modifications such as phosphorylation and/or acetylation events that are necessary to trigger transcriptional activation of the stabilized p53 are not induced by HPV-16 E7 expression. Alternatively, HPV-16 E7 may interfere with the transcriptional activation of p53. This has been suggested by earlier studies demonstrating that the expression of HPV-16 E7 could interfere with p53-dependent activation of reporter plasmids in transient assays. These experiments suggested that the ability of HPV-16 E7 to interfere with p53-mediated transcriptional activation might correlate with the capacity of HPV-16 E7 to interact with TATA-box binding protein, TBP (Massimi and Banks, 1997). Additional studies will be necessary to further address these issues. To determine whether HPV-16 E7 interferes with the transcriptional activation of p53, its effects on exogenous p53 were investigated. Reporter assays with increasing amounts of exogenous p53 were performed in a matched pair of control and HPV-16 E7 expressing IMR90 cells using an artificial p53 responsive reporter construct. These studies showed that exogenous p53 is transcriptionally active in HPV-16 E7 expressing cells while the transcriptional activity of endogenous p53 is impaired by the expression of HPV-16 E7 (Fig. 11). This indicates that the transcriptional inactivation of p53 by HPV-16 E7 might be a “long term” effect and therefore may not be observed in transiently transfected cells. Similarly the stabilization of p53 by HPV-16 E7 could never be observed in transient assays. This may indicate that the stabilization and transcriptional inactivation of p53 by HPV-16 E7 might be linked. Further experiments will be necessary to further illuminate this mechanism.

5.1.2 Susceptibility of p53 to mdm2 mediated degradation in HPV-16 E7 expressing IMR90 cells

In normal cells p53 stabilization is due to phosphorylation which inhibits mdm2 mediated degradation. The ubiquitin ligase mdm2 is a transcriptional target of p53 and is

thought to play a role as a feedback regulator to keep p53 activity in check (Wu *et al.*, 1993). During carcinogenic progression of high-risk HPV infected cells p53 is targeted for proteasomal degradation by a complex of the HPV-16 E6 oncoprotein the ubiquitin ligase E6-AP (Scheffner *et al.*, 1993; Scheffner *et al.*, 1995). Furthermore, it has been reported that mdm2 mediated p53 degradation is completely abrogated in HPV-positive cervical cancer cells and critically depends on E6/E6-AP (Hengstermann *et al.*, 2001). Given the fact that the only other viral protein consistently expressed in cervical cancer cells is HPV E7 it is thus conceivable that HPV-16 E7 is responsible for blocking mdm2-mediated p53 degradation in these cells.

Other oncoproteins including SV40 TAg (Tiemann and Deppert, 1994), Ad E1A (Lowe and Ruley, 1993), and c-myc (Hermeking and Eick, 1994) can stabilize p53. Since each of these oncoproteins results in dysregulation the G1/S transition it is plausible that aberrant activation of the pRB-regulated transcription factor E2F may cause p53 stabilization. One potential mechanistic link between E2F activation and p53 stabilization was suggested by the finding that $p14^{ARF}$, an inhibitor of mdm2-mediated p53 degradation (Zhang *et al.*, 1998) was E2F responsive (Bates *et al.*, 1998), and its cellular levels increased in response to Ad E1A and c-myc expression (de Stanchina *et al.*, 1998; Zindy *et al.*, 1998). Surprisingly, however, $p14^{ARF}$ levels do not increase upon HPV-16 E7 expression, and HPV-16 E7 mediated p53 stabilization was observed in mouse embryo fibroblasts deficient of the murine homolog of $p14^{ARF}$, $p19^{ARF}$ (Seavey *et al.*, 1999). Hence other pathways must exist that can interfere with either the ability of mdm2 to ubiquitinate p53 or the process of proteasomal p53 degradation.

A previous study reported increased transcription from the p53 responsive mdm2 promoter P2 in HPV-16 E7 expressing cells (Seavey *et al.*, 1999). Our analysis did not reveal any evidence for increased mdm2 mRNA expression in HPV-16 E7 expressing cells (Fig. 9B). Consistent with this, mdm2 protein levels were only slightly increased in HPV-16 E7 expressing cells (Fig. 12A). Hence we measured the transcriptional activation of a mdm2 P2 promoter driven reporter construct in control and HPV-16 E7 expressing IMR90 cells. Consistent with the results obtained with the artificial p53-responsive reporter (Fig. 9A), we did not detect increased activation of this reporter in HPV-16 E7 expressing cells, in fact, the transcriptional activity of the mdm2 P2 promoter

construct was decreased in HPV-16 E7 expressing cells (Fig. 12B). This supports the finding that the stabilized p53 is not transcriptionally active in HPV-16 E7 expressing cells without additional stimuli such as DNA damage, as was shown using gene expression profiling (Fig. 9B), transient reporter assays (Fig. 9A) and immunoblot analyses (Fig. 10). To investigate whether HPV-16 E7 inhibits the interaction of mdm2 and p53 or blocks the degradation of mdm2/p53 complexes, co-immunoprecipitation studies were performed. Our analyses revealed no evidence for a disruption of the mdm2/p53 interaction in normal or HPV-16 E7 expressing cells (Fig. 12A), suggesting that the interaction of mdm2 and p53 is not generally impaired in HPV-16 E7 expressing cells. This is in contrast to a previous study where mdm2 and p53 interaction was dramatically reduced in the presence of HPV-16 E7 (Seavey *et al.*, 1999). The two studies were performed with different strains of diploid normal human fibroblasts and it is of note that in the IMR90 cells used in this study, we did not observe a dramatic upregulation of mdm2 expression in HPV-16 E7 expressing cells (Fig. 9B, 12A) suggesting that either there are differences in the expression levels of HPV-16 E7 in the two studies or, that there may be differences in the susceptibility to p53 stabilization and activation between these strains of human fibroblasts. The finding that mdm2 is bound to p53 in HPV-16 E7 expressing IMR90 cells correlates with the finding that the stabilized p53 is not transcriptionally active since mdm2 binds to the transactivation domain of p53. Interestingly, however, our experiments revealed that the stabilized p53 in HPV-16 E7 expressing cells remains susceptible to proteasomal degradation by exogenous mdm2 as well as by the HPV-16 E6/E6-AP complex (Fig. 13). Additional studies will be necessary to delineate whether E7 interferes with mdm2-mediated ubiquitination of p53, or whether HPV-16 E7 affects the turnover of ubiquitinated p53.

Interestingly, mdmX a homologue of mdm2 that may act as a dominant negative inhibitor of mdm2 has been described. MdmX can bind p53 and stabilize it by preventing mdm2-mediated degradation through the proteasome (Jackson and Berberich, 2000; Sharp *et al.*, 1999). MdmX bound p53 was reported to be transcriptionally inactive (Shvarts *et al.*, 1997). These results suggest that mdmX may be an attractive candidate to act as a mediator of p53 stabilization in the presence of HPV-16 E7. Preliminary analyses revealed a 1.4 fold increase in mdmX mRNA levels in HPV-16 E7 expressing cells (Fig.

12C). Due to antibody limitations it could not be determined whether mdmX or a related protein may be present at higher levels in HPV-16 E7 expressing cells and may contribute to p53 stabilization in HPV-16 E7 expressing IMR90 cells.

5.1.3 Stabilization of the tumor suppressor p53 by the HPV-16 E7 oncoprotein

Stabilization and inactivation of p53 can occur through different mechanisms and it was important to investigate how HPV-16 E7 mediates the observed p53 stabilization and inactivation. It has been reported that wild-type p53 can be functionally inactivated by nuclear exclusion. This has been documented in different carcinomas (Bosari *et al.*, 1995; Moll *et al.*, 1995; Moll *et al.*, 1992; Schlamp *et al.*, 1997; Ueda *et al.*, 1995), and in addition, some viral oncoprotein proteins such as the Ad E1B-55kDa protein and the hepatitis B virus X protein can mislocalize p53 to the cytoplasm, thereby inhibiting its transcriptional activities (Elmore *et al.*, 1997; van den Heuvel *et al.*, 1993). However, subcellular fractionation studies showed that p53 in HPV-16 E7 expressing cells remains nuclear (Fig. 16,13). In some instances, p53 protein, which assumes a mutant conformation is stabilized. This was observed in many cancers that contain a p53 with a point mutation in its DNA binding domain (Cho *et al.*, 1994; Pavletich *et al.*, 1993). These point mutations oftentimes produce a change in the global conformation of the protein which can be monitored by a set of antibodies that recognize conformational epitopes (Gannon *et al.*, 1990; Milner *et al.*, 1987). Immunoprecipitation studies with these conformation specific antibodies (Fig. 15A) as well as treatment with geldanamycin, a compound that disrupts mutant p53/hsp90 complexes (Fig. 15B), demonstrated that the stabilized p53 in HPV-16 E7 expressing cells remains in a wild-type conformation. As mentioned earlier the phosphorylation of p53 at specific amino acid residues results in its stabilization. An analysis of the phosphorylation state of p53 by two-dimensional gel electrophoresis displayed no major differences in the number of phospho-forms in control or HPV-16 E7 expressing cells (Fig. 14). Taken together these results show that the p53 in HPV-16 E7 expressing cells is in the normal compartment of the cell, maintains a wild type conformation and can still bind to mdm2. Treatment of HPV-16 E7 expressing cells with the nuclear export inhibitor leptomycin B led to a further increase in p53 steady state levels, demonstrating that nuclear export of p53 is not

generally abrogated in HPV-16 E7 expressing cells (Fig. 17). The observation that p53 can be exported from the nucleus indicates that the stabilization might be due to an impairment of p53 degradation. This was investigated by treatment of control and HPV-16 E7 expressing cells with the proteasome inhibitor lactacystin. However, similar to the nuclear export inhibitor leptomycin B, lactacystin treatment resulted in increased p53 levels in normal and HPV-16 E7 expressing cells. These results suggest that proteasomal p53 degradation is not completely blocked in HPV-16 E7 expressing cells (Fig. 17). Another possibility is that HPV-16 E7 may interfere with a step that involves recognition of ubiquitinated forms of p53 by the proteasome. This process has not yet been studied in great detail, however, it has been postulated that ubiquitin binding proteins such as hPLIC that are associated with the proteasome may play a role in this process (Kleijnen *et al.*, 2000). Further experiments are necessary to investigate this hypothesis.

5.2 The “trophic sentinel” response of HPV-16 E7 expressing cells upon growth factor withdrawal

5.2.1 Involvement of the tumor suppressor p53 in the “trophic sentinel” response of HPV-16 E7 expressing cells

Cells expressing oncogenes such as HPV-16 E7 are predisposed to apoptosis upon suboptimal growth conditions (Jones *et al.*, 1997b). It is not clear, however, whether this “trophic sentinel” response is mediated by p53. The involvement of p53 in most other apoptotic responses, such as upon DNA damage, is well established, but the “trophic sentinel” response is not as well studied as the “DNA damage sentinel” response. It was shown previously that coexpression of HPV-16 E6 abrogates the HPV-16 E7 mediated apoptosis upon growth factor deprivation (Jones *et al.*, 1997b). This indicates that this apoptosis response might be p53 dependent. We previously showed that the stabilized p53 in HPV-16 E7 expressing cells is transcriptionally inert in proliferating cells. Since p53 can mediate apoptosis through transcription-dependent and –independent pathways, the possible transcriptional involvement of p53 in the ”trophic sentinel” response was determined. In order to become active as a transcription factor upon different stimuli p53 has to be stabilized and activated by posttranslational modifications such as phosphorylation. Therefore the phosphorylation of p53 at known residues was

investigated using phosphospecific antibodies. This analysis revealed no differences in the phosphorylation status of p53 in proliferating or apoptotic HPV-16 E7 expressing cells (Fig. 18). The observation that the phosphorylation status of p53 in control (LX) and HPV-16 E7 expressing cells (E7) in the presence of growth factors is unchanged (Fig. 18) is consistent with the finding that the number of phospho-forms observed in these cells by two-dimensional gel electrophoresis were unchanged (Fig. 14). These phospho-forms very likely represent phosphorylations at amino acid residues different than those investigated by phosphospecific antibodies. To investigate whether p53 in HPV-16 E7 expressing cells is capable of binding to its consensus sequence, a p53 EMSA was performed. The observation that the stabilized p53 in proliferating HPV-16 E7 expressing cells can bind to its DNA consensus sequence (Fig. 19) was somewhat surprising since it is not transcriptionally active (Fig. 9A,B, 10). This indicates that HPV-16 E7 might interfere with the assembly of the transcription initiation complex rather than impairing the DNA binding capacity of the stabilized p53. Further experiments including chromatin immunoprecipitations will be necessary to illuminate this mechanism. The finding that the DNA binding capacity of p53 is neither enhanced in HPV-16 E7 expressing cells nor in control cells upon growth factor deprivation (Fig. 19) indicates that its DNA binding activity is not increased during HPV-16 E7 mediated apoptosis. This correlates with the finding that p53 is not phosphorylated at the investigated serine residues in apoptotic HPV-16 E7 expressing cells (Fig. 18). In addition, analysis of known p53 target genes did not reveal an increase in the transcriptional activity of p53 in HPV-16 E7 mediated apoptosis upon growth factor withdrawal (Fig. 20). This cDNA array did not include the genes PERP, Noxa or p53AIP1, which were reported to be involved in p53 mediated apoptosis (Attardi *et al.*, 2000; Oda *et al.*, 2000a; Oda *et al.*, 2000b), and the possibility remains that these factors might be involved in mediating HPV-16 E7 dependent apoptosis upon growth factor withdrawal.

Since p53 may induce apoptosis through transcription-independent mechanisms as well (Bennett *et al.*, 1998; Ding *et al.*, 2000; Yan *et al.*, 1997), we investigated whether the observed HPV-16 E7 mediated cell death in response to growth factor deprivation is independent of p53 or whether transcription-independent mechanisms are involved. Surprisingly, transcriptional inactivation by a dominant negative p53 mutant as

well as HPV E6 induced degradation of p53 (Fig. 21A) abrogated HPV-16 E7 dependent apoptosis (Fig. 21B,C). This shows that HPV-16 E7 mediated apoptosis upon growth factor withdrawal differs from c-myc induced apoptosis in growth factor deprived cells. The c-myc induced apoptotic response was shown to be independent of p53 taking the same approach by coexpressing the carboxyl terminal p53 fragment (p53DD) that acts as a dominant negative mutant (Juin *et al.*, 1999). This indicates that the transcriptional activity of p53 is necessary for HPV-16 E7 mediated apoptosis (Fig. 28) although none of the investigated target genes are induced nor are any of the investigated serine residues on p53 are phosphorylated. This finding may indicate that p53 is transcriptionally activated by either phosphorylation of other, not investigated residues or is subject to other posttranslational modifications in apoptotic HPV-16 E7 expressing IMR90 cells. It is also a possibility that the dominant negative carboxyl terminal fragment of p53 alters the conformation of the transcriptionally inactive p53 heterotetramers and thereby abolishes transcription-independent mechanisms as well. It has been reported that the p53 homologes p63 and p73, are required for p53 dependent apoptosis in response to DNA damage (Flores *et al.*, 2002). Some mutant forms of p53 that act in a dominant negative fashion can interact with p63 and p73 (reviewed in Moll *et al.*, 2001). It is therefore possible that p63 or p73 functions are altered by the dominant negative mutant p53 used and may contribute to the observed apoptosis. Further studies using mouse embryo fibroblasts (MEFs) where p53, p63 and/or p73 are deleted will be useful to investigate the role of p53 and related family members in HPV-16 E7 mediated apoptosis.

5.2.2 Unknown mediators of HPV-16 E7 mediated apoptosis upon growth factor withdrawal

In addition to investigating the gene expression levels of known p53 target genes, gene expression profiling was used to determine changes in gene expression levels of genes involved in HPV-16 E7 mediated apoptosis upon growth factor withdrawal. The used cDNA array membrane contained 205 different genes and although the cDNA array analysis showed no increase in expression levels of any of the known p53-target genes, two genes were observed to be upregulated in HPV-16 E7 expressing cells. These two genes are IGFBP-2 and IGFBP-5. Expression levels of these proteins were further

increased in apoptotic HPV-16 E7 expressing cells upon growth factor withdrawal (Fig. 22A). This increase was verified by immunoblot (Fig. 22B). Since these proteins are involved in the regulation of the availability of the survival factor IGF-I (reviewed in Binoux, 1996), they may play a role in the regulation of the "trophic sentinel" response in HPV-16 E7 expressing cells (Fig. 28). It is known that in cells overexpressing the c-myc oncogene, the apoptotic response can be held in check by the addition of survival factors such as the insulin-like growth factor (IGF-I) (Harrington *et al.*, 1994a). It remains to be investigated whether this is also the case in HPV-16 E7 mediated apoptosis following growth factor deprivation. Further experiments using growth media without growth factors and supplementing it with different growth factors such as IGF-I, insulin, fibroblast growth factor (FGF) or others, will be performed to determine the role of different growth factors in HPV-16 E7 mediated apoptosis upon suboptimal growth conditions. IGFBP-2 and IGFBP-5 were only upregulated in apoptotic HPV-16 E7 expressing cells while the protein levels remained unchanged upon growth factor deprivation in IMR90 cell lines coexpressing a transcriptionally inactive form of p53 (p53DD) or HPV-16 E6 with HPV-16 E7 (Fig. 23). This indicates that upregulation of the two IGFBPs during HPV-16 E7 mediated apoptosis is dependent on the transactivation domain of p53 although it is not transcriptionally active. IGFBP-2 and –5 levels may also be lower in cells coexpressing HPV-16 E6 or p53DD with HPV-16 E7 due to lower HPV-16 E7 protein expression levels. A report showing that the IGFBP-2 gene has NFκB binding sites in its promoter (Cazals *et al.*, 1999) led to the investigation whether NFκB is activated in apoptotic HPV-16 E7 expressing IMR90 cells. A previous report indicated crosstalk between the activated transcription factors p53 and NFκB. The two transcription factors were shown to inhibit each other's ability to stimulate target gene expression depending of the apoptotic stimuli (Webster and Perkins, 1999). In contrast to these findings another study showed that the induction of p53 not only results in NFκB activation but that p53-dependent apoptosis is abrogated by the loss of functional NFκB (Ryan *et al.*, 2000). Although the results of these two studies are contradictory, they indicate that p53 and NFκB signal pathways are somehow connected. Since the DNA binding activity of NFκB was elevated in HPV-16 E7 expressing IMR90 cells upon growth factor withdrawal (Fig. 24) but the DNA binding activity as well as the

transcriptional activity of p53 remained unchanged in growth factor deprived cells (Fig. 19, 20). This could indicate that NFκB negatively regulates the transcriptional activity of p53. On the other hand the two proteins may crosstalk on a non-transcriptional level. Since NFκB is a transcription factor that exhibits both pro- and antiapoptotic activities, it is not clear whether it is activated to counteract apoptosis or to enhance it. The same concern has to be raised about the IGFBPs, which were reported to be pro- or antiapoptotic. Further experiments using compounds that block activation of NFκB will provide insight into the role of NFκB activation in HPV-16 E7 mediated apoptosis (Fig. 28).

5.2.3 The involvement of known apoptotic pathways in HPV-16 E7 mediated apoptosis upon growth factor deprivation

Caspases are cysteine proteases that are activated by proteolytic processing (Green and Kroemer, 1998). They have the capacity to activate each other, which results in the initiation of a caspase cascade. The caspase cascade is involved in most apoptotic responses. The involvement of caspases in the “trophic sentinel” response is not very well established. Therefore, the activity of the downstream “effector” caspase 3 and the upstream “initiator” caspases 8 were determined. The activity of caspase 3 was found to be elevated while caspase 8 was not activated in HPV-16 E7 expressing cells upon growth factor deprivation. Activation of caspase 3 without activation of caspase 8 suggests the involvement of the mitochondria in the HPV-16 E7 mediated apoptosis. To investigate whether the mitochondria are involved, cytochrome c release was determined by measuring the changes of the cytochrome c contents of the mitochondrial and the cytoplasmic fraction upon growth factor withdrawal. Higher cytochrome c contents were observed in the cytoplasmic fraction of apoptotic HPV-16 E7 expressing cells, which correlates with the elevated activity of caspase 3 (Fig. 28).

The apoptosis-inducing factor (AIF) was reported to induce apoptosis in a caspase independent manner by translocating to the nucleus and inducing DNA fragmentation (Susin *et al.*, 1999). AIF localization in proliferating and growth factor deprived control and HPV-16 E7 expressing cells was determined by immunofluorescence. We found that AIF remains in the mitochondrial fraction in growth factor deprived HPV-16 E7

expressing cells. This indicates that AIF is not involved in HPV-16 E7 mediated apoptosis upon growth factor deprivation.

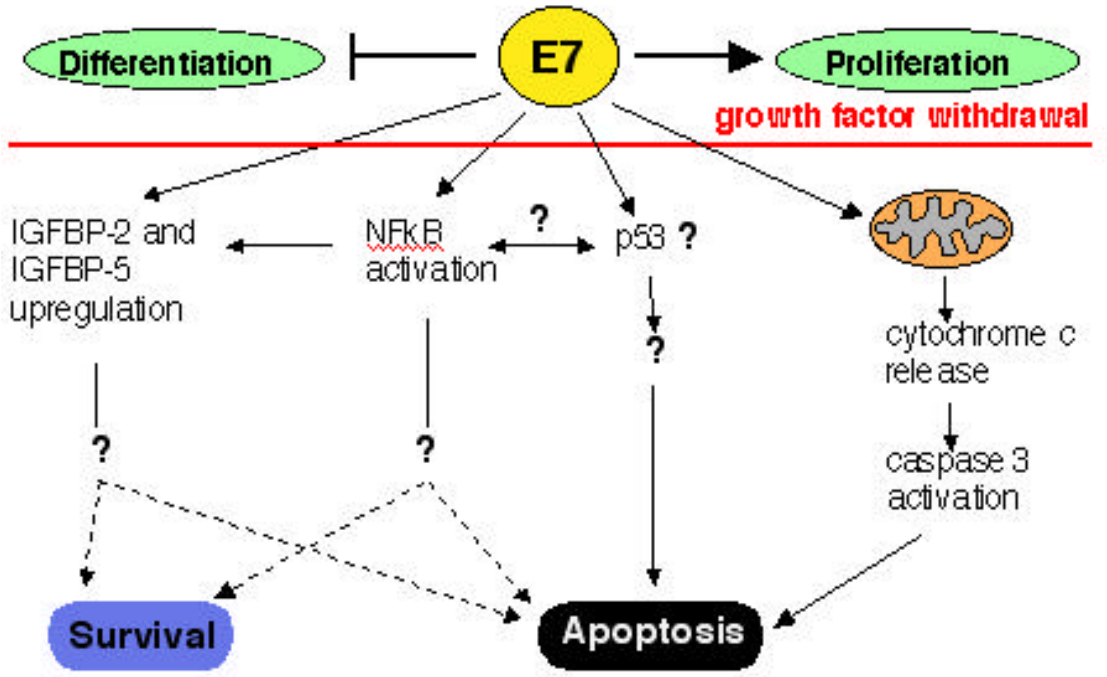

Figure 28: Model for HPV-16 E7 mediated apoptosis upon growth factor withdrawal. In proliferating cells results the expression of HPV-16 E7 in the inhibition of differentiation and the promotion of proliferation. Upon growth factor withdrawal undergo HPV-16 E7 expressing cells apoptosis. The "trophic sentinel" response in HPV-16 E7 expressing cells induces cytochrome c release from the mitochondria and results in caspase 3 activation, which triggers apoptosis. The role of p53 in HPV-16 E7 mediated apoptosis is not clear. P53 is not activated as a transcription factor but is required for apoptosis to ensue. One possibility is that p53 may counteract the prosurvival function of NFκB. It remains to be elucidated whether the upregulation of IGFBP-2 and -5 and the activation of NFκB are involved in HPV-16 E7 mediated apoptosis or whether this represents a cellular attempt to prevent apoptosis and signal survival.

6. Future directions

The further investigation of the stabilization and functional impairment of p53 by HPV-16 E7 includes the determination whether HPV-16 E7 can interfere with the ubiquitination of p53 by mdm2 by investigating the ubiquitination status of the stabilized p53 compared to control cells. The possible involvement mdmX in the abrogation of mdm2 mediated p53 degradation can be addressed as soon as better antibodies are available. The interference of HPV-16 E7 with the recognition of ubiquitinated p53 by the proteasome or impairment of the turnover of the ubiquitinated p53 could be other mechanistical possibilities for the stabilization. The finding that the stabilized p53 is transcriptionally inactive indicates that modifications different from those induced by DNA damage, may be involved in stabilization. These may include phosphorylations at residues that were not investigated or acetylation of the carboxyl terminus. The finding that the stabilized p53 is capable of binding DNA indicates that HPV-16 E7 may interfere with the assembly of the transcription initiation complex. This can be investigated by chromatin immunoprecipitation and subsequent identification of the bound components.

Future investigations evaluating the underlying mechanisms of HPV-16 E7 mediated apoptosis upon growth factor withdrawal will focus on the role of the IGFBP-2 and –5. It remains to be elucidated whether upregulation of these two proteins is involved in the induction of apoptosis or whether it is an antiapoptotic reaction of the cell in response to suboptimal growth conditions. This question may be addressed by adding recombinant IGFBP-2 and –5 to the growth media and monitor the apoptosis rates to observe an enhancement or a decrease in apoptosis rates due to increased exogenous IGFBP-2 or-5 levels. Alternatively the corresponding cDNAs could be expressed in cells either in the absence or the presence of HPV-16 E7. Similarly the pro- or antiapoptotic role of NFκB can be determined by blocking NFκB activation and monitoring apoptosis rates.

The involvement of p53 in HPV-16 E7 mediated apoptosis could be due to the transcriptional induction of target genes that were not investigated in this study including

PERP, Noxa, and p53AIP1. p53 might be subject to other posttranslational modifications such as acetylation. Another possibility is that a portion of the stabilized p53 may translocate to the mitochondria and thereby induce an apoptotic response. It is also possible that the p53 homologes p63 and p73 play a role in HPV-16 E7 mediated apoptosis. This could be investigated by using MEFs that are wild type or null for p53, p63 or p73 as well as MEFs missing combinations of two or all three p53 family members.

7. Summary

The expression of high-risk HPV-16 E7 in a cell without HPV-16 E6 results in the degradation of pRB but in the stabilization of p53. This increase is not due to increased transcription of p53, but results from an extension of its half-life (Jones and Münger, 1997). The data presented in this thesis show that the stabilized p53 in HPV-16 E7 expressing cells is not transcriptionally active in the presence or absence of growth factors although p53 seems necessary for HPV-16 E7 mediated apoptosis upon growth factor withdrawal. Furthermore, the stabilized p53 in proliferating HPV-16 E7 expressing cells can bind mdm2, is in a wild type conformation and is in the normal compartment of the cell. Nuclear export and proteasomal degradation of p53 are not impaired by HPV-16 E7 and it remains to delineate whether HPV-16 E7 inhibits the assembly of the transcriptional p53 initiation machinery or the ubiquitination of p53 and thereby inactivates and stabilizes p53.

It has been shown previously that cells expressing oncogenes such as HPV-16 E7 are predisposed to apoptosis upon suboptimal growth conditions. Investigations of the mechanisms underlying HPV-16 E7 mediated apoptosis upon growth factor withdrawal revealed the involvement of the mitochondria as demonstrated by cytochrome c release and caspase 3 activation. The apoptosis-inducing factor (AIF) remained mitochondrial and is not involved in mediating apoptosis in HPV-16 E7 expressing cells. An upregulation of IGFBP-2 and IGFBP-5 was observed in HPV-16 E7 expressing cells with a further increase in protein levels upon suboptimal growth conditions. These proteins have been shown to bind and control the cellular availability of the survival factor IGF-I. It remains to be determined whether the expression of these two proteins predisposes HPV-16 E7 expressing cells to apoptosis or whether the upregulation is an antiapoptotic mechanism triggered by the cell in response to deprivation of growth factors.

These results contribute to the better understanding of how cells react to deregulation of the cell cycle and uncontrolled proliferation induced by viral oncoproteins. Detailed knowledge about the effect of HPV-16 E7 in proliferating cells as well as growth factor deprived cells may lead to the development of a novel treatment for

HPV-positive cancers by rendering the cells susceptible to HPV-16 E7 mediated apoptosis.

Keywords: tumor supressor p53, Human papillomavirus type 16 E7 oncoprotein, apoptosis

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Acknowledgement

This work would not have been possible without the help and the support of a number of people.

First and foremost, I would like to thank Karl Münger for giving me the great opportunity to work in this lab and for being a wonderful supervisor. He demonstrated a level of patience, encouragement and support that was remarkable and without which I would not have achieved this level of success. His professional integrity and limitless enthusiasm have been inspiring. He always gave me the freedom to pursue my own ideas and to develop my own way of scientific thinking.

I am grateful to Prof. Dieter Paul for supervising my work from Germany and supporting me wherever he could. I thank Prof. Mueller, Prof. Otto and Prof. Resch for being on my thesis defense committee.

I thank my parents for always believing in me and supporting me.

I thank Denise Galloway, Moshe Oren, Michael Hinz, Sheila Steward for their kind gifts of reagents, Joseph Growney and Elsa Flores for technical advice, and Philip Hinds, Carl Maki and Valerie Zacny for critical comments on the project.

A big “thank you” to Michael Hinz who was a great supervisor and teacher during my diploma thesis and got me interested in cancer research in the first place.

Thanks to Christoph Grundner for sharing good and bad times. Miranda Grace, Siribang-on (Tammy) Pibooniyom, Jessica Kirshner, and Sonia Gonzalez helped me to laugh through some frustrating times in lab. I also thank my sister and all my friends for always being there for me.

I thank the DAAD (German Academic Exchange Service) for financial support through a graduate student research fellowship (Doktorandenstipendium im Rahmen des gemeinsamen HSP III von Bund und Ländern).

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt und nur die angegebenen Hilfsmittel benutzt habe. Diese Arbeit wurde bisher nicht an einer anderen Universität oder einem anderen Fachbereich als Dissertation eingereicht.

Weiterhin erkläre ich, dass die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

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