Inhibition of the transcription factor AP-1 in cervical cancer

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DECLARATION

1. I know that plagiarism is wrong. Plagiarism is to use anothers' work and to pretend that it is ones' own.

2. I have used the Cell Journal style for citation and referencing. Each significant contribution to, and quotation in this project from the work or the works of other people has been attributed, and cited and referenced.

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4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

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"I praise You because I am fearfully and wonderfully made; Your works are wonderful, I know that full well." Psalm 139: 14

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ABBREVIATIONS

Amp	Ampicillicin
AP-1	Activating Protein-1
AP-1-Luc	AP-1 Luciferase
APS	Ammonium Persulphate
ATF	Activating Transcription factor
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
Bp	Base pairs
bZIP	basic region leucine zipper
CDK	cyclin dependent kinases
CO_2	Carbon dioxide
CRE	cAMP responsive element
CMV	Cytomegalovirus
°C	Celsius
DAPI	4', 6-diamidino-2'-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ECL	Enhanced Chemoluminescence
ERK	extracellular signal-related kinase
FACS	Fluorescent activated cell sorting
FBS	Fetal Bovine Serum
Fra	Fos related antigen
HPV	Human Papilloma Virus

Hr	hour
HRP	Horse Radish peroxidase
HSIL	High-grade Squamous Intraepithelial Lesions
LB	Luria Broth
LSIL	Low grade Squamous Intraepithelial Lesions
JNK	c- Jun N terminal kinase
M	molar
MAF	Musculoponeurotic fibrosarcoma
MAPK	Mitogen –activated kinase
mg	milligram
min	minutes
ml	milliliter
mut -AP-1- Luc	mutated AP-1 Luciferase
NF-κB	nuclear factor κB
ng	nanogram
nm	nanomolar
nt	nucleotides
OD	Optical density
PAGE	Polyacylamide gel electrophoresis
PAP smear	Papanicolaou smear
PBS	Phosphate Buffered Saline
PLB	Passive lysis buffer
pLRT	retroviral plasmid
PMSF	Phenylmethanesulfonyl fluoride
RB	Retinoblastoma protein

RIPA	Radioimmunoprecipitation Assay
RNA	ribonucleaic acid
RNAi	RNA interference
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SE	Standard error
siRNA	short interfering RNA
TAD	Transactivation domain
TAM67	Transactivation Domain Mutant 67
TBS	Tris Buffered Saline
TBE	Tris Borate EDTA
TEMED	N,N,N,N- Tetramethyl-ethylene diamine
TK	Tyrosine Kinase
TPA	12-O-tetradecanoyl-phorbol 13-acetate
TRE	TPA responsive element
µl	microlitre
µg	microgram
UV	ultra violet
V	Volts

ABSTRACT

AP-1 is a dimeric transcription factor comprised primarily of Jun and Fos family proteins, that regulates numerous genes involved in cell proliferation, differentiation and oncogenesis. The expression of AP-1 is shown to play an important role in many human cancers and plays a key role in the regulation of the E6 and E7 oncoproteins of high-risk Human Papillomaviruses (HPV) that are etiologically associated with cervical cancer. The c-Jun and Jun B components of AP-1 were shown to be expressed at higher levels in cervical cancer patients compared to normal patient tissue while Jun D levels were largely unchanged. To define the role of AP-1 in cervical cancer, the effect of inhibiting AP-1 actvity was determined using a dominant-negative deletion mutant TAM67. CaSki cervical cancer cells with a doxycycline inducible TAM67 demonstrated that inhibition of AP-1 activity and expression resulted in an altered cell morphology, a significant decrease in cell proliferation and inhibition of colony formation. This was accompanied by a slower progression of TAM67 expressing cells through the cell cycle, with an accompanying increase in G2/M phase. An increase in the expression of the cell cycle regulatory protein, $p21^{CIP1}$, was observed that appeared independent of p53 expression. siRNA directed at inhibiting individual AP-1 components showed that Jun B was an important regulator of CaSki cell proliferation. These results suggest that AP-1 is involved in the cell proliferation and tumourigenic phenotype of cervical cancer cells, such as CaSki cells, possibly via a direct repression of cell cycle regulator $p21^{CIP1}$.

CHAPTER 1

INTRODUCTION

1.1 Oncogenesis

There exists a fine balance between the activity of proto-oncogenes which promote cell proliferation and tumour suppressor genes that regulate the progression of cells through the cell cycle (Hanahan and Weinberg, 2000; Shehata *et al.*, 2005). Disruption of either of these may result in oncogenesis which is a process involving three main stages; tumour initiation, promotion and progression (Digiovanni, 1992; Hanahan and Weinberg, 2000). The initiation stage, a step which occurs rapidly and at high frequency, occurs when an irreparable gene mutation is induced in target cells by exposure to physical, chemical or viral agents, (Young *et al.*, 2003). Tumour promotion results from the sustained exposure to extracellular tumour promoters such as growth signals, (Hanahan and Weinberg, 2000; Digiovanni, 1992). During this stage, rate limiting molecular events occur along signal transduction pathways resulting in the altered regulation of essential pathways and expression of proteins required for normal cellular functioning (Young *et al.*, 2003; Hahn, 2002; Blume-Jensen and Hunter, 2001). The cells lose their ability to sense and repair DNA damage and thus cells accumulate new additional mutations causing defects in normal cell functioning (Kaufmann and Kaufman, 1993; Hanahan and Weinberg, 2000). These abnormal cells acquire a growth advantage with characteristics of autonomous growth, increased proliferation and division without control and undergo selective expansion (Hanahan and Weinberg, 2000; Digiovanni, 1992). The resultant uncontrolled cell division when new cells are not needed by the body causes a mass of extra tissue or benign growth to form (Digiovanni, 1992). During tumour progression, the cells progress to become invasive or metastasic and lead to the formation of malignant lesions known as cancer (Hanahan and Weinberg, 2000; Digiovanni, 1992). Of the number of

proteins thought to be involved in the development of cancer, transcription factors such as AP-1 are thought to be essential to this process (Young *et al.*, 2003; Leaner *et al.*, 2007).

1.2 Cervical Cancer

Cervical cancer occurs when cells within the cervix become cancerous. Cancer of the cervix is overall the seventh most common cancer in the world with approximately 439 100 new cases occurring annually worldwide, resulting in an estimated 237 449 deaths as determined in 2002 (Ferlay *et al.*, 2004; Kamangar *et al.*, 2006; Parkin *et al.*, 2005). It is the second most common epithelial derived tumour that is found in women worldwide (Goldie, 2002; Parkin *et al.*, 2005).

The incidence and mortality rates in developing countries are almost double in comparison to rates found in developed countries where cervical cancer accounts for 15% of all cancers compared to 3.6% in developed countries (Kamangar *et al.*, 2006; Parkin *et al.*, 2005). The highest incidences of cervical cancer are found in Africa and Central/South America where on average approximately 87.3 cases per 100 000 population occur annually (Kamangar *et al.*, 2006).

The incidence of cervical cancer in South Africa is 38/100000, where its prevalence is higher in black females (SA Cancer registry). In 2002, an estimated 6742 new cases and as many as 4500 deaths were reported in S.A. (Ferlay *et al.*, 2004). Many of these cases should have been preventable through early detection and successful treatment.The high incidence of cervical cancer in developing countries is largely attributed to the lack of effective implementation of routine screening programs such as the Papanicolaou (PAP) smears used to detect precancerous lesions (Munoz *et al.*, 2003).

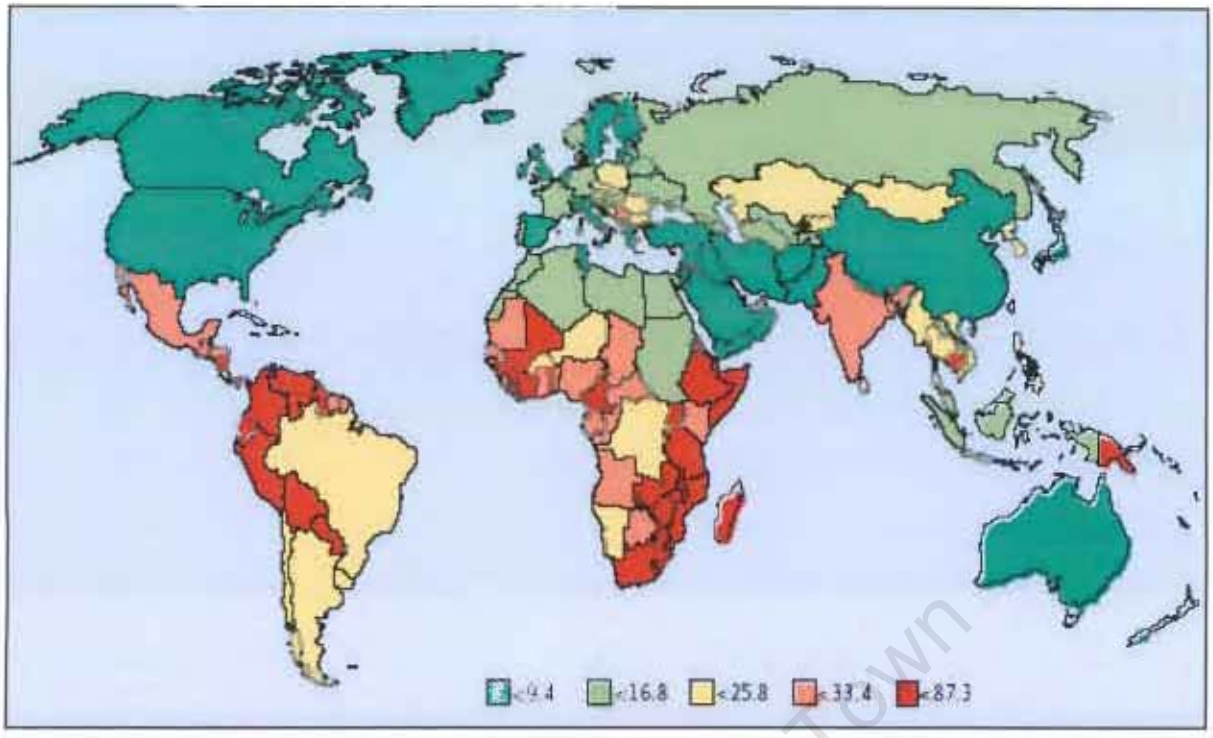


Figure 1.1. Incidence of cervical cancer worldwide. Numbers indicate cases per 100 000 population (Schiffman and Castle, 2005).

Recent developments in the treatment of cervical cancer include the development of a prophylactic quadrivalent HPV vaccine targeting HPV types 6, 11 of the low risk HPVs and high risk HPV types 16 and 18, most commonly associated with cervical cancer (Munoz *et al.*, 2004; Villa *et al.*, 2005). Clinical trials suggest that it is effective in preventing 90% of new infections and therefore should be able to decrease the number of cervical cancer cases (Villa *et al.*, 2005). The large number of issues surrounding the implementation of such a vaccine may, however, delay its cancer preventing benefits (Hantz *et al.*, 2006). Ongoing research into understanding the processes involved in cervical cancer development and identification of mechanisms, both viral and cellular, that associate with this disease is of importance.

1.2.1 The role of the Human Papillomavirus in cervical cancer

Infections with high risk Human Papillomaviruses (HPV) are etiologically linked with cervical cancer (Zur Hausen, 2002). There are a number of different types of HPV that infect

the genital epithelium and account for the most common sexually transmitted viruses (Howley, 1996; Walboomers *et al.*, 1999). The different types of HPVs are classified into low-risk or high-risk types due to their different abilities to transform the cervical epithelium. The low-risks types (6, 11, 42) are mainly associated with genital warts and benign lesions that do not progress to invasive cervical cancer whereas the high-risk types including HPV 16, 18, 31 and 45 are most commonly found in pre-neoplastic lesions that may progress to invasive cancer (Munoz *et al.*, 2003). High-risk HPV virus types have been associated with 80-90% of pre-cancerous lesions and invasive carcinoma (Zur Hausen, 2002). The most commonly cancer-associated types are HPV16 and HPV18 that are responsible for causing approximately 50% and 15% of all cervical cancer cases respectively (Munoz *et al.*, 2003).

High-risk HPVs have been described as the "necessary and initial factor" in cervical cancer (Walboomers *et al.*, 1999) and persistant infections with these HPVs is determined as the single best predictor of cervical cancer (Munoz *et al.*, 2003). Not all women infected with these high risk HPVs develop cervical cancer. HPV infections are largely transient with approximately 70% clearance 12 months after infection and more than 90% clearance within 2 years (Bosch *et al.*, 2002; Howley, 1996). However, a fraction of these HPV infections persist and initiate transformation events that may be followed by the formation of precancerous lesions (Bosch *et al.*, 2002; Longworth and Laimins, 2004). Cervical cancer develops via a progression from low grade squamous intraepithelial lesions (LSIL) to high-grade squamous intraepithelial lesions (HSIL) to invasive carcinoma (Bosch *et al.*, 2002). Continued viral replication and shedding in the actively proliferating basal epithelial cells initially causes mild dysplastic changes (LSIL) which may progress to a more severe HSIL after a long latency period. Many of the premalignant lesions often regress or are removed by the host immune response before progression occurs (Howley, 1996; Bosch *et al.*, 2002). If

these early dysplastic cells are not eliminated, the episomal circular HPV genome integrates into the host cell DNA at high frequency and causes the progression from HSIL to invasive cancer (Bosch *et al.*, 2002; Howley, 1996).

1.2.2. HPV E6 and E7 oncoproteins

Integration of the HPV genome into the host cell results in the sustained expression of the viral E6 and E7 oncoproteins (Howley, 1996); an early event that occurs in HPV associated oncogenesis. The resultant cellular transformation is due to the interaction of the viral proteins with the host cell transcriptional machinery (Chan *et al.*, 1990; Walboomers *et al.*, 1999; Werness *et al.*, 1990).

The viral E6 and E7 oncoproteins' modulation of the host cells' machinery is largely responsible for the differences in oncogenic potential among the HPV types (Werness *et al.*, 1990). The E6 and E7 of high-risk HPV types act as oncoproteins by disrupting the normal cell regulatory functions of cell cycle proteins and interacting with numerous host transcription factors to enhance or repress their activity (Dephilippis *et al.*, 2003; Howley, 1996; Rapp and Chen, 1998).

The E6 protein of high risk HPVs has been shown to bind to and accelerate the ubiquitin-mediated degradation of the tumour supressor p53, responsible for inhibition of cell proliferation (Scheffner *et al.*, 1993; Tommasino *et al.*, 2003). This inactivation is found in more than 85% of cervical cancer cases and results in 2-3 fold lower basal levels of p53 in cervical carcinoma cell lines in comparison to primary cells (Scheffner *et al.*, 1991). The E7 protein is responsible for binding to the active hypophosphorylated pRb tumor suppressor protein, causing the destabilization of the Rb/E2F complex and the release of the

transcriptional activator E2F that regulates the expression of genes required for cell cycle progression from G1 to S phase (Boyer *et al.*, 1996).

In addition, these oncoproteins interact with transcription factors such as activating protein-1 (AP-1) and NFκB which are important modulators of HPV gene expression (Li *et al.*, 1998; Soto *et al.*, 2000). AP-1 specifically has been reported to associate with the prolonged expression of E6 and E7 proteins and is essential for the malignant phenotype induced by E6 and E7 (Chan *et al.*, 1990; Chong *et al.*, 1991; Li *et al.*, 1998). AP-1 is involved in multiple mechanisms regulating HPV gene expression. The regulation of E6 protein expression occurs directly via a number of AP-1 binding sites in the E6 promoter region while the HPV16 E7 oncoprotein is reported to bind to and transactivate c-Jun/AP-1 induced transcription (Antinore *et al.*, 1996; Chan *et al.*, 1990). Inhibition of AP-1 activity causes a decrease in E7 and Ras co-induced transformation and protects against E7 enhanced tumourigenesis in mouse skin (Antinore *et al.*, 1996; Young *et al.*, 2002). The expression pattern of AP-1 has also been shown to correlate with HPV gene expression in stratified epithelial cells of the cervix (Kyo *et al.*, 1997). There is therefore significant evidence in the literature for a potential role of transcription factors such as AP-1 in the development of cervical cancer.

1.3 Activating Protein-1 (AP-1) transcription factor

The AP-1 transcription factor exists as a combination of dimers composed primarily of members of the Jun family of proteins comprising of c-Jun, JunB, JunD that dimerise to form homodimers or hetereodimers with the Fos family proteins (c-Fos, Fos B, Fra-1 and Fra-2) (Glover and Harrison, 1995; Shaulian and Karin, 2002). These proteins belong to the basic-region leucine zipper (bZIP) protein group that contain three highly conserved domains: a leucine zipper (bZIP) (LZ) domain, a basic DNA binding domain (DBD) and the

transactivation domain (TAD) (Figure 1.2.A). Dimerisation occurs through the leucine zipper motif that positions the adjacent basic regions of each bZIP domain, to form a bimolecular DNA binding domain shown in Figure 1.2.B (Glover and Harrison, 1995).

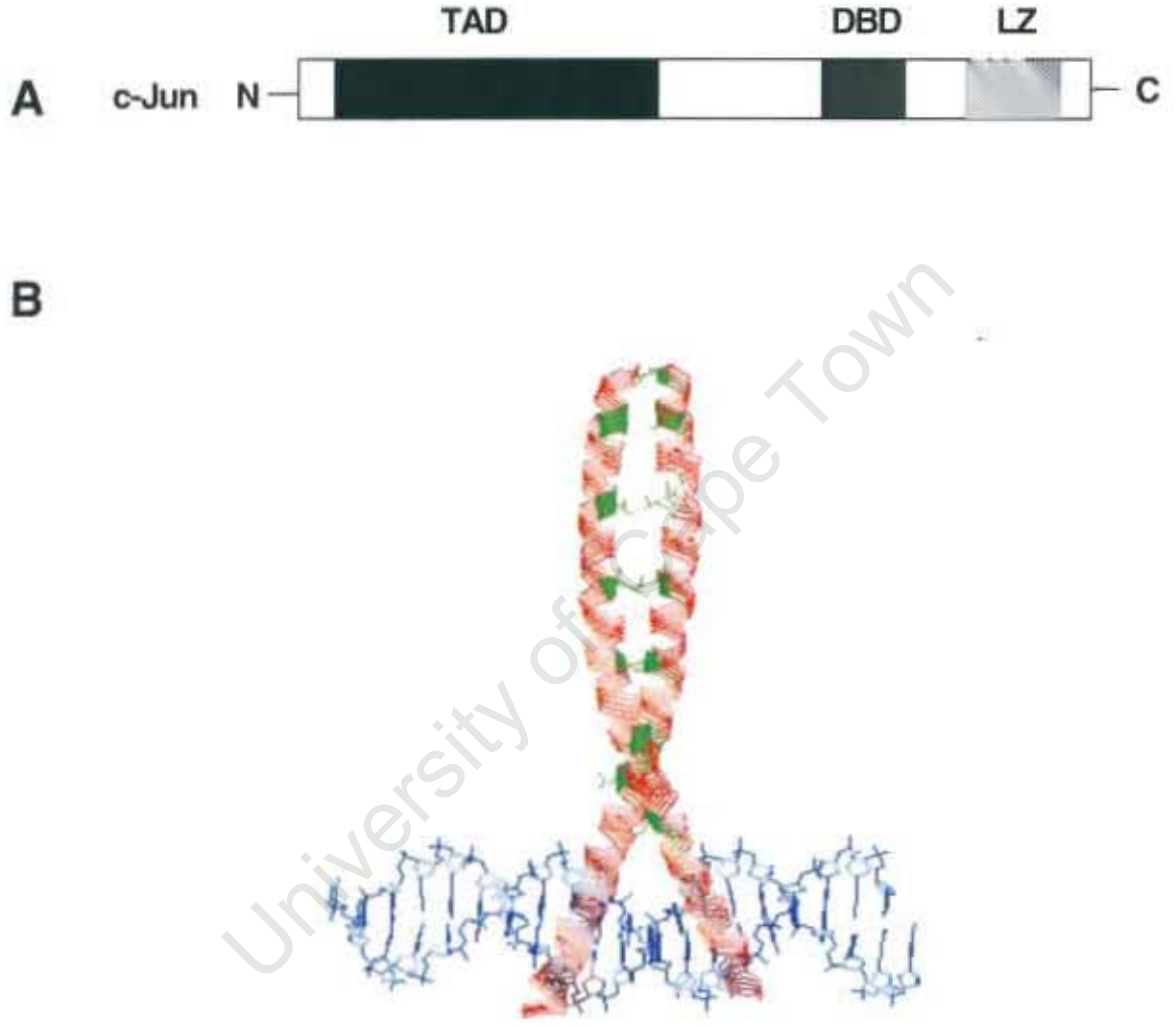


Figure 1.2. Structure of AP-1 proteins. (A). The structure of basic-region leucine zipper (bZIP) protein group proteins represented by c-Jun. Diagram depicting the three highly conserved domains, a leucine zipper (bZIP) (LZ) domain, a basic DNA binding domain (DBD) and the transactivation domain (TAD). (B). The structure of AP-1 bound to DNA. The AP-1 dimer is formed by Jun and Fos heterodimers (shown in red) bound to DNA (shown in blue). It holds a leucine zipper motif where the leucine residues (shown in green) form a zipper-like structure between the two helices (Glover and Harrison, 1995).

Jun proteins are able to form stable homodimers that are capable of binding to DNA and mediating gene expression, while Fos proteins require a Jun protein for dimerisation and

transcriptional activation. However, the Jun-Fos heterodimers are found to be far more stable and have greater DNA binding activity than the Jun homodimers (Hartl *et al.*, 2003; Ryseck and Bravo, 1991). Heterodimers with related bZIP proteins such as Activating transcription factor (ATF) and the Musculoponeurotic fibrosarcoma (MAF) proteins may also occur, as well as interactions with other transcription factors such as p65 and NFκB that increases the combinatorial potential of AP-1 within cells (Angel and Karin, 1991; Eferl and Wagner, 2003).

The AP-1 transcription factor activates or represses transcription by binding to heptameric AP-1 binding sites TGAG/CTCA also known as TPA 12-O-tetradecanoylphorbol-13-acetate response elements (TREs) and cAMP response elements (CRE) made up of an octameric consensus sequence of TGACGTCA, found in the promoter and enhancer regions of target genes (Karin *et al.*, 1997; van Dam and Castellazzi, 2001). An AP-1 complex of Jun-Fos dimers has been shown to have the highest affinity to the TRE binding sites, whereas the Jun-ATF dimers show greater affinity for the CRE element (Ryseck and Bravo, 1991; van Dam and Castellazzi, 2001; Angel and Karin, 1991; Shaulian and Karin, 2002). AP-1 has been reported to regulate numerous genes involved in many biological processes such as cell proliferation, apoptosis and differentiation (Angel and Karin, 1991; Hartl *et al.*, 2003; Shaulian and Karin, 2002). AP-1 is involved in regulating both basal and stimulus activated gene expression, as several members of the AP-1 proteins contain an N-terminal transactivational domain (TAD) that is phosphorylated or dephosphorylated in response to various stimuli (Angel and Karin, 1991; Shaulian and Karin, 2002). Activation is induced by various growth factors, hormones, stress, cytokines, ROS and UV radiation that signal through mitogen activated protein kinase (MAPK) pathways including Extracellular-signal-related kinase (ERK), Jun amino-terminal kinase (JNK) and p38 MAPK cascades (Shaulian

and Karin, 2002). The combinational diversity of AP-1 proteins making up the AP-1 complex and other cellular regulatory factors present in cells appears to play an important role in determining which genes are regulated and influences how the specific cell types respond to a stimulus (Dunn *et al.*, 2002; Young and Colburn, 2006).

1.3.1 Jun Family proteins

The Jun family proteins (c-Jun, Jun B and Jun D) are highly conserved and have high sequence homology but are found to have distinct expression and biologic activity within different cellular contexts (Jochum *et al.*, 2001; Mechta-Grigoriou *et al.*, 2001). c-Jun, a major component of the AP-1 transcription factor complex was the first oncogenic transcription factor discovered as the 39 kD cellular counterpart of the viral oncoprotein v-Jun (Bohmann *et al.*, 1987; Maki *et al.*, 1987). c-Jun is an immediate-early gene that is expressed in many cell types at low basal levels (Eferl and Wagner, 2003). Activation occurs rapidly and transiently in response to various stumuli (Ryseck and Bravo, 1991; Shaulian and Karin, 2002). c-Jun is able to mediate a positive auto-regulation of the c-Jun gene via two AP-1 binding sites within the enhancer region (Angel *et al.*, 1988). Activation of c-Jun occurs post translationally by phosphorylation at positions Ser63 and Ser73 within its TAD by JNKs (Rinehart-Kim *et al.*, 2000; Wisdom *et al.*, 1999; Behrens *et al.*, 2000). c-Jun is seen as a "master regulatory gene" as it affects a variety of biological processes, in a cell type dependent manner (Rinehart-Kim *et al.*, 2000; Wisdom *et al.*, 1999). The overexpression of c-Jun in 3T3 fibroblasts and vascular endothelial cells is shown to induce apoptosis (Bossy-Wetzel *et al.*, 1992; Wisdom *et al.*, 1999), whereas in cultured fibroblasts and hepatoblasts, c-Jun acts a positive regulator of cell growth (Schreiber *et al.*, 1999). Many of the cell-cycle regulatory proteins are regulated by c-Jun including cyclin D1, cyclin A cyclin E, p53, $p21^{CIP1}$ and $p19^{ARF}$ (Shaulian and Karin, 2001).

Similarly, Jun B is an early response gene that regulates numerous genes in response to growth factors (Ryder *et al.*, 1989; Schutte *et al.*, 1989). Whereas c-Jun is able to mediate transcription of target genes containing a single AP-1 binding site, Jun B requires multiple sites for activation (Chiu *et al.*, 1989). Jun B lacks Ser 63 and 73 required for phosphorylation but holds two threonine residues within its TAD for activation (Kallunki *et al.*, 1996). c-Jun and Jun B have been shown to work both antagonistically or co-operatively in processes such as cellular transformation and proliferation (Jochum *et al.*, 2001; Leaner *et al.*, 2003). c-Jun promotes cell proliferation, while Jun B suppresses (Jochum *et al.*, 2001; Mechta-Grigoriou *et al.*, 2001). Other studies have demonstrated that AP-1 dimers composed of c-Jun and Jun B regulate transcriptional targets to bring about the cellular transformation in Rat1A fibroblasts (Leaner *et al.*, 2003). Recent studies have also shown that Jun B can replace c-Jun in cellular proliferation and that they can act together in tumourigenesis (Gurzov *et al.*, 2007; Passegue *et al.*, 2002).

Jun D is found to be more constitutively expressed and is the most ubiquitously expressed of all the AP-1 proteins (Hirai *et al.*, 1989). Jun D lacks the transactivational domain and demonstrates more of an anti-proliferative and tumour suppressing activity than c-Jun and Jun B (Castellazzi *et al.*, 1991; Leaner *et al.*, 2003). Overexpression of Jun D in fibroblasts causes suppression of cell proliferation and acts antagonistically to transformation mediated by Ras (Pfarr *et al.*, 1994).

1.3.2 AP-1 and cell cycle regulatory proteins

The negative regulation of AP-1 on the tumour suppressor functions of p53 can occur both directly and indirectly. AP-1 is shown to negatively regulate p53 directly by binding to sequences in the p53 promoter or indirectly by causing the inactivation of a p53 downstream

target $p21^{CIP1}$ (Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). Like p53, $p21^{CIP1}$ is another important cell-cycle regulatory molecule that is regulated by AP-1. AP-1 has been shown to cause either the activation or repression of $p21^{CIP1}$ by different mechanisms depending on the cellular context (Kardassis *et al.*, 1999; Wang *et al.*, 2000). Such regulation by AP-1 on these key cell regulatory molecules suggests an important role of AP-1 in cell cycle progression and consequently an important contributing factor in oncogenesis (Shaulian and Karin, 2001).

1.3.3. AP-1 and Oncogenesis

Tumourigenesis involves cell transformation that may lead to invasive growth, angiogenesis and metastasis (Hahn, 2002). The transactivation of AP-1 has been shown to be important for oncogenic transformation in mouse epidermal cells and tumour progression in mouse and human epidermal keratinocytes (Behrens *et al.*, 2000; Young *et al.*, 2002). The AP-1 proteins, c-Jun, c-Fos and Fos B are highly effective in transforming cells while Jun B, Fra 1 and Fra 2, have weaker transforming activity and Jun D has none at all (Eferl and Wagner, 2003; Jochum *et al.*, 2001; Leaner *et al.*, 2007; Smeal *et al.*, 1991).

AP-1 activity is induced by oncogenic H-Ras and v-Src and other tumour promoters, linking AP-1 to cellular transformation (Shaulian and Karin, 2002). Overexpression of the Fos and Jun proteins is able to induce transformation in chicken embryo fibroblast and immortalised rat fibroblasts Rat1a (Rapp *et al.*, 1994; Suzuki *et al.*, 1994). For transformation of primary rat embryo cells, c-Jun co-operates with activated Ras oncoproteins and is essential for Ras-induced transformation of immortalised rat fibroblasts (Alani *et al.*, 1991; Schutte *et al.*, 1989). c-Jun acts an oncogene itself, as it is able to transform Rat1a fibroblasts and chicken embryo fibroblasts cells alone, while Jun B is unable to transform cells alone but is able to

induce transformation and immortalisation when co-expressed with activated Ras (Schutte *et al.*, 1989; Smeal *et al.*, 1991; Suzuki *et al.*, 1994).

While the AP-1 proteins are mainly oncogenic and implicated in many tumourigenic processes such as angiogenesis, metastasis, deregulated proliferation, apoptosis and survival (Angel and Karin, 1991; Ozanne *et al.*, 2007; Wisdom *et al.*, 1999), some of the proteins of AP-1 such as Jun B, Jun D and c-Fos have been shown to be involved in tumour suppression (Eferl and Wagner, 2003; Kameda *et al.*, 1993; Schutte *et al.*, 1989).

The constitutive activation of AP-1 required for tumour formation in mammalian and avian cells also occurs in distinct human tumour cells and is therefore suspected to play a role in human oncogenesis (Hartl *et al.*, 2003). AP-1 transcription factor is found to be overexpressed in various cancers such as breast cancer, ovarian cancer, endometrial cancer, colon cancer and lung cancer (Neyns *et al.*, 1996; Vleugel *et al.*, 2006; Bamberger *et al.*, 2001; Wang *et al.*, 2002; Risse-Hackl *et al.*, 1998). It is also found to play an important role in melanomas of the skin and osteosarcomas of the bone (Young *et al.*, 1999; Wang *et al.*, 1995). In breast cancer cells, the overexpression of the c-Jun component of AP-1 is shown to be associated with a more aggressive phenotype and its activation is found to be associated with proliferation and angiogenesis in invasive breast cancer (Smith *et al.*, 1999; Vleugel *et al.*, 2006). The association of AP-1 expression with HPV E6/E7 oncoproteins suggests a role for AP-1 in HPV induced carcinogenesis in both the skin and cervix (Kyo *et al.*, 1997; Li *et al.*, 1998; Young *et al.*, 2002).

1.4. Dominant negative mutant TAM67 (Transactivating mutant 67)

TAM67 is a 29 kD dominant-negative mutant of c-Jun that lacks the major transactivation domain of c-Jun coded by amino acids 3-122 on the N-terminal of c-Jun (Alani *et al.*, 1991; Brown *et al.*, 1993) (Figure 1.3). It still holds the DNA binding domain and the bZIP domain required for dimerisation and thus TAM67 is still able to dimerise with other members of the bZIP family and dimers containing TAM67 are still able to bind to DNA but they have little or no transcriptional activity (Brown *et al.*, 1993; Brown *et al.*, 1994).

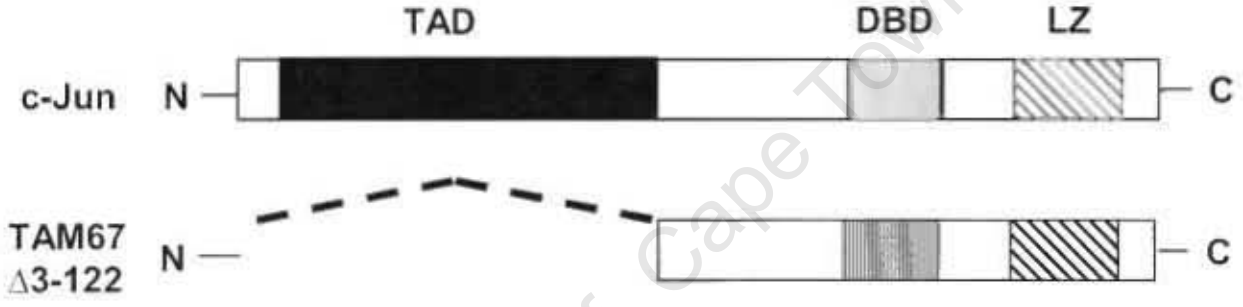


Figure 1.3. The structure of TAM67 showing the lack of transactivational domain of c-Jun. Schematic diagram depicting the N-terminal transactivation domain (TAD) and the C-terminal DNA binding domain (DBD) and Leucine Zipper (LZ) domain of the bZIP domain. Adapted from Brown *et al.*, (1994).

In this action, TAM67 acts as a global inhibitor of the AP-1 complex in cells and provides an effective molecular approach to study c-Jun/AP-1 activity (Brown *et al.*, 1996; Hennigan and Stambrook, 2001; Smith *et al.*, 1999). This mutant protein is unable to stimulate the transcription of AP-1 responsive gene expression and is unable to transform c-Jun/ras and TPA/ras induced transformation (Brown *et al.*, 1994). The expression of TAM67 has been shown to effectively inhibit the transactivating activity of AP-1 and affect the growth of human breast cancer cells and ovarian cancer cells (Neyns *et al.*, 1999; Smith *et al.*, 1999).

The two probable mechanisms describing the dominant negative action of TAM67 include a "quenching mechanism" or a "blocking mechanism", where TAM67 either sequesters endogenous Jun and Fos proteins into low activity AP-1 complexes in cells or TAM67 dimers form and prevent functional AP-1 complexes from binding to target DNA. Both mechanisms result in the inhibition of AP-1 mediated gene expression. The DNA binding of Jun/Jun homodimers and Jun/Fos heterodimers is effectively inhibited by the blocking mechanism of TAM67 (Figure 1.4).

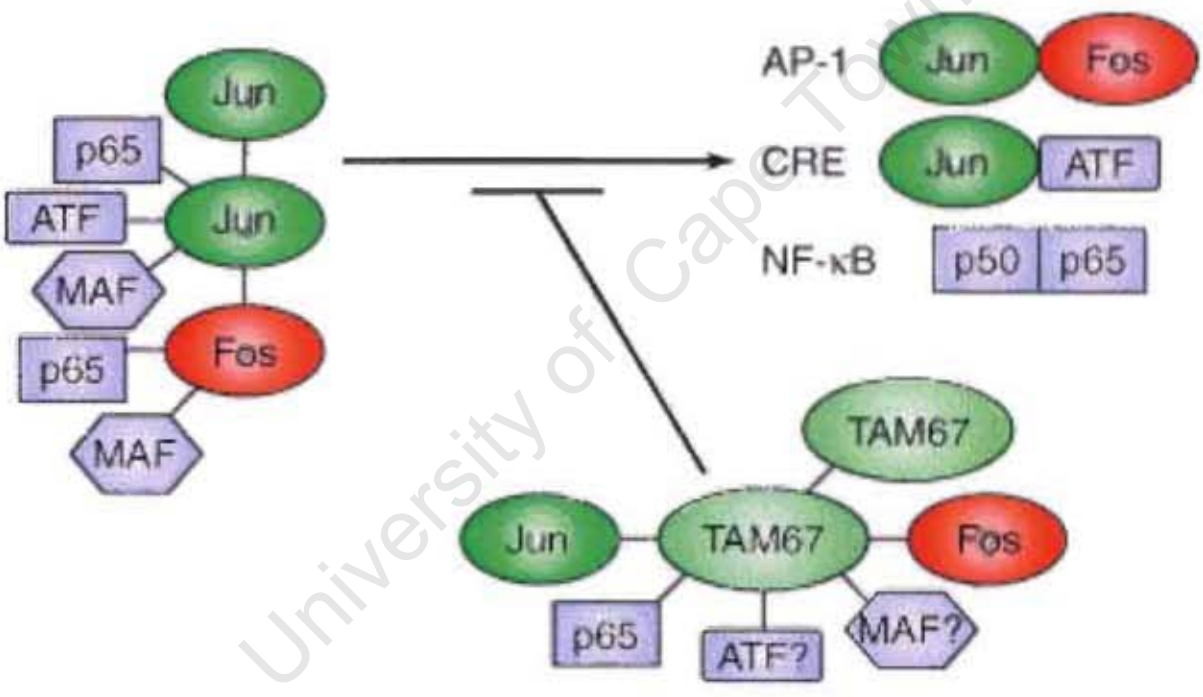


Figure 1.4. Action of TAM67 on endogenous AP-1 proteins. TAM67 is able to form homodimers that block endogenous AP-1 proteins from binding to DNA or TAM67 forms dimers with endogenous AP-1 proteins sequestering them into low AP-1 complexes within cells. Diagram from Young *et al.*, (2003).

TAM67 has been widely used to elucidate the role of the transcription factor AP-1 in a variety of biological systems. For example, TAM67 is able to reverse the transformed phenotype induced by ornithine decarboxylase and Ras-overexpression which is associated with constitutively active AP-1. It is also able to revert human tumour cells to a less progressed

phenotype (Kielosto *et al.*, 2004; Li *et al.*, 1997). TAM67 has also been shown to effectively inhibit TPA-induced AP-1-dependent transcriptional activity responsible for matrigel invasion by keratinocytes and inhibition by TAM67 has also been shown to inhibit tumour promotion in transgenic mice (Domann *et al.*, 1994; Dong *et al.*, 1994; Dong *et al.*, 1997). These studies suggest that TAM67 is an effective inhibitor of AP-1s' function in invasion and tumourigenesis. Similarly, TAM67 expression effectively suppressed anchorage independent growth of HPV immortalized human keratinocytes and protected mice from HPV-induced 16E7 enhanced skin tumourigenesis; suggesting AP-1 as a target for prevention of HPV induced oncogenesis (Li *et al.*, 1998; Young *et al.*, 2002). Together these studies suggest that TAM67 is an effective tool to study the inhibition of AP-1 in tumourigenesis.

Modulating the activity of AP-1 therefore appears to be a promising molecular approach to develop novel preventative agents for cancer therapy (Leaner *et al.*, 2007; Young *et al.*, 2003). As full inhibition of AP-1 may disrupt many important physiological processes required for normal growth and differentiation, inhibition of AP-1 may allow the identification of altered AP- 1 mediated genes relevant to the oncogenesis process (Hartl *et al.*, 2003).

1.5. Significance

Understanding the processes and mechanisms involved in oncogenesis is important for the identification and functional characterisation of associated gene targets that may be required for the selective growth advantage of cancer cells. We propose that the expression of AP-1 in cervical cancer cells may have multiple effects that assist with cellular transformation and by inhibition of AP-1 activity in human cervical cancer cell lines we may be able to characterize its role in the development of cervical cancer. We anticipate that AP-1 activity may be a

requirement for a more aggressive phenotype in cervical cancer cells and therefore its inhibition with TAM67 may block those processes essential for the development of this disease including deregulated cell growth. A clearer knowledge of the role of transcription factors such as AP-1 in changes associated with cervical cancer may ultimately help in the development of early detection strategies and more effective treatments regimes for this disease.

1.6. Project Aim

The goal of this project is to investigate the effect of inhibiting the functional activity of the transcription factor AP-1 in cervical cancer

Specific aim 1: To determine the biological relevance of the functional inhibition of transcriptional factor AP-1 in the development of cervical cancer

Specific aim 2: To determine the mechanisms through which AP-1 inhibition affects the biology of cervical cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Patient material

Cervical cancer tissue was obtained from patients admitted to Groote Schuur Hospital, Cape Town, South Africa for the treatment of cervical dysplasia and carcinoma. Patients' ages ranged from 29 to 76 years. Cervical cancer tissue was obtained from cervical cancer biopsies and normal cervical tissue material was obtained from patients admitted for hysterectomies due to gynecological complications, excluding cervical abnormalties. Tissue specimens were obtained with consent from the patients and the collection was approved by the Research Ethics Committee of the University of Cape Town (REC REF: 153/2004).

2.2. Cell Culture

The six cervical cancer cell lines used include; CaSki, (HPV 16 positive) HeLa (HPV18 positive), SiHa (HPV16 positive), Me180 (HPV68 positive), Ms751 (HPV18, HPV45 positive), and C33A (HPV negative containing a p53 codon 273 CGT to TGT mutation) (Geisbill *et al.*, 1997; Wu *et al.*, 1997) and were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Highveld Biological Lyndhurst, South Africa) supplemented with heat inactivated 10% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, California, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a 95% air and 5% CO_2 incubator and used at 60-80% confluency.

2.2.1. Culturing and Passage of cells

Cells were maintained in 100 mm dishes at 60-80% confluency. For passage, cells were

washed with 2 ml trypsinisation solution and the solution was aspirated off. Cells were incubated in 4 ml trypsinisation solution for two minutes at 37°C. The dishes were shaken gently until the cells lifted and 4 ml of serum containing media was added. Cell freezes were routinely made by resuspension of cells in 2 ml cell freeze media and stored at -70°C for a short period and then moved to liquid nitrogen.

2.2.2. Mycoplasma Testing

Mycoplasma testing was performed 2-3 times per year to determine mycoplasm contamination as these organisms cannot be viewed under the microscope by eye and may interfere with tissue culture results. Contamination with mycoplasms is detected as speckled DNA in the cytoplasm/ membrane after nuclear staining detected under the microscope (Fleckenstein *et al.*, 1994). Briefly, cells were cultured in antibiotic free medium for a week to allow growth of mycoplasms. The cells were then trypsinised as usual and spun down for 5 minutes at 300 g. Cells were resuspended in 2 ml penicillin and streptomycin free 10% FBS DMEM media. Coverslips were flamed in ethanol and placed in 60 mm dish. 100 µl of resupended cells were pipetted onto the coverslip and allowed to adhere at 37°C. After four hours of incubation, a further 3 mls of media was added and the cells were incubated overnight. Prior to staining, the cells were fixed onto the coverslips with 5 ml of fixing solution after a few seconds (Appendix B). The coverslips were washed with tap water and dried. Once dry, 0.5 µg/ml of Hoeschst No33258 nuclear stain was added to the cover slip for 30 seconds. The stain was rinsed off and the coverslip was placed face down onto a slide with mounting fluid. A drop of oil was placed onto a slide for viewing under the fluorescent microscope at 100X magnification.

2.3. Plasmids

A reporter plasmid containing 4X AP-1-Luc was used to measure AP-1 activity and a mutated 4X AP-1-Luc promoter construct was used as a control. The 4X AP-1 promoter construct contains four wild type AP-1 sites fused to a luciferase gene whereas the mut 4X AP-1-Luc contains a promoter construct of four mutated AP-1 binding sites. The expression vector pCMV-TAM67 encoding a dominant negative mutant of c-Jun, TAM67 gene was used to express TAM67 and an empty pCMV vector was used as a control. These plasmids were kindly provided by Dr M. J. Birrer, National Institute of Health (NIH), USA. A -2500/+50 p21-Luc promoter construct kindly donated Dr S Prince (Division of Human Biology, UCT) was used to measure p21 promoter activity and a promoterless pGL3 vector (Promega, Madison, USA) was used as a control. pRL-TK-Luc construct (Promega) containing the Renilla luciferase gene under the control of a Tyrosine kinase promoter was used to control for transfection efficiency.

2.4. Antibodies

Primary polyclonal rabbit antibodies directed against mouse c-Jun (SC44), $p21^{CIP1}$ (H164), Jun B (SC73), Jun D (SC74) and β-tubulin (SC9104), all purchased from Santa Cruz Biotechnologies Inc (Santa Cruz, California, USA), were used. Primary rabbit phosphorylated Ser73 antibody from Cell Signaling Technologies (Danvars, Massachusetts, USA) was used and a primary monoclonal mouse anti-human p53 antibody (D-O7) from Dako Cytomation (Glostrop, Denmark) was used. Secondary anti-rabbit or anti-mouse goat antibody, conjugated with horse-radish perioxidase (HRP) obtained from Pierce (Pierce Biotechnology, Rockford, USA) and Biorad (Bio-Rad Laboratories (Pty) Ltd, Rosebank, South Africa) were used.

2.5. Western Blot analysis

2.5.1. Protein harvest and quantification

Total protein was harvested from cells in RIPA lysis buffer (Appendix B) with 1X complete protease inhibitors (Roche Applied Science, Mannheim, Germany), 0.1 mM Na_2VO_4, a phosphatase inhibitor and 0.1 M Phenylmethylsuphonyl fluoride (PMSF). Cells were washed with 2 mls 1X PBS and scraped off in RIPA buffer solution. The lysed cells were transferred to 1.5 ml microcentrifuge tubes and placed onto ice to prevent degradation. The cell lysates were sonicated at maximum pulse for a few seconds and centrifuged at 8000 g for 10 minutes at 4°C to remove debris. Protein concentrations were determined using the Bicinchoninic Acid (BCA) assay obtained from Pierce (Rockford, USA). The BCA assay is based on the formation of Cu^{2+} protein complex under alkaline conditions and the subsequent reduction of Cu^{2+} to Cu^{1+} by cystine, tryptophan, tyrosine, and the peptide bond. BCA forms a purple blue complex with Cu^{1+} and the absorbancy is read at 595 nm (Stoscheck, 1990). A protein standard curve was determined by dilutions of BSA (Bovine Serum Albumin) and used to calculate the protein concentrations. Samples were stored at -70°C until required.

Proteins from cervical cancer and normal tissues collected from patients at Groote Schuur Hospital (Cape Town, South Africa) were isolated with Trizol reagent (Gibco, Life Technologies) and kindly provided by Pauline van der Watt (Division of Medical Biochemistry, UCT). The extraction of proteins from homogenised patient tissue sample using Trizol reagent was followed according to the manufacturer's instructions.

2.5.2. SDS-Polyacrylamide Gel electrophoresis

For gel electrophoresis, 30 µg - 50 µg of proteins (except for p53 blots where 100 µg was loaded) were electrophoresed on 15% and 12% SDS polyacrylamide gels (SDS-PAGE) using

BioRad MINI Protein II system. 4X Laemmli loading buffer was added to the harvested protein sample and denatured by heat treatment for 2 minutes at 94°C. Laemmli buffer contains SDS that denatures proteins to give all the proteins a similar random coil shape and coats them with a negative charge giving the protein a uniform charge-mass ratio. The proteins' migration through the gel is therefore based purely on their molecular weight. The buffer contains β-mercaptoethanol, a reducing agent that disrupts the disulphide bonds to unfold the protein further and glycerol making the sample dense for effective loading into the wells. Bromophenol blue dye allows for visualisation (Gallagher *et al.*, 1997). The upper 4% stacking gel allows the proteins to enter the gel, followed by a separating gel that separates proteins according to their size (Appendix B). The matrix of these gels is formed by a polyacrylamide crosslink network of polymerised monomer acrylamide (Gallagher *et al.*, 1997). Proteins were electrophoresed at a voltage of 200V for 45 minutes in 1X Running Buffer (Appendix B). 5 µl of pre-stained Amersham Full-Range Rainbow molecular weight marker (RPN8000 from Amersham Bioscience, Germany) or Biorad Kaleidoscope Marker (BioRad) with protein sizes ranging from 250-10 kD was loaded to determine protein sizes (Appendix A).

2.5.3. Immunoblotting

Proteins were subsequently transferred to nitrocellulose membranes (Hybond XL™ ECL™ (Amersham Biosciences) using a tank system in 1X Transfer buffer at 100V for 1.5 hours. A cassette was assembled to form a transfer sandwich in the following order: sponge on cathode side, Whatman 3M filter paper, gel, nitrocellulose membrane, Whatman 3M filter paper, sponge on anode side of the cassette. The cassette was placed in the correct orientation into the transfer tank and filled with 1X Transfer buffer and an ice block to prevent overheating during transfer.

2.5.4. Gel staining

To detect successful transfer, the gels were stained with Coomassie brilliant blue solution for 2 hours at room temperature followed by destaining in destaining solution overnight (Appendix B). Coomassie Brilliant Blue binds non-specifically to proteins through ionic interactions, hydrogen bonding, Van der Waals forces and hydrophobic interactions that enable the detection of 0.3-1 µg/protein band (Ausubel and Frederick, 1987).

2.5.5. Immunodetection

The membranes were blocked in 5% milk for an hour at RT to prevent any nonspecific binding of the antibody to the membrane. Primary antibodies were incubated overnight at 4°C with gentle shaking. Primary rabbit antibody directed against c-Jun (SC44) was diluted 1:1000 in 5%BSA, Jun B (SC73) at 1:500 in 5% milk , Jun D (SC74) at 1:1000 in 5% BSA, $p21^{CIP1}$ (H164) at 1:200 in TBS-T, primary mouse anti-p53 (D-O7) at 1:1000 in TBS-T, β-tubulin (SC9104) at 1:1000 in TBS-T and anti-phospho c-Jun (Ser73) diluted 1:500 in 5% BSA were used in Western blot analysis. Membranes were washed 3 times for 10 minutes in 1XTBS-Tween (0.05%) to remove unbound antibody and then incubated at room temperature for 1 hour with a HRP-conjugated secondary anti-rabbit antibody from Biorad diluted at 1:5000 in 5% milk or a 1:2000 in TBS-T using HRP-conjugated secondary anti-rabbit or anti-mouse antibody from Pierce. Membranes were then washed 3X for 10 minutes with 1X TBS-T (0.05%), followed by detection. Detection of immunoreactive bands was performed using Lumiglo® Chemoluminescence Substrate or Lumiglo® Reserve Chemiluminescence Substrate from KPL Inc (Gaithersburg Maryland, USA). Detection Reagents were prepared by mixing substrate A to substrate B according to the manufacturers' instructions and added to the membrane for 1 minute. In the presence of hydrogen perioxide and luminol in the substrate mixture, the HRP conjugated to the secondary antibody converts luminol to light

emission which can be detected on X-ray film (Walker *et al.*, 1995). The membranes were exposed to X-ray film (AGFA) for varying exposure times and the film was subsequently developed for detection of antibody specific reactive bands. Gentle stripping of the blots for reprobing was performed with 1 M Glycine HCl pH 2.5. Blots were incubated for 7 minutes in 1 M Glycine HCl pH 2.5 and turned over for a further 7 minutes. The low pH disrupts the binding of the previous antibodies and then the membranes are neutralised with 1/10 of volume 1M Tris HCl pH 7.5. β-tubulin levels were determined to correct for protein loading. Densitometric analysis was performed with Chem Genius densitometer (Bio Imaging System, Vacutec) and GeneSnap® scanner and GeneTools® analysis (Cambridge, England).

2.6. Stable Transfection of TAM67

Doxycycline (Sigma Aldridge) inducible TAM67 CaSki clones were established previously (M.Maritz, Honours project) using the retroviral plasmid pLRT containing an inducible TAM67 construct. Stable TAM67 expressing pools and clones were selected in the presence of 5 µg/ml blasticidin (Sigma Aldridge) and screening for TAM67 protein expression was performed by Western blot analysis after induction with 1 µg/ml doxycycline. CaSki-TAM67 expressing cells were maintained in 10% FBS DMEM containing 5 µg/ml blasticidin and freezes were frequently made and stored at -80°C.

2.7. Reporter Gene Assays

2.7.1. AP-1 luciferase assays

Thirty thousand cells/well were plated in 24 well plates and incubated for 24 hours in 10% FBS/DMEM with antibiotics. Before transfection, media was aspirated off and 500 µl of 10% FBS/DMEM media lacking antibiotics was added to the cells to facilitate transfection. To determine AP-1 activity, cells were co-transfected with 100 ng of 4X AP-1-Luc and 20 ng of

pRL-TK-Luc Renilla plasmid using Transfectin Lipid Reagent (BioRad) according to the manufacturers' recommendations at a ratio of 3:1, transfectin: plasmid DNA. As a control, cells were transfected independently with 100 ng of 4X mutated AP-1-Luc plasmid. A 50µl transfection mix containing the following, Transfectin reagent, plasmid DNA, and the transfection efficiency control Renilla plasmid (pRL-TK-Renilla-Luc) was made up with serum-free media and incubated at RT for 20 minutes before dropwise addition to the cells. Transfected cells were incubated for six hours followed by a media change. The cells were then incubated for a further 24 hours before the cell lysates were harvested. Cells were washed in 500 µl ice cold 1X PBS and harvested by addition of 120 µl of 1X Passive Lysis Buffer (PLB) (Promega). Cells were incubated in PLB at room temperature with shaking for 15 minutes and stored at 4°C overnight. The specially formulated PBL, enables the rapid lysis of cells without scraping (Sherf *et al.*, 1996). 40 µl of cell lysates in PLB were then transferred to 96 well opaque plates used for luminescence analysis. Luciferase activity was measured using the Dual Luciferase™ Reporter Assay System (DLR™) (Promega) on the Veritas Microplate Luminometer, Turner Biosystems (California, USA). The DLR™ system allows the measurement of experimental firefly (*Photinus pyralis*) and control Renilla (*Renilla reniformis*) luciferase activity, sequentially from a single sample (Sherf *et al.*, 1996). To determine the Firefly luciferase luminescence, 40 µl of Luciferase Assay Reagent was added to the lysates and activity was measured. The reaction is quenched and the Renilla luciferase reaction is simultaneously initiated, by addition of 40 µl of Stop and Glo® Reagent (Sherf *et al.*, 1996). Media from untransfected cells was also measured to serve as a background control. Transfections were performed in triplicate and firefly luciferase activity was expressed relative to Renilla luciferase activity as the mean ±SE.

To determine inhibition by transient transfection, cells (30 000 cells /well) were plated in a 24

well plate and incubated for 24 hours before transfection. Cells were co-transfected with 100 ng 4X AP-1-Luc plasmid and 20 ng pRL-TK-Luc Renilla plasmid with varying concentrations (50 ng and 100 ng) of pCMV-TAM67 plasmid. Cells were co-transfected with 50 ng or 100 ng of empty pCMV as a negative control and independent transfection with 100 ng of 4X mutated AP-1-Luc served as a control. For stable clones, 3 X 10^4 CaSki and CaSki-TAM67 expressing clones were plated in a 24 well plate and incubated in 1 µg/ml doxycycline for 24 hours before transfection. Co-transfections of 100 ng 4X AP-1-Luc plasmid and 20 ng pRL-TK-Luc Renilla plasmid were performed and luciferase activity was measured as described above.

2.7.2. p21 promoter luciferase assays

Thirty thousand CaSki cells/well were plated in a 24 well plate and incubated in 10% FBS DMEM for 24 hours before transfection. To determine p21 activity, 100 ng 2500/+50 p21-Luc plasmid was cotransfected with 20 ng pRL-TK-Luc Renilla plasmid and luciferase activity was measured as described in section 2.7.1.

To determine the effect of TAM67 on p21 promoter activity, cells were co-transfected with 100 ng 2500/+50 p21-Luc plasmid and 50 ng or 100 ng of pCMV-TAM67 plasmid. For stable clones, 3 X 10^4 CaSki and CaSki-TAM67 expressing cells were plated in a 24 well plate and incubated in 1 µg/ml doxycycline for 24 hours after transfection. 20 ng of pRL-TK-Luc Renilla plasmid was co-transfected and luciferase activity was measured as previously described.

2.8. Cell Proliferation Analyses

2.8.1. Adherent cell proliferation analysis

Two thousand cells/well were plated in a 96 well plate in quadruplicate in the presence of 1 µg/ml doxycycline to induce the expression of TAM67. Cell proliferation was measured over 6 days using the MTT Assay (Cell proliferation Kit 1, Roche) according to the manufacturers' instructions. 10 µl of MTT reagent was added to the cells and incubated for four hours prior to the addition of solubilisation buffer (10% SDS in 0.01 M HCl) and incubated overnight. The MTT assay is a colourimetric assay that is based on the colour change brought about by the reduction of the yellow tetrazolium salt MTT reagent (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide in PBS) (Roche) to purple formazan crystals when cleaved by metabolically active cells (Vistica *et al.*, 1991). The absorbance at a wavelength of 595 nm provides an indication of cells actively growing and was measured on the Biotek Instruments, EL800, (Highland Park, USA) using Gene5 software (BioTek Instruments).

2. 8.2. Non-adherent cell proliferation analysis

For non-adherent cell proliferation and to observe colony formation, cells were stained with 1 mg/ml of p-iodo-nitrotetrazolium violet (Sigma Aldridge, Aston Manor, South Africa) for 16 hr at 37°C to detect live cells in colonies. The colonies were photographed by bright field microscopy at a magnification of 200X using the Carl Zeiss Axiocam High Resolution Camera on an Axiovision Fluorescence microscope (Muchen-Hallbergmoos, Germany). For quantitation, 10 000 cells/well were grown in 1.5% methylcellulose in 10% FBS containing media on polyheme poly(2-hydroxyethyl methacrylate) coated 96 well plates to prevent adhesion to dish. The wells were coated with 120 µl of polyheme and dried overnight before plating. Viable cells grown in non-adherent conditions were quantitated using the MTT reagent as described above.

2.9. Cell Cycle Analysis

To determine the cell cycle distribution, 7.5 X 10^5 cells were plated in 60 mm dishes in triplicate. After growth in 10% FBS DMEM for 24 hours, the cells were synchronized by serum depletion in 0.5% FBS DMEM for 24 hours and incubated for a further 24 hours, all in the presence of 1 µg/ml doxycycline. The cells were washed with ice cold 1X PBS, trypsinised and pelleted at 300 g for 5 mins using the Beckman J6-Centrifuge (Beckman Coulter Inc., Fullerton, USA). The pelleted cells were resuspended in 2 mls 10% FBS DMEM, counted and fixed with up to 10 mls 96% ethanol. The cells were stored at -20°C for 1 week to allow effective fixation which is essential to allow Propidium Iodide to penetrate the intact cell membrane. The harvested cells were then cetrifuged out of ethanol, washed and recentrifuged twice with ice cold 1X PBS. One million cells were transferred to a 1.5 ml microcentrifuge tube and incubated in 0.2 ml 1X PBS containing 0.5 µg/ul RNase A (Sigma) for 15 minutes at 37°C to remove RNA present. Finally cells were stained with 0.01 M Propidium Iodide for 20 mins at RT prior to analysis. Propidium Iodide is a fluorescent dye that intercalates between the bases of the double helix of DNA and RNA so to allow accurate quantification of DNA content (Krishan, 1975), RNA is degraded by the addition of RNase. The cells were sorted by Fluorescent Activated Cell Sorting (FACS) on a Beckman Coulter Cytomics Flow Cytometer 500 (Beckman Coulter Inc. Fullerton, USA). The Propidium Iodide fluoresces at a wavelength of 488 nm and therefore is detected in the FL3 channel. To compute the population of cells in G1, S and G2/M phase of the cell cycle, the Watson pragmatic mathematical model in Flowjo Flow cytometry analysis software (Tree Star Inc, Oregon, USA) was used. This model fits the cell cycle data by making no assumptions about the S-phase distribution and fits it exactly (Watson *et al.*, 1987).

2.10. Immunofluorescence

Coverslips were flamed in 95% ethanol and placed in 6 well plates. Fifty thousand cells were grown on coverslips for 48 hrs in presence of 1 µg/ml doxycycline. Media as aspirated off and cells were washed in 1X PBS. Cells were fixed in 4% paraformaldehyde for 45 mins and then permeabilised in 0.5% Triton-X-100 in 1X PBS for 5 minutes. The reaction was quenched in 50 mM NH_4Cl for 5 mins. NH_4Cl ions are able to quench the free aldehyde groups to prevent non specific background binding (Fox *et al.*, 1985). Cells were blocked in 0.2% gelatin for 30 mins and then incubated with primary antibodies for 45 mins in a humidified chamber. Both primary $p21^{CIP}$ (H164) (Santa Cruz) and p53 (D-O7) (Dako Cytomation) specific antibodies were diluted at 1:50 in 0.2% gelatin. Cells were washed three times in PBS and then incubated for 45 minutes with a Cy3-conjugated secondary antibody or an Alexa-488 conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc, Westgrove, USA) kindly provided by Dr. C Maske (Division of Anatomical Pathology, UCT). After two 5 min washes in PBS, cells were incubated with 100 ng/ml DAPI (4',6-diamidino-2-phenylindole dihydrochloride) for 5 mins and mounted onto slides with Mowiol. The slides were viewed with the Carl Zeiss Axiovision Fluorescence microsope (Germany) under 400X magnification using selective filters at 543 nm and 488 nm for the fluorophores Cy3 and Alexa-488 respectively and a filter at 405 nm was used for the nuclear stain, DAPI. For confocal microscopy, the Carl Zeiss Laser Scanning Microscopy (LSM) Confocal microscope (Germany) with similar filters was used.

2.11. RNA interference (RNAi)

Short interfering RNA (siRNA) inhibition of Jun gene expression was performed using siRNA directed specifically against human c-Jun (SC 29223), Jun B (SC 35726), Jun D (SC 35728) and siRNA containing a scrambled sequence, Control-A (SC 37007). Control-A

siRNA was used to discount any effects due to non- specific gene silencing of the siRNAs. All siRNAs were obtained from Santa Cruz Biotechnology. For siRNA inhibition, CaSki cells were transiently transfected with 20 nM or 80 nM siRNA.

2.11.1. Western blot analysis of siRNA inhibition

One hundred and fifty thousand CaSki cells were plated in 10% FBS/DMEM in a 35mm dish and allowed to settle overnight. Before transfection, media was aspirated off and 1 ml fresh 10% FBS/DMEM media lacking antibiotics was added to the cells to facilitate transfection. A 50 µl transfection mix containing Transfectin reagent (BioRad) and siRNA at a ratio of 2.5:1 was made up in serum-free media and incubated at RT for 30 minutes. Cells were transfected by dropwise addition of 50 µl transfection mix to the cells and incubated for 6 hours before media was changed. Cells were incubated for 24 hours, 48 hours and 72 hours after addition of new media and protein lysates were extracted and used in Western blot analyses as described in section 2.5.

2.11.2. Cell proliferation analysis of siRNA inhibition

The effect of individual siRNAs on cell proliferation was performed with 2000 CaSki cells plated in 96 well plates and allowed to settle overnight before transfection of siRNA. Cells were transfected with a transfection mix containing Transfectin reagent and siRNA at a ratio of 2.5:1, made up with serum free media and incubated at RT for 30 minutes before addition to cells. 20 nM of the different siRNA was used per transfection and cell proliferation was determined over four days after transfection using MTT assay as described in section 2.8.

2.12. Identification of potential AP-1 binding sites in p21 promoter

Transcription factor binding sites within a promoter region are identified using the

MatInspector Release Professional software that uses the Matrix Family Library Version 6.3. (Cartharius *et al.*, 2005) to identify individual binding sites within the promoter of specific interest. Whether these sites are functionally important in the regulation of the promoter still requires to be assessed. A 5134 bp p21 promoter sequence was compared with sequences in the library database and potential AP-1 binding sites were identified.

2.13. Transformation of competent cells with plasmid constructs

2.13.1. Preparation of competent cells

For large scale preparation of plasmids, competent cells were prepared. 5 ml of LB was inoculated with 50 µl glycerol stock of *Eschericia coli* XL1-Blue cells and incubated overnight at 37°C with vigourous shaking. 100 ml of LB was inoculated with 1 ml of overnight culture in a 500 ml flask and incubated for 3-4 hours to an OD reading of 0.4 - 0.6 at 600 nm. Cultures were transferred to 50 ml tubes and chilled on ice, followed by centrifugation at 4000 g for 10 mins at 4°C to pellet the cells. The medium was decanted and cells were resuspended in 10 ml of ice-cooled 0.1 M $CaCl_2$ and placed on ice. Cells were centrifuged at 4000 g for 5 mins at 4°C and resuspended in 10 ml 0.1 M $CaCl_2$ and recentrifuged as before. The cells were resuspended in 2 ml 0.1 M $CaCl_2$ for every 50 ml of original culture and incubated at 4°C overnight. Cells were placed on ice for 15 mins after addition of 70 µl of DMSO and 100 µl was transferred to prechilled microcentrifuge tubes before storage at -70°C. The efficiencies were checked by transformation of serial dilutions of plasmid. 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng of plasmid were added to 100 µl of competent cells and incubated on ice for 30 mins. The cells are then heat shocked at 42°C for 2 mins, added to 900 µl LB medium and incubated at 37°C for an hour in a waterbath. 100 µl of each transformation reaction was plated onto duplicate selective LB plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Colonies were counted and the average efficiency

of 1.8 X 10^6 cfu/ug DNA was calculated from the dilutions of the plasmid.

2.13.2. Transformation of plasmids

To prepare pRL-TK-Luc Renilla,, wt 4X AP-1-Luc and mutated 4X AP-1-Luc, CMV, CMV-TAM67, -2500/+50 p21-Luc, 2 µg of each plasmid was used to transform to 50 µl of thawed competent cells and mixed by gently flicking. The tubes were placed on ice for 20 minutes before a 2 min heat shock in a 42°C waterbath. The tubes were returned to ice for 2 minutes. 950 µl SOC medium was added and cells were incubated for 1.5 hrs at 37°C with shaking at 150 rpm. 100 µl of each transformation was plated onto selective 100 µg/ml ampicillin- or 100 µg/ml chloramphenicol- containing LB plates. DNA was isolated using the Qiagen Plasmid Maxi Kit (California, USA) as follows: Overnight starter cultures of 5 ml were diluted into 500 mls and grown at 37°C overnight with vigourous shaking. Cells were harvested by centrifugation at 6000 g on a Beckman ™ JA-10 centrifuge for 15 mins at 4°C. The supernatant was removed and the pelleted cells were resuspended in 10 ml resuspension Buffer P1 containing 0.1 mg/ml RNAse A. For lysis, 10 ml of lysis Buffer P2 was added and mixed thoroughly by inverting 4-6 times for 5 mins at RT, until the solution became slightly clear and viscous. To neutralise, 10 ml of chilled Buffer P3 was then added, incubated on ice for 20 min and mixed thoroughly by inverting up to 10 times as the solution became cloudy. The samples were then centrifuged at 20000 g for 30 mins at 4°C and the supernatant containing the plasmid DNA was then centrifuged again for 15 mins at 4°C. The supernatant is then transferred to an equilibrated Qiagen-tip 500 spin column and the plasmid DNA was allowed to enter the silica membrane by gravity flow. The membrane was then washed twice with 30 mls wash buffer QC to remove all contaminants. DNA was eluted with 15 ml of Buffer QF. The eluted DNA was then precipitated by addition of 10.5 ml RT isopropanol and centrifuged at 15000 g for 30 min at 4°C. The DNA pellet was then washed with 70% ethanol

and centrifuged at 15000 g. The pellet was air dried for 5-10 mins and redissolved in 50 µl TE buffer, pH 8.

2.13.3. Glycerol stocks

Colonies were inoculated into 5 ml selective LB medium containing 100 µg/µl of ampicillin and grown till stationery phase (with an OD 600 nm of 1-2) at 37°C with vigourous shaking. 800 µl of cells was then added to 200 µl sterile glycerol and stored at -80°C.

213.4. Spectrophotometric analysis of DNA

Absorbance of DNA was measured at 260 nm and 280 nm using the Beckman DU 650 spectrophotometer (Beckman Coulter Fullerton, USA). The Beer-Lamberts Law equation of A260 nm = Ecl was used for quantification of the nucleic acids using a 1 cm pathlength spectrophotometer cuvette (l) and the extinction co-efficient (E) at A260 nm of 50 µg/ml for dsDNA. The concentrations were determined as follows: DNA c(µg/ml) =A260nm X 50 µg/ml x dilution factor X 1 cm)/1000 µl. A 1:500 dilution was used so concentration was determined by c (µg/ml) = A260nm X 50 µg/ml X 10. Quality of DNA was assessed by ratio of A260nm/280nm. A ratio of between 1.8 and 2 indicates good quality nucleic acids (Glasel, 1995).

2.14. Statistical Analysis

The data is represented as the mean ± Standard Error (SE) as calculated by the division of the standard deviation by the square root of the number of samples ($\sigma^2\sqrt{n}$). The statistical significance of the differences was determined by the two tailed Student's t-test unless otherwise stated. The Mann Whitney T-Test was used to statistically analyse c-Jun, Jun B and Jun D expression levels in patient material. Statistical analysis was performed in Microsoft

Excel and Graph Pad Prism (GraphPad Software, San Diego, USA). A p value of ≤ 0.05 was considered statistically significant.

CHAPTER 3

THE EFFECTS OF AP-1 INHIBITION ON THE BIOLOGY OF CERVICAL CANCER CELLS

3.1. INTRODUCTION

The biological response of a cell relies on the intricate system of signal transduction pathways that transmit signals required for processes including growth, movement and cell death. A key factor involved in regulating signal transduction is the activation of transcription factors responsible for the transcriptional regulation of target genes in the nucleus. These events have a significant impact on processes such as cell proliferation, differentiation, cell movement and cell death and thereby regulate the overall responses of the cell (Blume-Jensen and Hunter, 2001; Darnell, Jr., 2002). Cancer cells acquire similar functional capabilities leading to biological changes that result in increased proliferation and the potential for invasion and metastasis (Hahn, 2002). The activation of transcription factors is largely involved in this modulation (Darnell, Jr., 2002; Hanahan and Weinberg, 2000). AP-1 is one such transcription factor that is involved in a vast array of cellular responses including proliferation and survival (Shaulian and Karin, 2001; Shaulian and Karin, 2002). Due to the ability of AP-1 transcription factor to influence numerous biological processes and tumourigenic processes (Jochum *et al.*, 2001; Karin *et al.*, 1997; Shaulian and Karin, 2001; Shaulian and Karin, 2002) examining its contribution to the oncogenic phenotype of cancer cells is of interest, particularly in its potential as a therapeutic target (Leaner *et al.*, 2007; Young *et al.*, 2003).

The use of cell culture model systems has proven to be an effective way to study transcriptional mechanisms associated with disease progression. The use of human immortal

cancer cell lines provides a useful system within which to study the biological roles of specific proteins and the identification of phenotypes associated with cancer (Masters, 2000). In this study, the biological relevance of the transcriptional factor AP-1 in the development of cervical cancer was investigated with the use of cervical cancer cells lines.

Two approaches are often used to examine the role of specific proteins of interest within cell lines. The first approach involves the identification of the cell lines with little or no protein of interest which can be used as model within which the gene can be over expressed. The second approach involves identifying a cell line with high levels of expression of a gene of interest allowing for inhibitory studies to be performed. The overexpression or inhibition of target genes can be performed by both transient transfections using artificial promoter constructs and or by generating stable transfectants in which the protein of interest is over-expressed or inhibited. Transient transfections serve as useful way to determine immediate effects, however there is limited time after transfection during which the effects can be investigated. The generation of stable transfectants allows for the investigation of more long term effects on, for example, the biological changes that may occur as a result of overexpression or inhibition of a particular gene and is thus able to provide a better reflection of what is happening biologically within the cells.

Within this study, a dominant negative mutant of AP-1 known as TAM67 was used to inhibit AP-1 in cervical cancer cells. TAM67 acts as a global inhibitor of AP-1 within cells, by disrupting endogenous AP-1 activity (Brown *et al.*, 1994). Both transient and stable AP-1 inhibition by TAM67 was investigated in cervical cancer cells. The effects of TAM67 inhibition of AP-1 allows for the deciphering of AP-1's contribution to a number of biological endpoints such as cell growth, cell cycle progression and tumourigenic markers, including

non-adherent growth of cervical cancer cells.

An alternative way in which to study the effects of inhibition of a specific gene includes the use of RNA interference (RNAi) technologies. RNAi makes use of a naturally occurring gene silencing phenomenon that occurs in cells (Elbashir *et al.*, 2001). The use of sequence specific short interfering RNA (siRNA) provides an effective method for specific gene silencing. In this study we used both dominant negative mutants and siRNAs to inhibit AP-1 activity in cervical cancer cells. TAM67 inhibits all potential AP-1 complexes containing c-Jun, Jun B, Jun D, while siRNA directed specifically against the individual Jun Family proteins (c-Jun, Jun B, Jun D) were used to determine their individual roles in cervical cancer cells.

3.2. RESULTS

3.2.1. AP-1 expression in cervical cancer

Previously published studies identified a correlation of high AP-1 expression levels with a number of cancers including breast, endometrial and colon (Bamberger *et al.*, 2001; Vleugel *et al.*, 2006; Wang *et al.*, 2002; Wang *et al.*, 1995; Young *et al.*, 1999). To determine the expression levels of components of the AP-1 complex in cervical cancer patient material, Western blot analysis of proteins extracted from normal cervical and cervical cancer tissue specimens collected at Groote Schuur Hospital was performed using antibodies specific to the Jun family proteins, c-Jun, Jun B and Jun D. Western blots revealed that c-Jun expression levels appeared to be overall higher in cervical cancer tissue compared to normal specimens with 7/9 (78%) cancers showing high levels of c-Jun expression (Fig. 3.1.A). In comparison only 2/7 of the normal tissue (28%) showed relatively high c-Jun expression. Quantification of c-Jun expression in patient material by densitometric scanning of western blots and normalising c-Jun levels relative to that of β-tubulin, a housekeeping gene, in the same protein lysate, revealed an overall trend towards higher c-Jun levels in cervical cancer patient material compared to the normal patient tissue (Fig 3.1.B).

High Jun B expression was also identified in cervical cancer patient tissue compared to that of normal patient tissue with 12/15 (80%) cancers showing high Jun B expression in comparison to 2/8 (25%) normal tissues showing moderately high Jun B expression levels (Fig 3.2.A). Quantification relative to β-tubulin showed an overall increase in Jun B levels in the cancers compared to the normal (Figure 3.2.B). The difference in Jun B expression levels was found to be statistically significantly higher using the Mann Whitney T Test (p value <0.01) (Figure 3.2.B). In contrast to c-Jun and Jun B, Jun D expression appeared to be at similar levels in

cervical cancer tissues compared to normal patient tissue showing moderate levels of expression relative to β-tubulin in most of the cancers (14/15) and normal tissue (6/8) (Figure 3.3.A). Quantification of Jun D levels relative to β-tubulin revealed no difference in the overall Jun D levels in normals compared to cancer (Figure 3.3.B). This finding is consistent with published reports that Jun D is constitutively expressed (Hirai *et al.*, 1989). The determined expression levels of the AP-1 components and the relatively high levels of c-Jun and Jun B in cervical cancer specimens suggest that the transcription factor AP-1 may have relevance to the phenotype of this disease.

The expression levels of c-Jun, Jun B and Jun D was also determined in a panel of six cervical cancer cell lines including CaSki, HeLa, SiHa, Me180, Ms751 and C33A. Variable c-Jun and Jun B expression was observed in the six cervical cancer cell lines tested, with CaSki cells having the highest expression levels of these AP-1 components (Fig. 3.4.A, B). Surprisingly, Jun B levels were highly variable in the cancer cell lines with SiHa cells showing no detectable Jun B by Western blot analyses (Fig. 3.4.B). This variation is consistent with that observed with patient material. Similar amounts of Jun D protein were detected in the cervical cancer cell lines tested, with a slightly lower expression level in CaSki cells (Fig. 3.4.C). These results show that the highest c-Jun/AP-1 and Jun B/AP-1 expression was detected in CaSki cells. This cell line was therefore used to explore the potential role of AP-1 in cervical cancer.

3.2.2. Inhibition of AP-1 in cervical cancer cells using the dominant negative mutant, TAM67

To determine the potential role of AP-1 in cervical cancer cells, the dominant-negative mutant of AP-1, called TAM67, that has previously been shown to inhibit AP-1 activity and function was used (Brown *et al.*, 1993; Domann *et al.*, 1994).

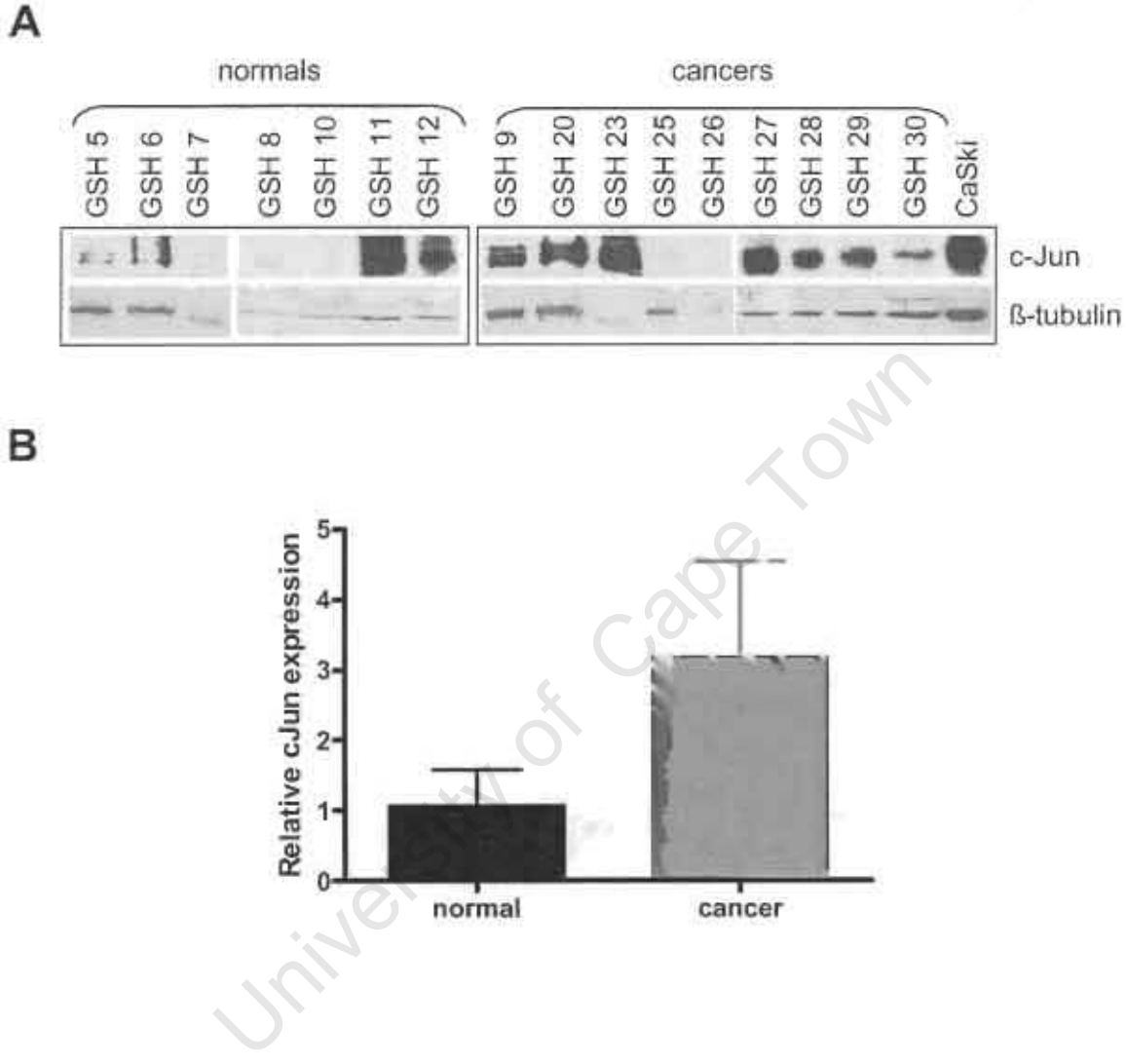


Figure 3.1. c-Jun/AP-1 expression in normal cervical tissue and cervical cancer tissue. (A). Western blot analysis showing c-Jun expression in normal and cervical cancer tissue. Twenty µg of protein isolated from patient material was subjected to Western blot analysis for the presence of c-Jun using an anti-c-Jun specific antibody that detects a 39 kD protein (see Materials and Methods). The blots were stripped and reprobed with β-tubulin as a control for loading. (B). Relative c-Jun expression levels in normal cervical tissue and cervical cancer tissue. c-Jun and β-tubulin levels were quantified by densitometric scanning and c-Jun levels relative to β-tubulin were expressed as arbitrary densitometric units. To correct for variations between blots, CaSki protein extracts were included on all blots. Results shown represent the mean ±SE.

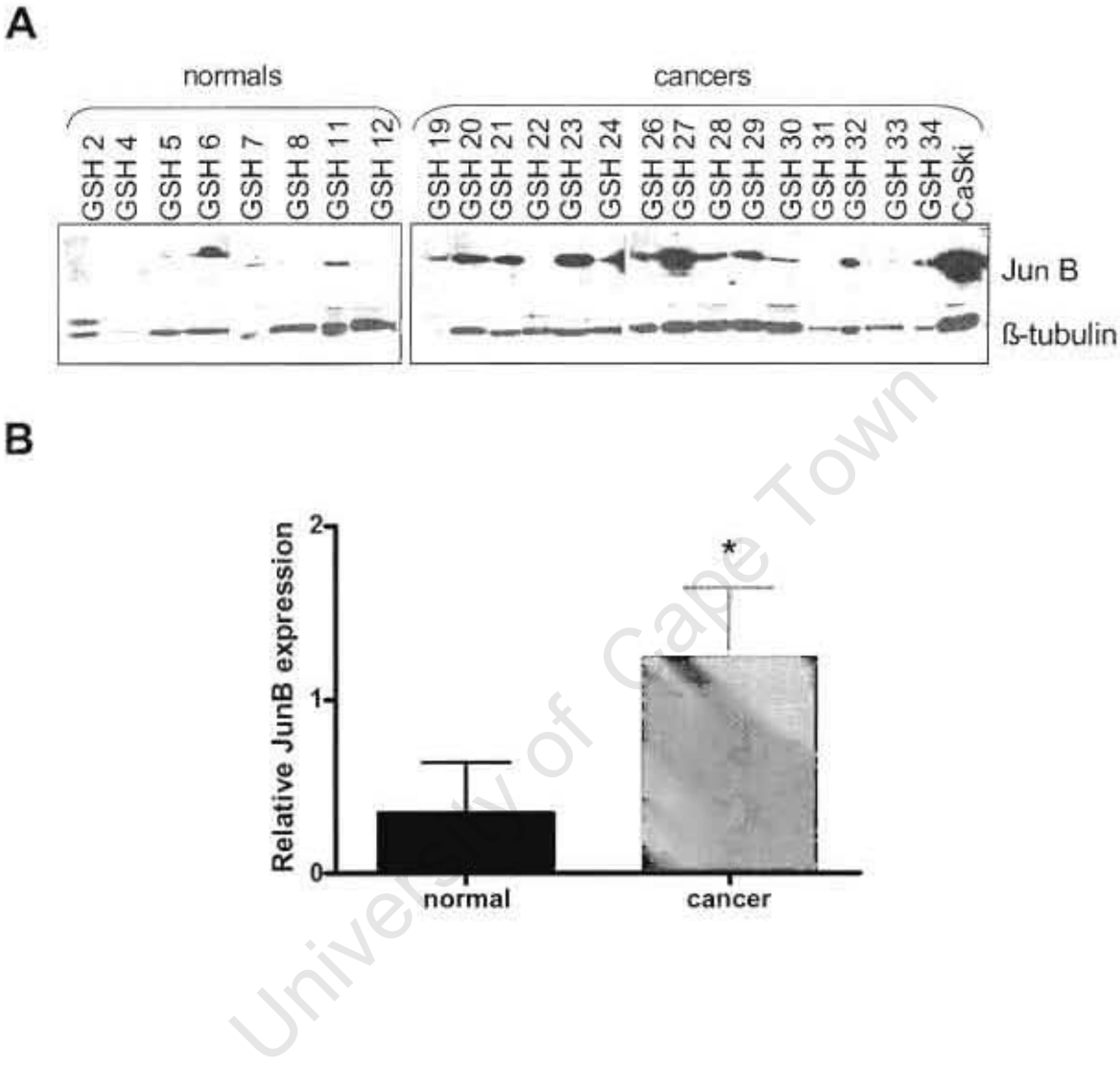


Figure 3.2. Jun B/AP-1 expression in normal cervical tissue and cervical cancer tissue. (A).Western blot analysis showing Jun B expression in normal and cervical cancer tissue. Twenty µg of protein isolated from patient material was subjected to Western blot analysis for the presence of Jun B using an anti-Jun B specific antibody (see Materials and Methods). The blots were stripped and reprobed with β-tubulin as a loading control. (B). Relative Jun B expression levels in normal cervical tissue and cervical cancer tissue. Quantification of Jun B and β-tubulin levels by densitometric scanning is shown as arbitrary densitometric units. To correct for variations between blots, CaSki protein extracts were included on all blots. Results shown represent the mean ±SE.* ($p<0.01$).

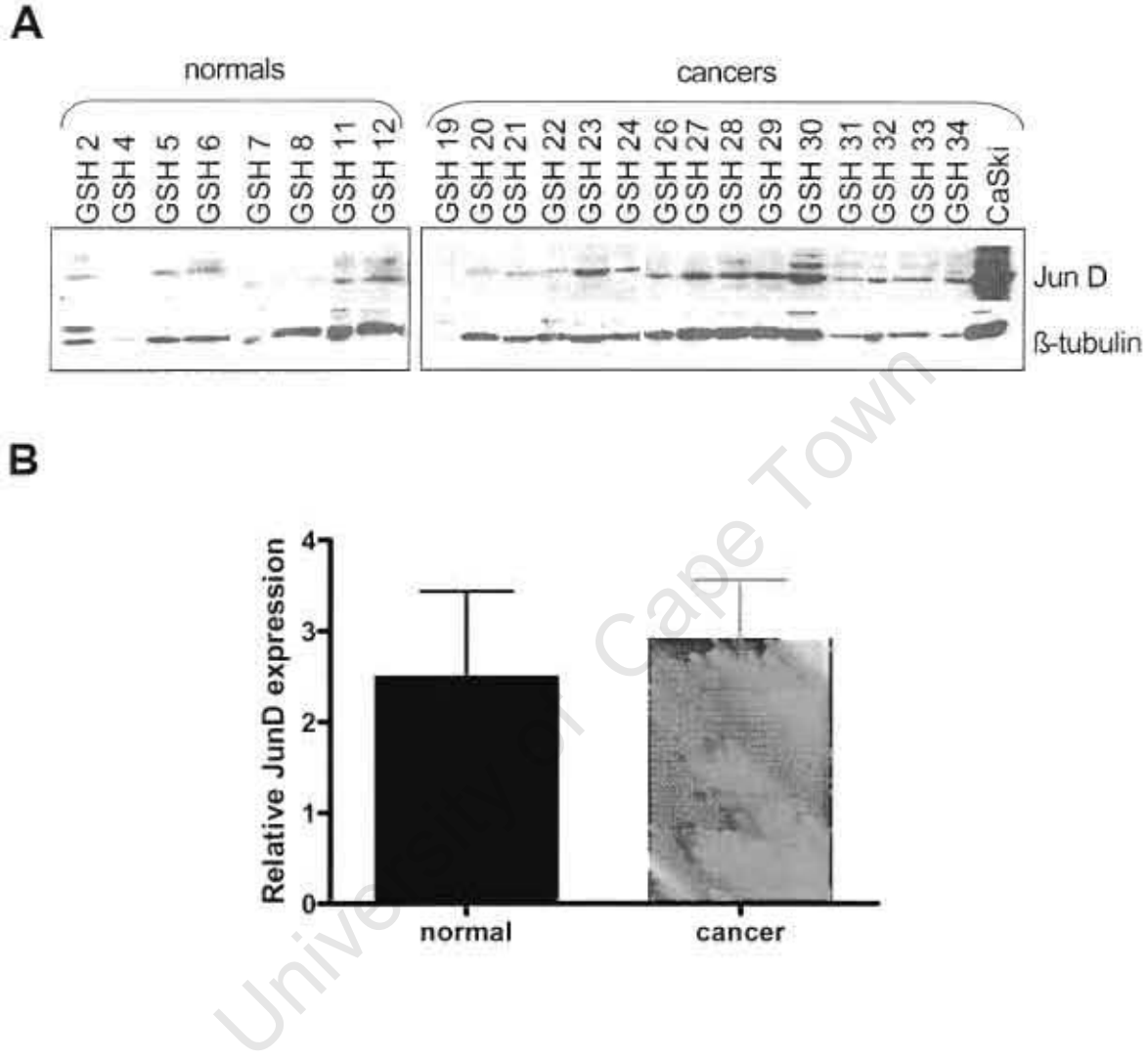


Figure 3.3. Jun D/AP-1 expression in normal cervical tissue and cervical cancer tissue. (A). Western blot analysis showing Jun D expression in normal and cervical cancer tissue. Twenty µg of protein isolated from patient material was subjected to Western blot analysis for the presence of Jun D using an anti-Jun D specific antibody (see Materials and Methods). The blots was stripped and reprobed with β-tubulin as a loading control. (B). Relative Jun D expression levels in normal cervical tissue and cervical cancer tissue. Quantification of Jun D levels relative to β-tubulin by densitometric scanning is shown as arbitrary densitometric units. To correct for variations between blots, CaSki protein extracts were included on all blots. Results shown represent the mean ±SE.

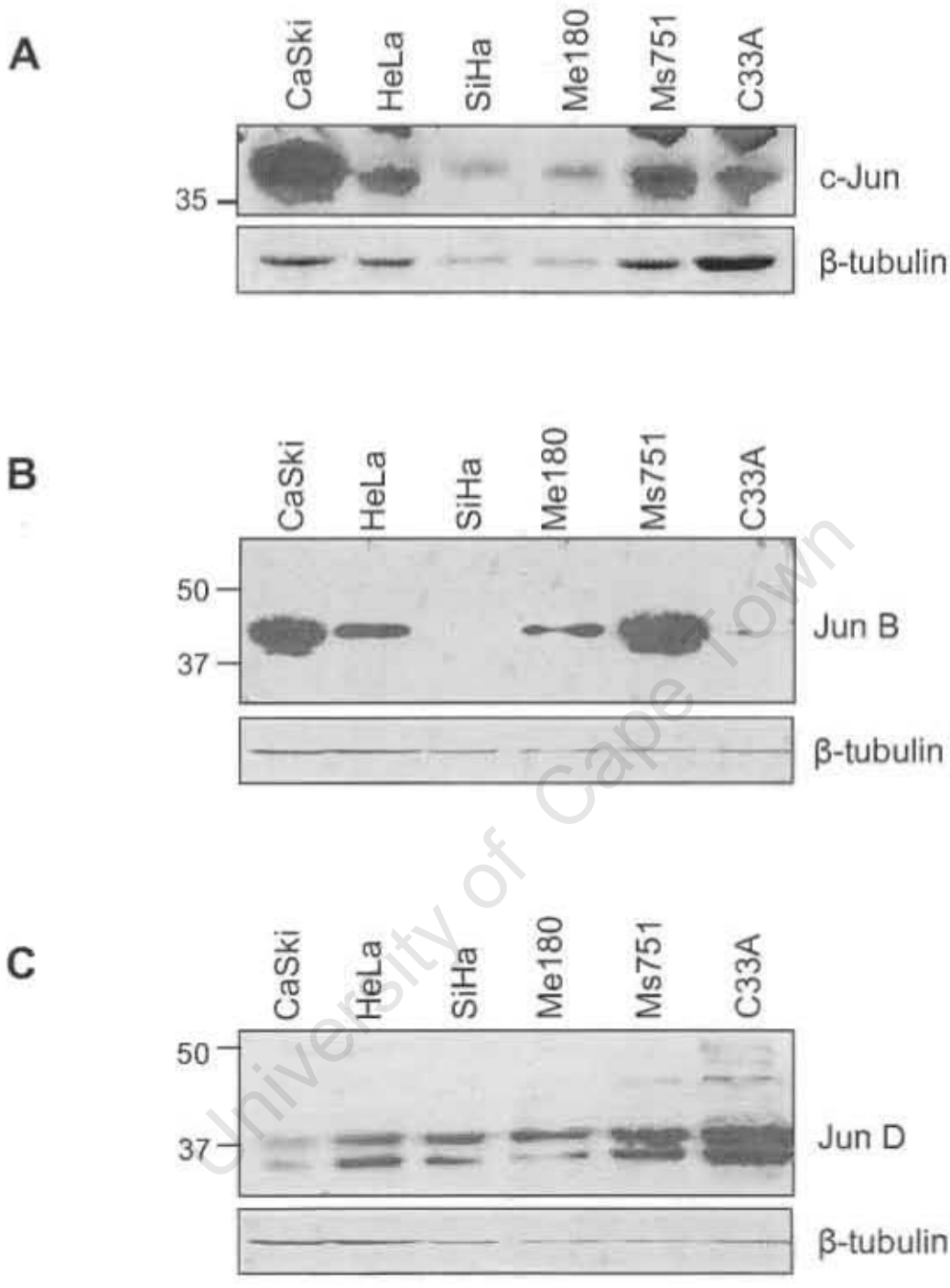


Figure 3.4. c-Jun, Jun B and Jun D in cervical cancer cell lines. Protein isolated from cervical cancer cell lines were subjected to western blot analysis for the presence of (A) c-Jun, (B) Jun B and (C) Jun D. β-tubulin was used as a loading control.

TAM67 was introduced into CaSki cells either via transient transfection or by preparing clonal cells with TAM67 stably included in the cells genome. Transient transfections allowed for assaying immediate, short term effects while stable clones allowed for studying more long term effects (Figure 3.5).

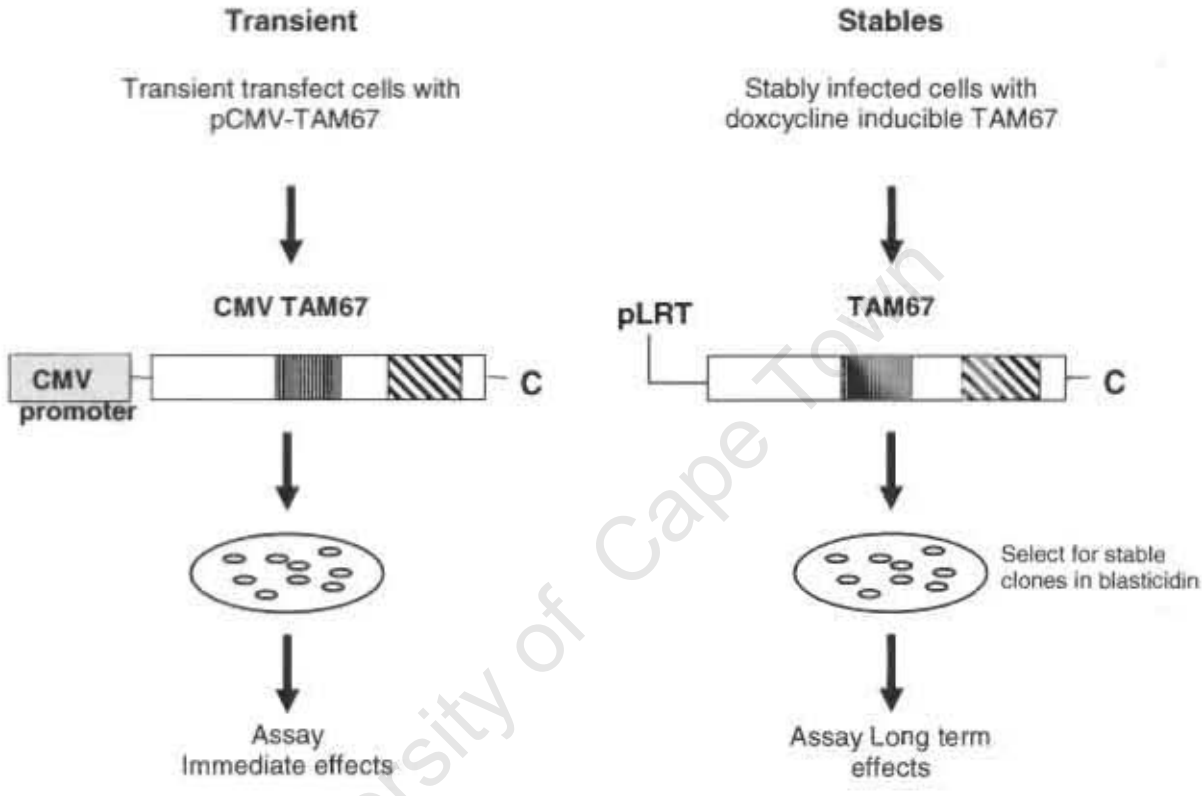


Figure 3.5. Approaches used to express TAM67. Transient and stable expression of the dominant-negative mutant, TAM67 used to assay short term and long term effects of AP-1 inhibition in cervical cancer cells.

For transient transfections, an expression plasmid, pCMV-TAM67, where TAM67 is driven by Cytomegaloviral promoter, a strong promoter allowing the expression of genes in mammalian cells, was used (Brown *et al.*, 1993; Schmidt *et al.*, 1990). Stable TAM67 clones were generated using a plasmid with inducible TAM67, pLRT-TAM67 (Kinoshita *et al.*, 2003).

3.2.2.1. Effect of AP-1 inhibition by transient transfection of TAM67

The ability of TAM67 to inhibit AP-1 was initially investigated in CaSki cells. Transient transfections of CaSki cells with the increasing concentrations (0.05, 0.1, 0.5, 1 and 2 μg/ml) of pCMV-TAM67 expression vector, was performed to determine the plasmid concentration required for detectable amounts of TAM67. After transfection, protein was isolated and the presence of TAM67 was analysed using an antibody directed to the carboxyl terminus of c-Jun, thus allowing this antibody to detect both endogenous full length c-Jun at 39kD and TAM67 that lacks the transactivation domain of c-Jun as a 29kD protein (Brown, 1993, Bohmann *et al* 1987). Optimal TAM67 expression was observed in protein lysates of cells transfected with 0.5 μg/ml pCMV-TAM67 (Figure 3.6). A decrease in c-Jun/AP-1 expression levels was detected with an increase in TAM67 expression (Figure 3.6). This finding is consistent with published reports showing that c-Jun/AP-1 autoregulates its own expression, hence inhibition of the AP-1 complex is expected to interfere with c-Jun expression (Angel *et al.*, 1988). TAM67 could be detected with as little as 100 ng pCMV-TAM67 expression vector (Figure 3.6). Interestingly, transient transfections beyond 0.5 μg/ml did not necessarily result in higher TAM67 expression. This is likely due to variations in transfection efficiencies.

Having established that TAM67 was being expressed in transient transfection approach, the ability of TAM67 to inhibit AP-1 promoter activity within cervical cancer cells was determined. A promoter construct containing four AP-1 binding sites fused to a luciferase reporter gene (4X AP-1-Luc) was co-transfected with pCMV-TAM67 by transient transfections. Functional AP-1 complexes present in the cell are able to bind to the 4X AP-1-Luc promoter construct and thus drive the expression of luciferase. Luciferase activity was therefore used as a measure of AP-1 activity. The results showed that TAM67 could effectively inhibit AP-1 activity in CaSki cells by as much as 94% (Figure 3.7.A). Similarly,

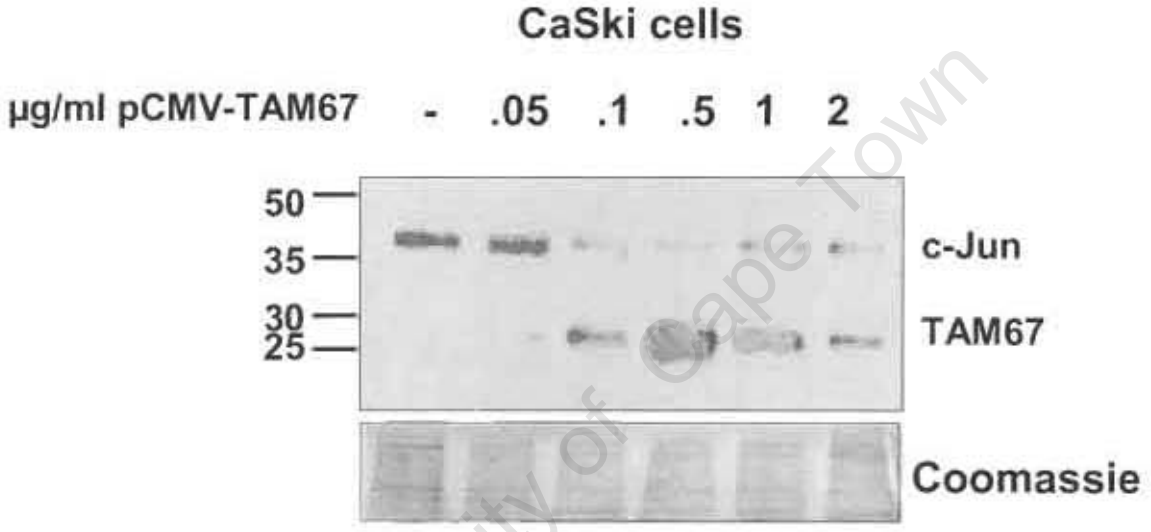


Figure 3.6. TAM67 and c-Jun/AP-1 expression in transiently transected CaSki cervical cancer cells. CaSki cells were transfected with varying concentrations of the pCMV-TAM67 expression. Thirty µg of protein was subjected to Western blot analysis. A band in the region of 39 kD is representative of c-Jun, while TAM67 is expressed as an approximately 29 kD protein. Coomassie staining of the gel after transfer shows similar protein loading.

TAM67 was an effective inhibitor of AP-1 activity in other cervical cell lines, including Me180 and SiHa cells (Figure 3.7.B and 3.7.C). To control for transfection efficiency, Renilla luciferase expression under the control of the TK promoter, pRL-TK-Renilla was used. To discount any inhibitory effects due to the vector or higher DNA amounts, the pCMV empty vector was co-transfected with the 4X AP-1-Luc promoter construct. pCMV had little effect on AP-1 activity in all three cell lines, suggesting that the inhibition on the 4X AP-1 activity was specifically due to the expression of the dominant negative TAM67. A promoter construct containing 4X mutated AP-1 binding sites (mut 4X AP-1-Luc) served as a negative control for AP-1 binding and no activity was detected using this construct (Figure 3.7).

Taken together, the transient transfection of TAM67 into cervical cancer cells showed this dominant-negative mutant of AP-1 could inhibit both endogenous expression of AP-1 components (Figure 3.6), as well as the activity of this transcription factor in cervical cancer cell lines (Figure 3.7).

CaSki cells were identified as the cervical cancer cell line within which to pursue further studies with regard to the biological relevance of AP-1 activity in cervical cancer. In order to determine the long term effects of AP-1 inhibition, stably transfected CaSki cells with an inducible TAM67 were generated (M.Maritz, Honours project). The plasmid construct, pLRT-TAM67 contained a marker conferring for blasticidin resistance thus allowing the isolation of stable clones. Doxycycline-inducible TAM67 expression was screened in eight clones by Western blot analysis (Figure 3.8.A). Pools of TAM67 expressing cells and individual clones were used in further analysis in order to discount potential clonal variation.

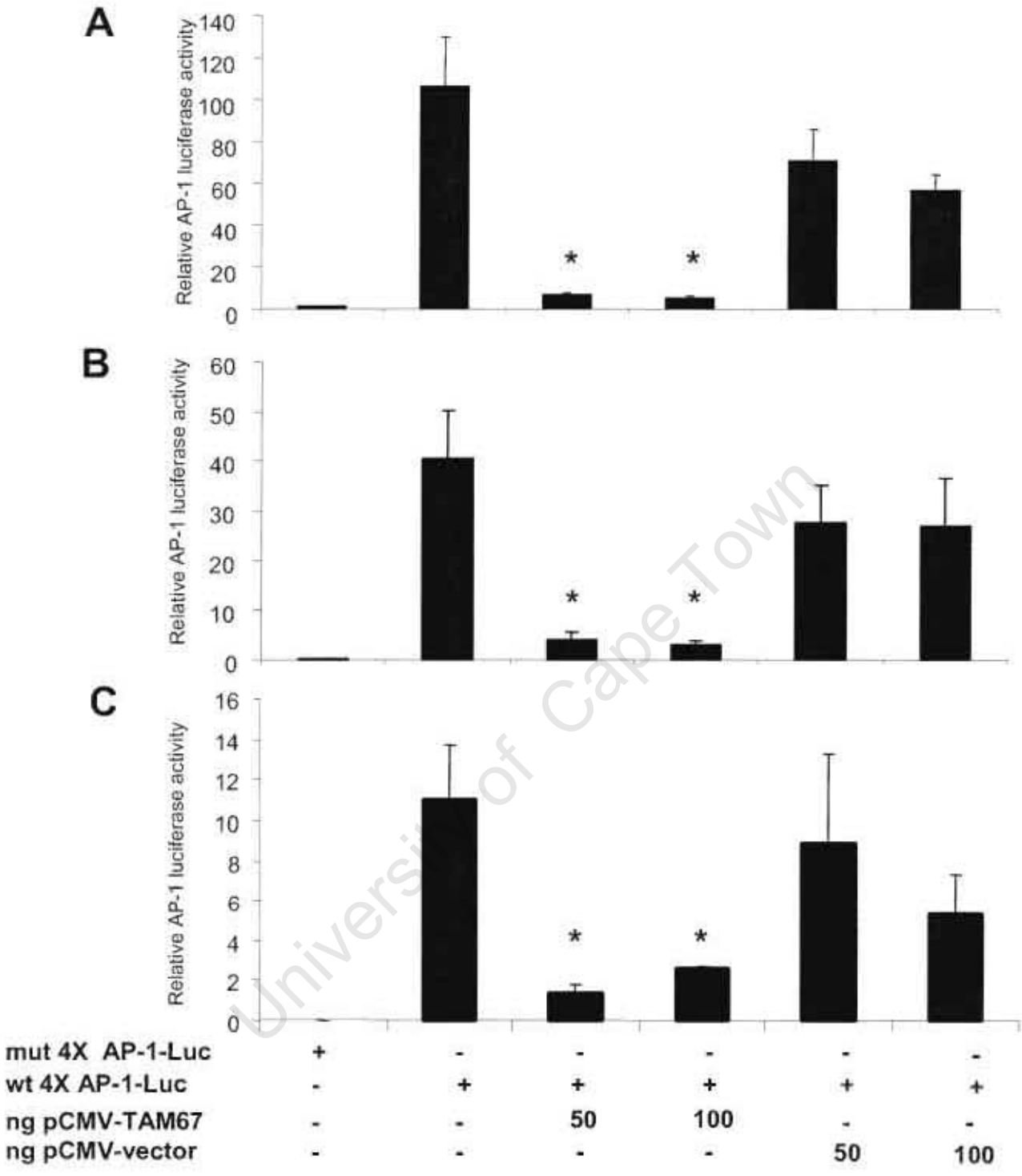


Figure 3.7. TAM67 inhibition of AP-1 promoter activity in transient transfection of CaSki, Me180 and SiHa cervical cancer cells. pCMV-TAM67 or pCMV only was transiently co-transfected with wild type AP-1 binding site promoter construct (4XAP-1) into CaSki (A), Me180 (B) and SiHa (C) cells.TAM67 causes a decrease in transcriptional activity of AP-1 in (A) Me180 cells and (B) SiHa cells. A pLR-TK-Luc Renilla luciferase vector was cotransfected to correct for transfection efficiency. Activity was determined by the Dual Luciferase™ Reporter Assay. Results shown are the mean ±SE of experiment performed in triplicate and repeated at least twice. TAM67 expression resulted in a significant decrease in promoter activity. *p value ≤ 0.05.

3.2.2.2 Effect of inducible TAM67 expression on cervical cancer cells

Doxycycline-inducible TAM67 expression in two CaSki-TAM67 expressing clones, Clones 5 and Clones 7, was subsequently used to investigate the effects of AP-1 inhibition in cervical cancer. The cells were incubated for 48 hours in the absence or the presence of 1 µg/µl doxycycline and screened for the presence of the 29 kD TAM67 protein. A substantial induction of TAM67 expression was detected in the presence of doxycycline (Figure 3.8.B). c-Jun forms part of a positive autoregulatory loop and regulates itself by binding to an AP-1 site within its promoter (Angel *et al.*, 1988). Considering the fact that TAM67 is an inhibitor of AP-1 activity, the effect of TAM67 expression on endogenous c-Jun/AP-1 expression was observed on the same Western blot (as with earlier detection in transient transfections with pCMV-TAM67). The results show that in the presence of doxycycline, an increase in TAM67 expression was accompanied by a decrease in endogenous c-Jun levels (Figure 3.8.B). Faint bands corresponding to TAM67 were also detected in the absence of doxycycline, indicating a leakiness of the inducible TAM67 construct. For this reason the majority of the experiments that follow were investigated by comparing CaSki parental cells and CaSki-TAM67 clones all grown in the presence of 1 µg/µl doxycycline.

The expression levels of other members of the AP-1 family of proteins namely Jun B and Jun D, were determined in the CaSki-TAM67 expressing cells by western blot analysis. Jun D showed a similar decrease in expression as c-Jun, while the expression of Jun B showed no change (Figure 3.9). The ability of TAM67 to decrease the expression of Jun D is possibly due to the disruption of positive regulation of its promoter by AP-1 (Berger and Shaul, 1998). Jun B expression is not reported as being regulated by AP-1.

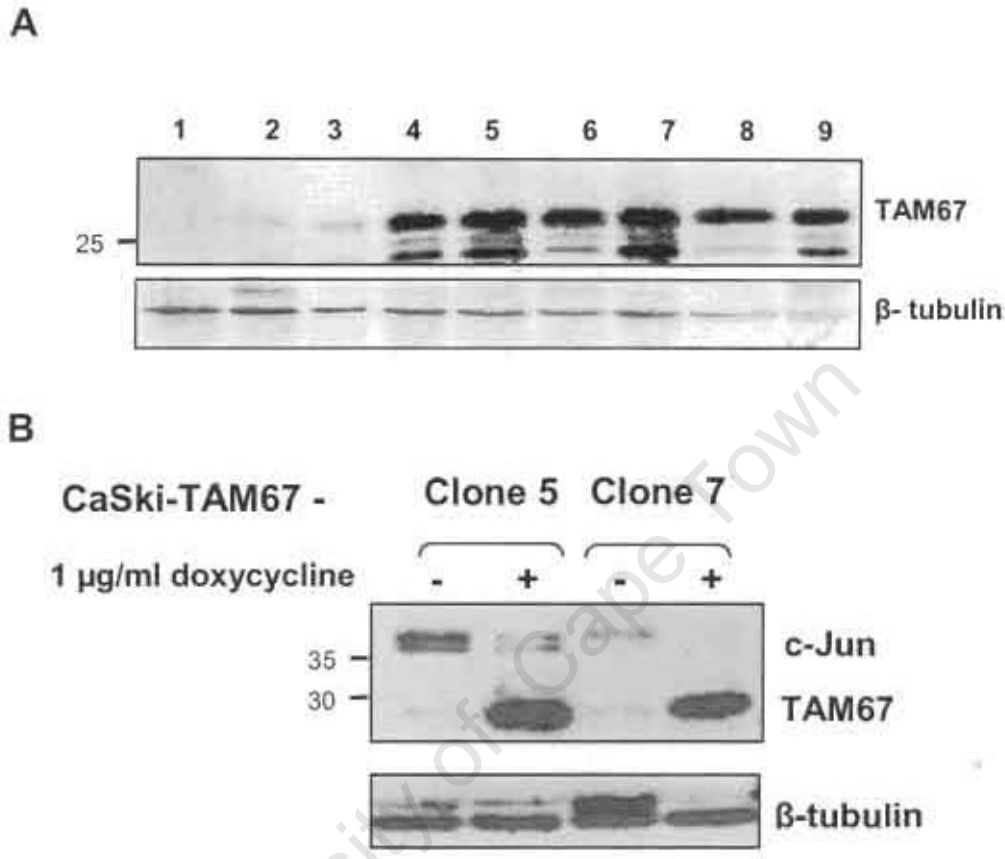


Figure 3.8. Expression of TAM67 in stably transfected Caski-TAM67 clones. (A).TAM67 expression in eight clones. Protein lysates were extracted from CaSki parental and eight individual CaSki-TAM67 expressing clones all grown in the presence of doxycycline for 48 hours. 50 µg protein was subjected to immunoblotting for the presence of TAM67 at 29 kD. Lower bands present are indicative of protein degradation. Lane 1: CaSki, Lanes 2-9: Caski-TAM67 Clones 1-8. (B). Doxycycline inducible TAM67 expression in CaSki-TAM67 expressing clones 5 and 7 grown in the presence of doxycycline for 48 hours. The presence of c-Jun and TAM67 at approximately 39 kD or 29 kD respectively is detected. β-tubulin was used as a control for loading.

The phosphorylation of c-Jun at Ser 63 and Ser73 have been reported to be essential for transcriptional activity (Kallunki *et al.*, 1996). To determine whether TAM67 is effective in inhibiting the expression of activated c-Jun, western blot analysis was performed using lysates of control CaSki and CaSki-TAM67 expressing cells. An antibody directed against phosphorylated Serine 73 of c-Jun was used as an indicator of activated c-Jun. Inhibition of AP-1 in TAM67 expressing clones was accompanied with a decrease in both total c-Jun and phosphorylated levels of c-Jun in both CaSki-TAM67 Clone 5 and CaSki-TAM67 Clone 7 (Figure 3.10).

3.2.2.3. Doxycycline - inducible TAM67 inhibits AP-1 promoter activity

To investigate the inhibition of stably transfected doxycycline-inducible TAM67 on the AP-1 activity in CaSki cells, CaSki and CaSki-TAM67 expressing cells grown in the presence of doxycycline were transfected with 100 ng of the 4X AP-1 promoter luciferase construct (4X AP-1-Luc). The AP-1 activity observed in the CaSki-TAM67 clones was compared to the AP-1 activity in the parental CaSki and a significant reduction in activity as observed in the CaSki-TAM67-Clones, Clone 5 and Clone 7 (Figure 3. 11).

These results support the earlier findings of the transient transfections of TAM67 which showed that this dominant negative mutant is an effective inhibitor of AP-1 activity in cervical cancer cells. Thus experiments to determine the effects of this inhibition on the biology of these cells were performed.

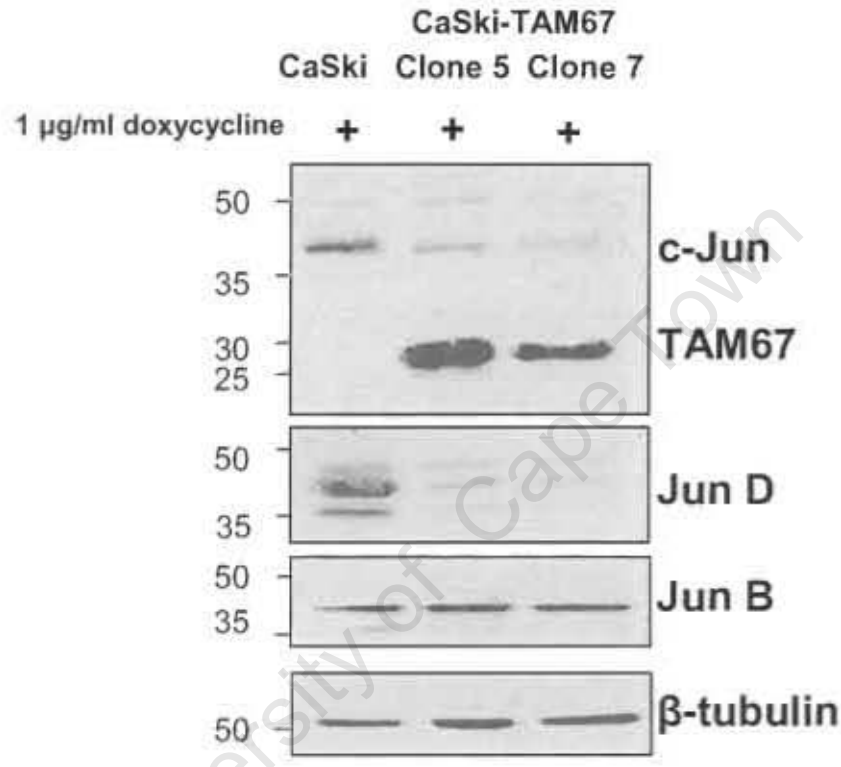


Figure 3.9. Effect of TAM67 inhibition on Jun Family proteins. Protein lysates extracted from CaSki parentals and CaSki-TAM67 expressing clones 5 and 7 grown in the presence of doxycycline for 48 hours. Thirty µg proteins were analysed by Western blot analysis for the presence of TAM67, c-Jun, Jun B and Jun D.

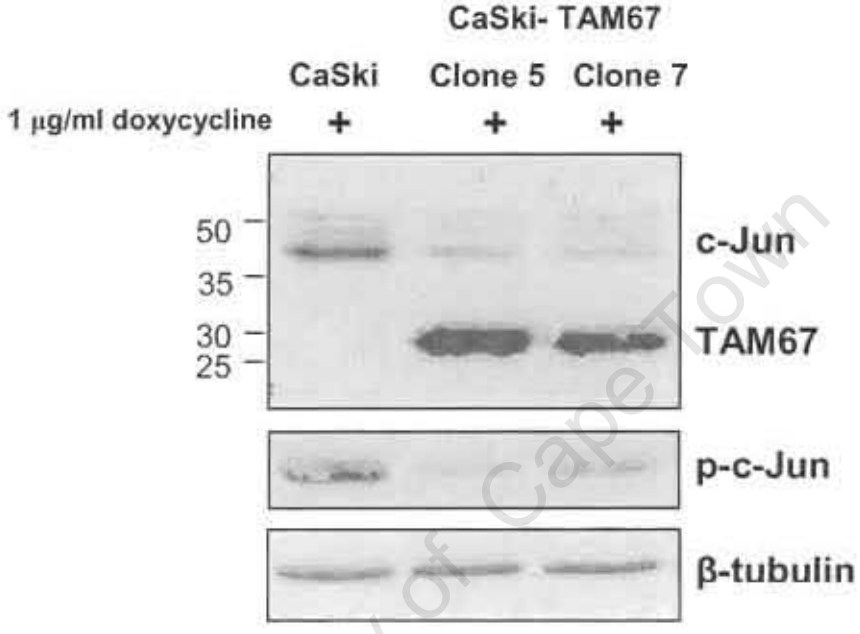


Figure 3.10. Effect of TAM67 on activated c-Jun. Protein lysates were extracted from CaSki and CaSki-TAM67 expressing clones 5 and 7 grown in the presence of doxycycline. Thirty µg protein was assayed by Western blot analysis for the presence of activated c-Jun using a phospho c-Jun specific antibody (anti-p-Ser73-c-Jun). β-tubulin was used as a loading control.

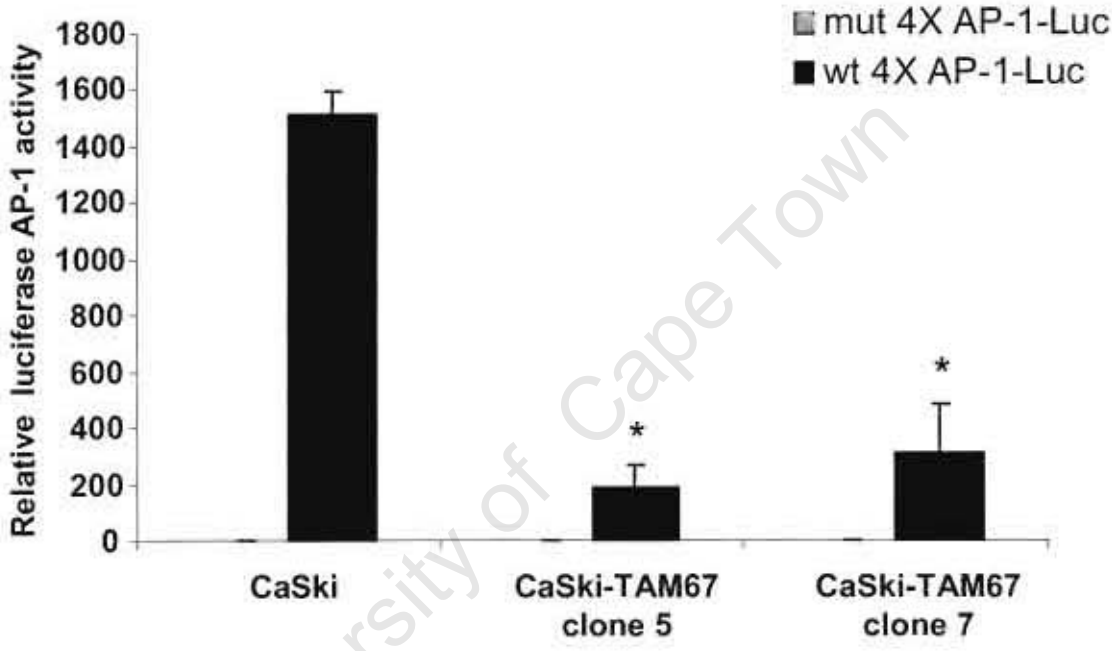


Figure 3.11. TAM67 inhibition of AP-1 activity in CaSki-TAM67 expressing clones. CaSki, CaSki-TAM67 Clone 5 and CaSki-TAM67 Clone 7 grown in the presence of doxycycline were transiently transfected with 100 ng 4X AP-1-Luc promoter constructs and luciferase activity was determined using the Dual Luciferase™ Reporter Assay system. The results were expressed as relative AP-1 dependent firefly luciferase activity normalized to Renilla luciferase activity as a control for transfection efficiency. The results show the mean ±SE of the experiment performed in triplicate and repeated at least two times (*$p<0.05$)

3.2.3. AP-1 inhibition affects the biology of cervical cancer cells

3.2.3.1. Effects of AP-1 inhibition on adherent cell growth

TAM67 resulted in an altered cell morphology of CaSki-TAM67 expressing cells, displaying a larger cell size with a more flattened and irregular shape (Figure 3.12.A). TAM67 expressing cells also appeared to be growing at a slower rate compared to the parental CaSki cells suggesting that inhibition by TAM67 was affecting the proliferation of cervical cancer cells.

To determine whether inhibition of AP-1 had any effect on cervical cancer cell proliferation the proliferation of the CaSki-TAM67 Clone 5 and Clone 7 cells was compared to that of the parental CaSki cells over five days using the MTT Assay. Cell proliferation for the TAM67 expressing clones was significantly lower, indicating that inhibition of AP-1 activity by TAM67 affects adherent cell proliferation. A significant decrease in adherent cell proliferation was identified in both CaSki-TAM67 Clone 5 and Clone 7 compared to the proliferation of the parental CaSki cells after five days (Fig. 3.12.B).

3.2.3.2. Effect of AP-1 inhibition on non-adherent cell proliferation

A characteristic of transformed cells is their ability to grow in an anchorage-independent or non-adherent manner and to form colonies (Fukazawa *et al.*, 1995; Macpherson and Montagnier, 1964).

To determine if inhibition of AP-1 by TAM67 affects the ability of cervical cancer cells to grow non-adherently, cells were grown in 1.5% methylcellulose on polyheme coated dishes to prevent adhesion to the dish. After 5 days under these conditions, cells were stained with 1 mg/ml p-iodo-nitrotetrazolium violet and colony formation viewed under bright field

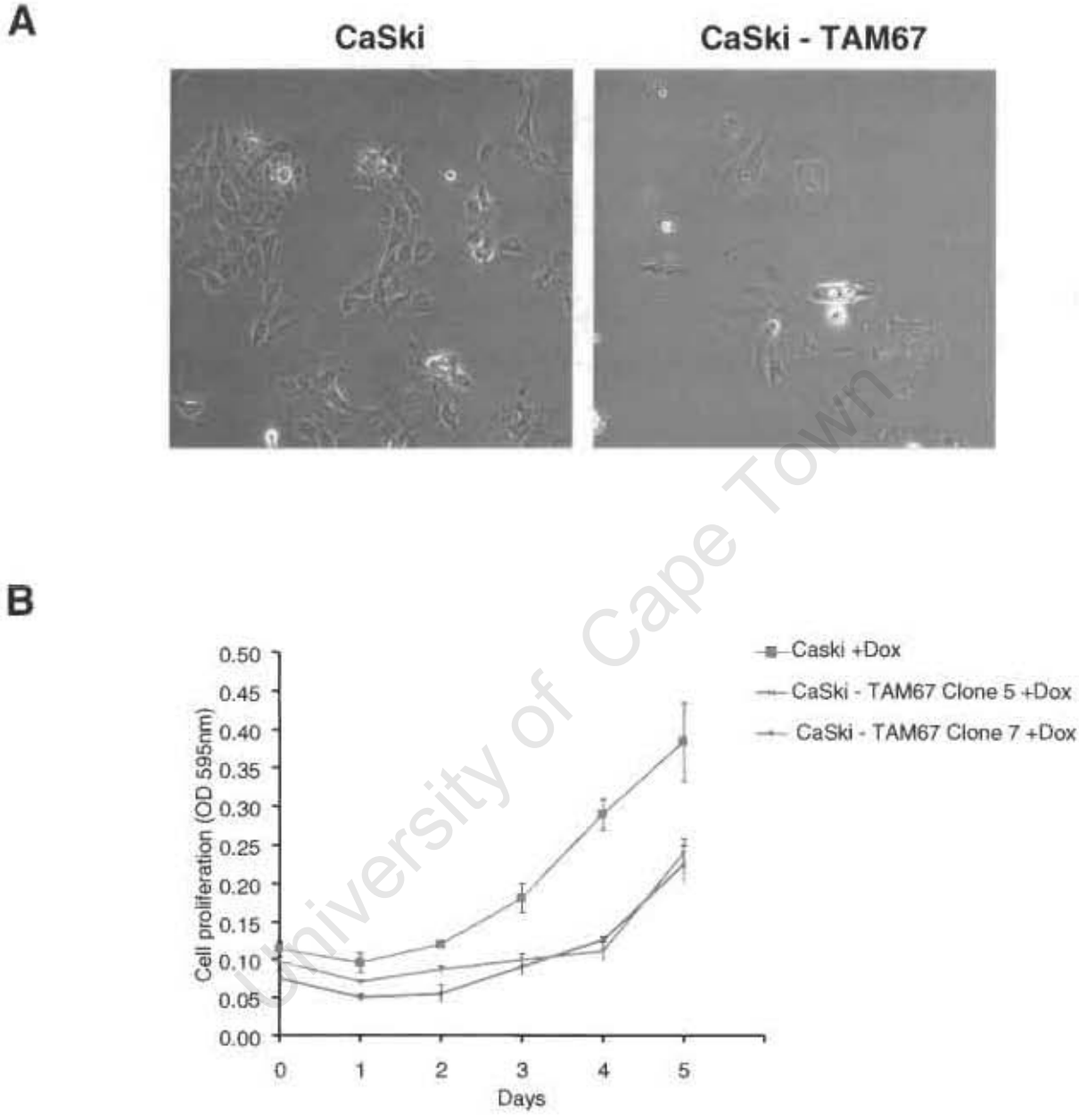


Figure 3.12. TAM67 effects on adherent cell growth. (A). TAM67 expression results in altered cellular morphology. The cell morphology of CaSki parental cells and TAM67 expressing CaSki cells was observed under phase contrast microscopy at 100X magnification. (B). Inhibition of AP-1 by TAM67 causes an inhibition of adherent cell proliferation Adherent cell proliferation of CaSki and CaSki-TAM67 expressing clones 5 and clone 7 was measured over five days using MTT assay (see Materials and Methods). The results show the mean ±SE of the experiment performed in quadruplicate. The experiment was repeated at least three times.

microscopy. After 6 days, both TAM67 expressing clones, CaSki-TAM67 Clone 5 and Clone 7 showed a clear decrease in colony formation in comparison to the large spherical colonies observed in the parental CaSki cells (Figure 3.13). The results shown are from experiments performed in triplicate. To quantitate these observations, concurrent MTT assays were performed. Quantitation of non-adherent proliferation by MTT assay confirmed that inhibition of AP-1 activity by TAM67 caused a significant decrease in the ability of CaSki cells to proliferate in non-adherent conditions (Figure 3.14). These results suggest a possible role for AP-1 in the tumourigenic phenotype observed in cervical cancer cells.

3.2.4 Inhibition of individual AP-1 components using siRNA

Having established that AP-1 activity is required for the phenotype (cell morphology, adherent cell proliferation, colony formation and non adherent proliferation) led to the next investigation of which members of the individual Jun family of proteins were required for the biology of cervical cancer cells. While TAM67 could inhibit all AP-1 complexes, inhibition of individual Jun proteins, c-Jun, Jun B, Jun D could be done with specific siRNA. This was achieved by transfecting individual Jun siRNA into CaSki cells. A control siRNA, Control-A siRNA was included in the analyses. The siRNAs were all 20-25nt oligomers purchased from Santa Cruz Biotechnology, with the control siRNA made up of a scrambled sequence of 20-25 nucleotides long that does not target any specific gene for degradation (Elbashir *et al.*, 2001). To determine whether the siRNAs were effective in inhibiting their respective Jun family proteins, CaSki cells were transfected with each siRNA and expression levels were determined by Western blot analysis. The concentrations of siRNA required and the time taken to inhibit protein expression were optimised using c-Jun siRNA. CaSki cells were transfected with two different concentrations of c-Jun siRNA, 20 nM and 80 nM, followed

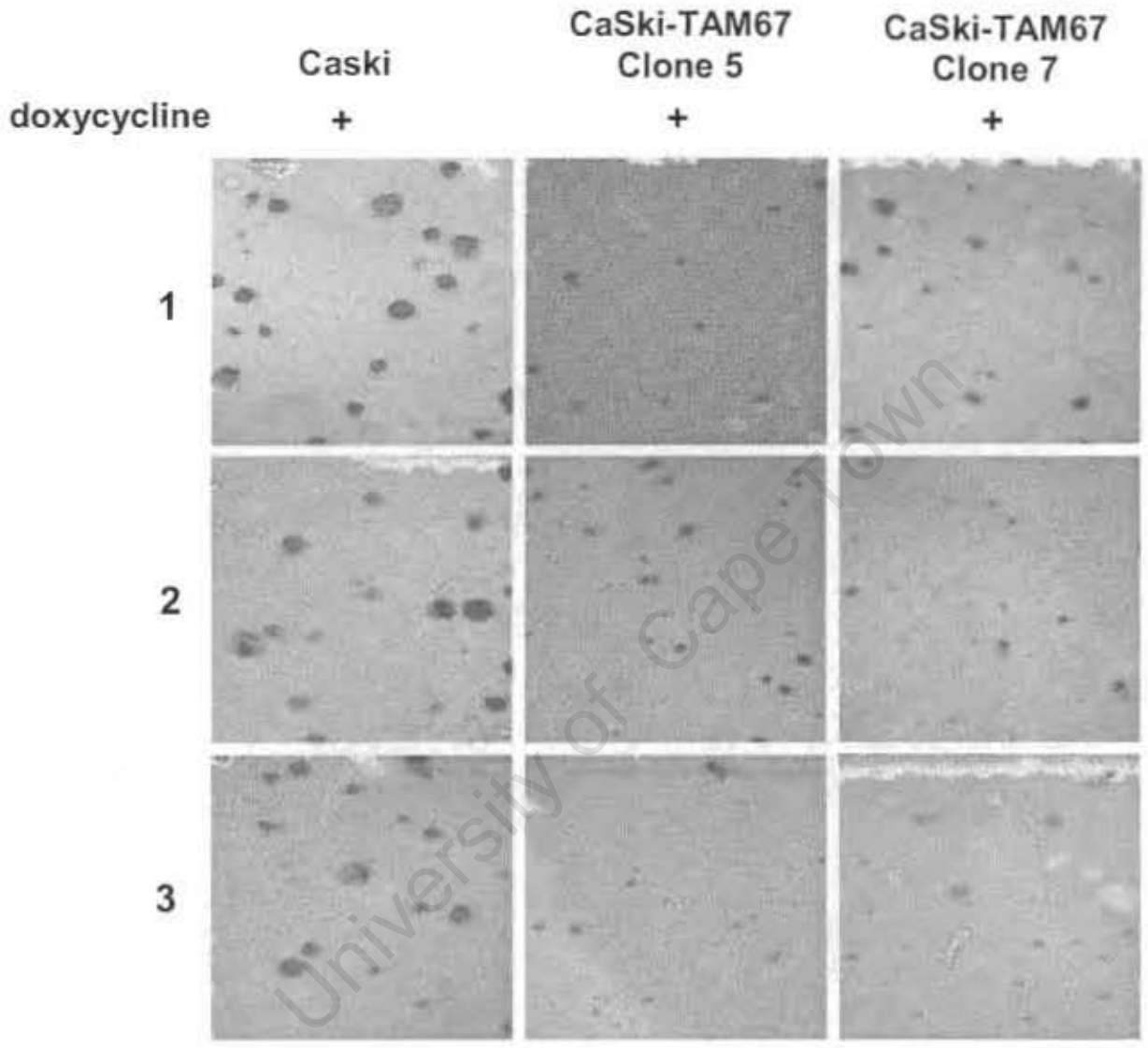


Figure 3.13. Inhibition of AP-1 by TAM67 causes inhibition colony formation. Cells were grown in 1.5% methylcellulose/10% FBS on polyheme coated dishes to prevent adhesion to dish and incubated for 6 days. Colony formation was observed by staining the cells with 1mg/ml of p-iodo-nitrotetrazolium violet for 16hr at 37°C to detect living cells in colonies. The colonies were viewed under bright field microscopy at a magnification of 200X and three fields of view were taken as represented by 1, 2, 3. Experiments were performed in triplicate.

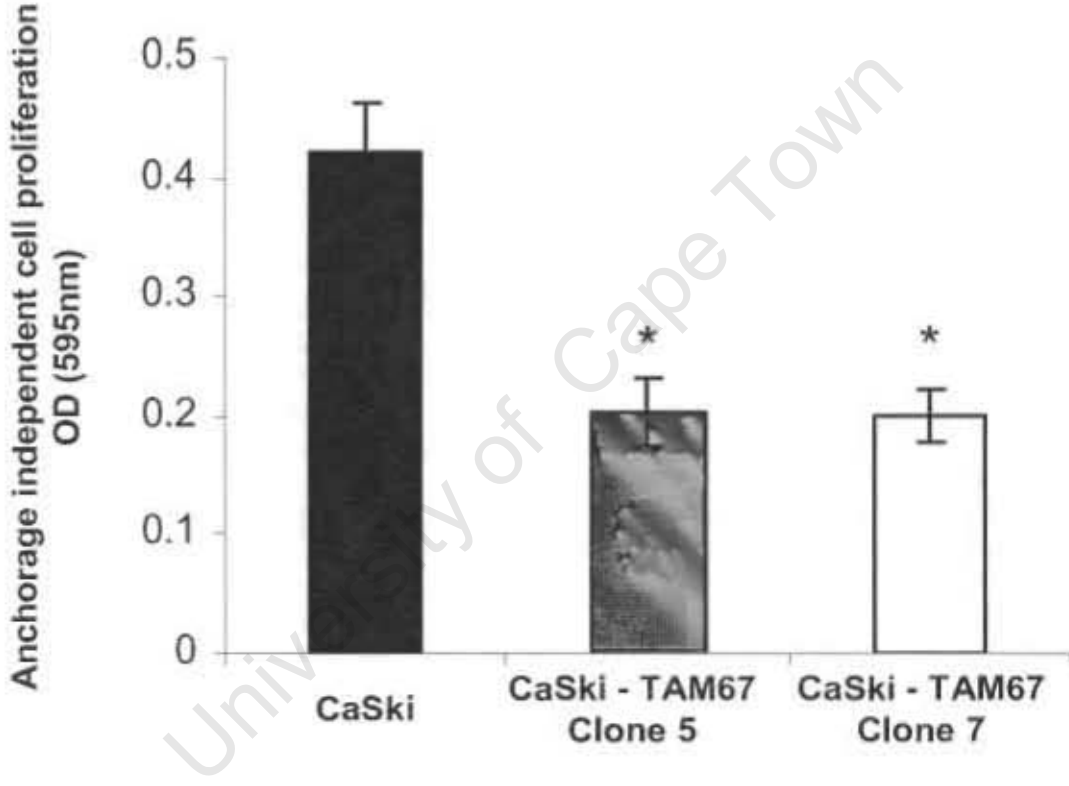


Figure 3.14. Inhibition of AP-1 by TAM67 causes inhibition of non-adherent cell proliferation. Cells were grown in 1.5% methylcellulose on polyheme coated dishes and non-adherent cell proliferation quantified using the MTT proliferation assay (see Materials and Methods). Results represent the mean ±SE of the experiments performed in quadruplicate after 6 days in culture. * $p < 0.05$.

by incubation for 24, 48 and 72 hours. Western blot analysis showed that both concentrations were effective in inhibiting c-Jun expression and a decrease in expression levels for all three time points compared to that of CaSki control cells with no siRNA (Figure 3.15.A). Transfection with both 20 nM and 80 nM Control-A siRNA showed no change in c-Jun expression, showing that the decrease by c-Jun siRNA was due to c-Jun specific targeting by the c-Jun siRNA (Figure 3.15.A).

Similarly, CaSki cells transfected with 20 nM Jun B siRNA, showed decreased Jun B expression after 24 and 48 hrs in comparison to controls (Figure 13.15.B). A similar decrease in Jun D expression levels was obtained in CaSki cells transfected with 20 nM Jun D siRNA for both 24 hrs and 48 hrs (Figure 3.15.C). To determine if the inhibition observed with these siRNA was selective for their specific Jun family proteins, the blots were reprobed for the expression of the other Jun family proteins not specifically targeted. Jun B siRNA caused no decrease in the expression levels of c-Jun and Jun D (Figure 3.15.B). Similarly, c-Jun and Jun D siRNA were also found to be specific (data not shown) indicating that the siRNA targeted for the individual Jun family proteins cause selective inhibition of the Jun protein targeted.

To examine the role of the individual Jun proteins in adherent proliferation of cervical cancer cells, CaSki cells were transfected individually with 20 nM of c-Jun, Jun B, Jun D siRNA and 20 nM of each siRNA transfected together. As controls, CaSki cells were transfected with no siRNA and Control-A siRNA to control for any effects on proliferation due to non specific silencing. The effect of the different siRNAs on cell proliferation of CaSki cells was compared over four days using the MTT assay. Transfections with the three siRNA together showed a significant decrease in cell proliferation in comparison to that of the controls

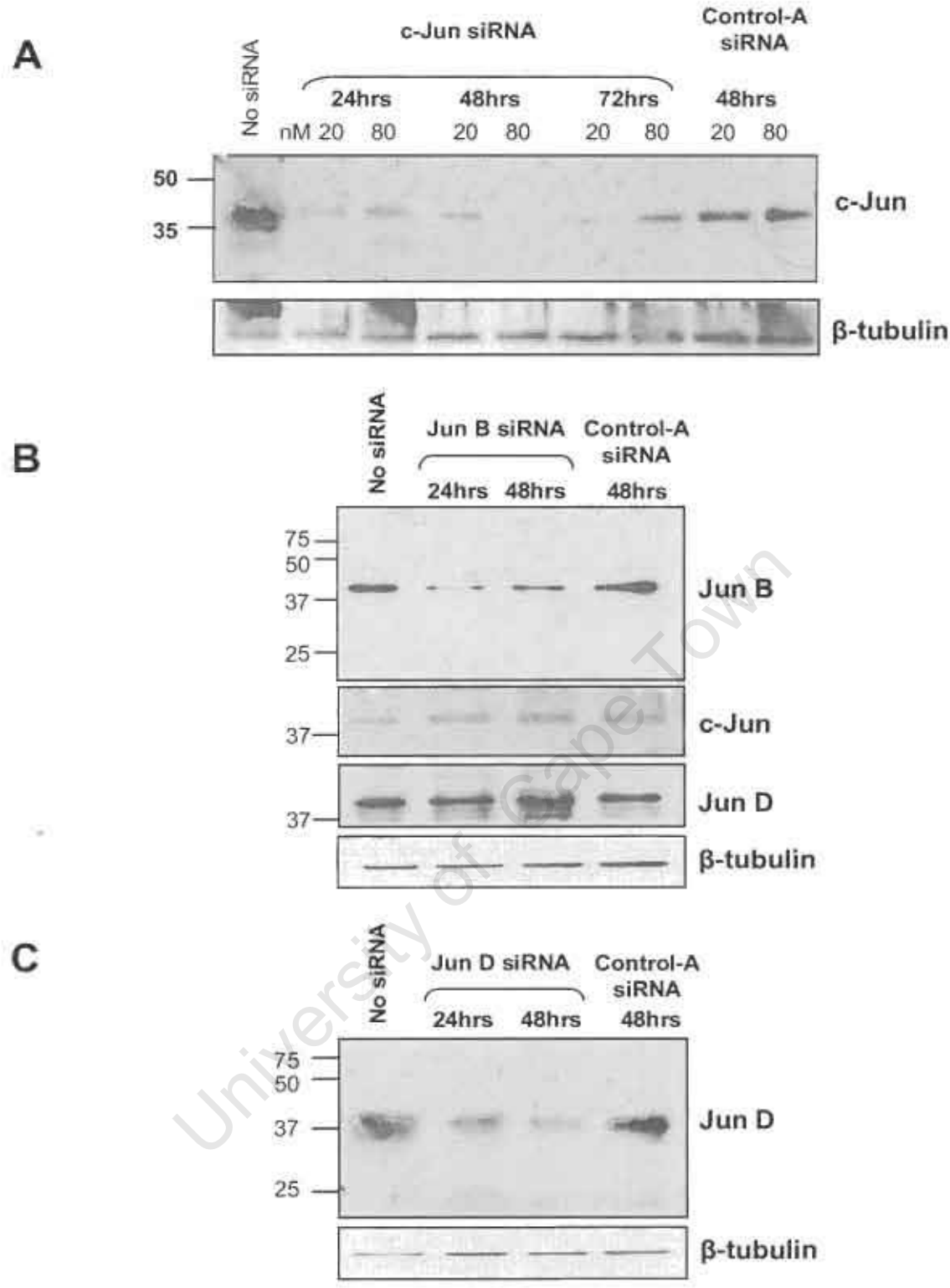


Figure 3.15. Effects of specific c-Jun, Jun B and Jun D siRNA inhibition on expression levels in CaSki cells. (A). CaSki cells were transfected with 20 nM and 80 nM c-Jun siRNA and Control-A siRNA, followed by 24 hr, 48 hr and 72 hr incubations. Proteins were subjected to Western blot analysis and probed for c-Jun. Blots were reprobed with β-tubulin to control for loading. (B). CaSki cells were transfected with 20 nM Jun B siRNA and Control-A siRNA and incubated for 24hrs and 48 hrs. Western blot analysis for Jun B and c-Jun, Jun D and β-tubulin is shown. (C). CaSki cells were transfected 20 nM Jun D siRNA and Control-A siRNA and incubated for 24 hrs and 48 hrs. Western blot analysis for Jun D and β-tubulin is shown. Individual siRNAs inhibit the expression of individual Jun family members.

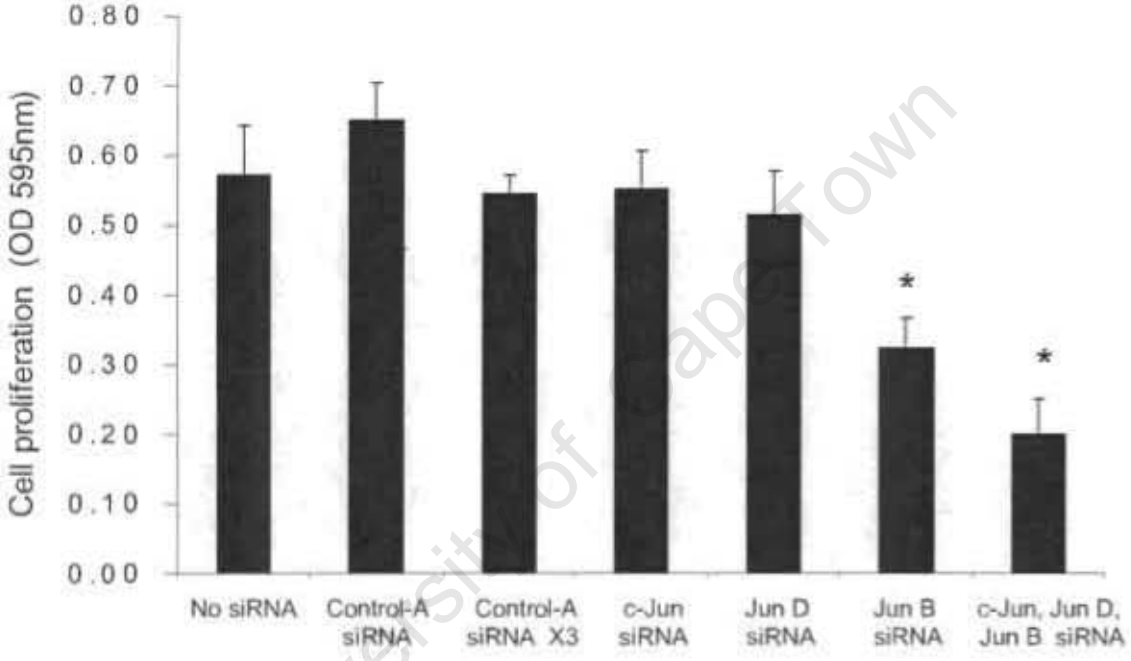


Figure 3.16. Effect of inhibition of individual Jun family members of CaSki cell proliferation. Adherent cell proliferation after four days of cells transfected with 20 nM siRNA specifically targeted to c-Jun , Jun B, Jun D and all three siRNA. CaSki controls; no siRNA and transfection with Control-A and Control-A X3 using MTT assay (see Materials and Methods). The results show the mean ±SE of the experiment performed in quadruplicate. The experiment was repeated at least two times. *$p \leq 0.05$.

(Figure 3.16). This decrease was similar to that observed for TAM67 inhibition of all AP-1 complexes. No significant decrease in cell proliferation was obtained for individual transfections with c-Jun and Jun D siRNA (Figure 3.16). A significant decrease in cell proliferation was however observed with the Jun B siRNA (Figure 3.16). These results suggest that the requirement of individual Jun proteins for the proliferation of CaSki cervical cells differs and more specifically, that Jun B is the likely AP-1 component necessary for the proliferation of these cells.

3.3 DISCUSSION

The study of transcription factors such as AP-1 is important for our understanding of the changes that are associated with cells that become transformed or cancerous, as they play a large role in regulating the biological responses of the cell (Shaulian and Karin, 2001).

Cells express a collection of AP-1 dimer combinations that are capable of binding to AP-1 sites and activate AP-1 dependent gene expression (Dunn *et al.*, 2002). In this study, the detection of AP-1 dependent transcriptional activity was determined using an artificial promoter reporter plasmid containing four AP-1 sites (TGACTCA) fused to a luciferase gene (4X AP-1-Luc) which served as a good measure of AP-1 activity. The CaSki cervical cancer cell line was selected as the cell line with which to study the role of AP-1 in cervical cancer by inhibiting its activity with a dominant negative mutant as well as siRNA technology.

The dominant negative mutant TAM67, has been shown to be a useful tool to study the role of the AP-1 transcription factor in cells owing to its ability to inhibit all AP-1 complex combinations (Brown *et al.*, 1996; Young *et al.*, 2003). In this study TAM67 was shown to inhibit AP-1 transcriptional activity in cervical cancer cells when administered transiently using the pCMV-TAM67 and in doxycycline inducible TAM67 expressing clones. A five fold molecular ratio over endogenous c-Jun expression needs to be achieved in order to obtain a complete block of AP-1 transcriptional activity (Neyns *et al.*, 1999; Rapp *et al.*, 1994). The inhibition by TAM67 detected in both the transient assay and in the doxycycline inducible TAM67 expressing clones suggested that this ratio was achieved in both systems. The pCMV-TAM67 makes use of the strong CMV promoter for expression in mammalian cells and during transient transfection multiple copies of the plasmid in the episomal form may be taken

up allowing for TAM67 expression. This ratio is therefore likely to be reached (Hennigan and Stambrook, 2001; Schmidt *et al.*, 1990). The ability of TAM67 to inhibit AP-1 activity in stable transfections is more difficult to control as TAM67 expression is dependent on the site of integration, the number of copies integrated and the direction of integration, which may be responsible for the variation in the expression of TAM67 obtained in the eight clones screened. However, high TAM67 expression was obtained in a number of clones including CaSki-TAM67 Clone 5 and Clone 7.

The expression of TAM67 in cervical cancer cells was accompanied by a decrease in the expression of endogenous c-Jun in both the transient transfections of TAM67 and within CaSki-TAM67 expressing clonal cells. This is presumably due to TAM67 disrupting the auto-regulatory loop of c-Jun regulating its own expression through AP-1 sites within its promoter (Angel *et al.*, 1988). Similar decreases in endogenous c-Jun were observed by transient transfection of pCMV-TAM67 and in inducible TAM67 expressing 3T3 fibroblasts (Kielosto *et al.*, 2004). The ability of TAM67 to decrease endogenous c-Jun is attributed to it achieving high levels ofexpression, responsible for altering the composition of the AP-1 complexes (Kielosto *et al.*, 2004). Thus a high enough expression level of TAM67 appears to have been obtained in CaSki cells to cause the observed decrease in endogenous c-Jun. Two mechanisms, a "blocking mechanism" and a "sequestering mechanism", have been described to explain how the dominant negative activity of TAM67 is brought about (Brown *et al.*, 1994). Should TAM67 act via the formation of TAM67 homodimers to block endogenous AP-1 DNA binding, higher concentrations of TAM67 may be required as the Jun-Jun homodimers and thus TAM67 homodimers, have a 3-fold lower affinity for DNA than the Fos-Jun heterodimers (Hennigan and Stambrook, 2001; Ryseck and Bravo, 1991; van Dam and Castellazzi, 2001).

A decrease in phosphorylated c-Jun observed in CaSki-TAM67 expressing cells is likely as a result of decreased levels of total c-Jun. This decreased phosphorylation of c-Jun may further indicate that TAM67 is an effective inhibitor of AP-1 activity as phosphorylation of c-Jun at Serine 73 and Serine 63 is required for its full activity (Behrens *et al.*, 2000). Inhibition of phosphorylated c-Jun by TAM67 in human carcinoma cell lines has previously been shown (Fan *et al.*, 2001). Phosphorylation of AP-1 requires the presence of functional homodimers and heterodimers and therefore the ability of TAM67 to inhibit phosphorylation of c-Jun can be explained by the fact that TAM67 forms defective heterodimers with the endogenous AP-1 proteins and therefore inefficient phosphorylation takes place (Brown *et al.*, 1994; Fan *et al.*, 2001; Kallunki *et al.*, 1996). The effect of TAM67 on phosphorylation at Ser 63 could not be investigated due to difficulties encountered in western blot analysis using the antibody directed against phosphorylation at Ser 63 but a similar decrease as obtained for Ser 73 is anticipated. The decrease observed in CaSki-TAM67 cells, however, does not indicate a change in the level of phosphorylation but rather a reflection of decreased amounts of total c-Jun protein caused by TAM67.

TAM67 has been previously been shown to inhibit Jun D equally well. The expression of TAM67 inhibits Jun D mediated NF-κB transactivation in hepatocytes (Bhoumik *et al.*, 2004; Rahmani *et al.*, 2001). Jun D positively auto regulates its promoter (Berger and Shaul, 1998) explaining the decrease in Jun D expression in the CaSKi-TAM67 clones. While no decrease in Jun B expression was observed, TAM67 has the ability to affect Jun B transcriptional activity as it does for c-Jun and Jun D (Brown *et al.*, 1994; Brown *et al.*, 1996).

Different effects of the individual Jun family proteins c-Jun , Jun B and Jun D on cell growth and transformation have been established suggesting that they play distinct roles in cellular

growth and cellular transformation (Castellazzi *et al.*, 1991; Leaner *et al.*, 2003). TAM67 has the ability to alter the activity of most endogenous AP-1 protein complexes and therefore its effects can not be attributed to individual components in the AP-1 complex (Brown *et al.*, 1994). The use of siRNA directed specifically to the individual Jun Family proteins, allows for the determination of the potential roles of individual Jun proteins on the biological characteristics of cells. Gene silencing is a naturally occurring process in mammalian cells. When long dsRNA molecules enter the cell, they are cleaved by a Dicer complex into 19-21bp small interfering RNA duplexes with two nucleotide 3' overhangs on each strand (Elbashir *et al.*, 2001; Zamore *et al.*, 2000). These siRNAs are then incorporated into a RNA Induced Silencing Complexes, an endo-ribonuclease-containing complex known as RISC, where the ATP-dependent unwinding of the siRNA duplex by RNA helicase takes place and causes RISC to become activated. The activated RISC is guided by the now single stranded siRNA to its complementary target mRNA and binds to it. The bound mRNA is subsequently cleaved and rapidly degraded, resulting in gene silencing. Thus this phenomenon can be utilised to induce specific gene silencing by directly delivering small siRNA targeted to a specific gene into the cells (Aigner, 2006). Both approaches of using a dominant negative TAM67 and siRNA were effective in elucidating the role of the transcription factor AP-1 in cervical cancer cells.

CHAPTER 4

MECHANISMS ASSOCIATED WITH INHIBITION OF AP-1 IN CERVICAL CANCER CELLS

4.1. INTRODUCTION

Having established that inhibition of AP-1 in cervical cancer cells resulted in changes in cell biology, the mechanisms associated with these changes was further investigated. One of the most obvious biological changes observed when inhibiting AP-1 activity was a reduction in cell proliferation. The proliferation of cells is tightly controlled as they move through the cell cycle. The cell cycle involves progression through several phases which are tightly regulated by a large number of cell cycle regulatory molecules (Niculescu *et al.*, 1998). Alterations in the expression or function of the critical genes that tightly regulate cell cycle checkpoints can subsequently lead to altered proliferation and cellular transformation, an early step in carcinogenesis (Dash and El-Deiry, 2004; Kaufmann and Kaufman, 1993).

Cells enter the cell cycle in the Gap 1 (G1) phase and prepare to duplicate their DNA in the S phase (Figure 4.1). If the cells are functioning normally, detected DNA damage is corrected and preparation for the cell division occurs in the Gap 2 (G2) phase. During M phase, mitosis occurs as the chromatids and daughter cells separate. The cells then either re-enter into G1 phase or exit the cell cycle into quiescence in Go phase (Stillman, 1996). The DNA content changes from haploid (2n) to diploid (4n) as progression through the cell cycle occurs and this change can be measured to obtain a DNA profile which represents the distribution of cells within the different phases (Watson *et al.*, 1987). Entry into each phase is regulated at cell cycle checkpoints that occur late in G1 (G1/S checkpoint) and late G2 (G2/M checkpoint) to

ensure that accurate DNA replication and division occurs within S phase and M phase respectively (Dash and El-Deiry, 2004; Stillman, 1996). These checkpoints allow time for DNA repair to occur to ensure precise cell cycle execution and involve a series of sequential events that includes activation and subsequent inactivation of cyclin dependent kinases (Cdks) and cyclins (Harper and Adams, 2001). If the damage detected is not corrected, the cell halts its progression and cell death may occur via initiation of apoptosis.

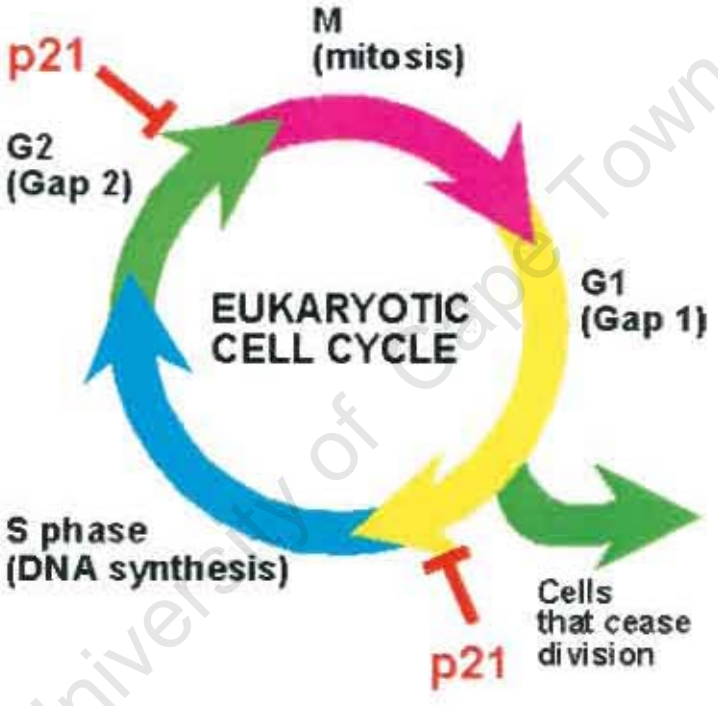


Figure 4.1. The eukaryotic cell cycle. The four distinct phases of the cell cycle are represented by the G1 Gap 1 phase, the S phase, the G2 Gap 2 followed by Mitosis. Important cell regulatory molecules such as $p21^{CIP1}$ regulate the cell cycle at the G1/S and G2/M checkpoints. Diagram adapted from Collins *et al.*, (1997).

There are a number of important regulatory proteins that control the progression through the cell cycle. Of these the tumour suppressors p53 and $p21^{CIP1}$ are well described (Levine , 1997; Niculescu *et al.*, 1998). $p21^{CIP1}$ is a key cell cycle regulator responsible for the inhibition of a number of cyclins and cyclin dependent kinases complexes (Cdks) and is found to play a role

in both the G1/S phase and G2/M transitions (Figure 4.1) (Chang *et al.*, 2004; Duli *et al.*, 1998; Niculescu *et al.*, 1998). AP-1 plays a role in cell cycle progression in numerous cell types where increased AP-1 activity has been associated with increased progression through the cell cycle (Schreiber *et al.*, 1999; Shaulian and Karin, 2001). AP-1 has been shown to regulate the expression of $p21^{CIP1}$ both positively and negatively depending on the cellular context (Chang *et al.*, 2004; Kardassis *et al.*, 1999; Wang *et al.*, 2000). An important transcriptional upstream regulator of $p21^{CIP1}$ is the tumour suppressor protein p53. Different studies however have shown that the transcriptional regulation of $p21^{CIP1}$ expression by AP-1 occurs by both p53-dependent mechanisms and p53-independent mechanisms (Datto *et al.*, 1995; Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). Based on these previously published reports and the findings that inhibition of AP-1 with TAM67 inhibits cell proliferation in this study, it was hypothesised that the cell cycle regulatory proteins such as $p21^{CIP1}$ and p53 may have altered expression in cervical cancer cells when AP-1 is inhibited.

To address the mechanisms through which AP-1 inhibition by TAM67 resulted in changes in the growth of cervical cancer cells, the following investigations were carried out. The cell cycle profiles of CaSki control cells and TAM67 expressing CaSki clones were determined and the effects of AP-1 inhibition by TAM67 on the cell cycle regulatory proteins $p21^{CIP1}$ and p53 were examined.

4. 2. RESULTS

4.2.1. AP-1 inhibition results in an altered cell cycle profile

The altered morphology and growth inhibition observed in the CaSki-TAM67 clones suggested that inhibition of AP-1 was affecting the cell cycle distribution of these cells. The distribution of cells within the different phases of the cell cycle was determined by fluorescently activated cell sorting (FACS) using Propidium Iodide staining to measure the DNA content. The proportion of cells in the G1, S phase and G2/M phases of the cell cycle was analysed. The cell cycle distribution of CaSki parental cells was compared with the profiles obtained for CaSki-TAM67 expressing Clone 5 and Clone 7. Cells were grown in 10% FBS DMEM for 24 hours and then synchronized by serum depletion in 0.5% FBS DMEM for 24 hours and then incubated for an additional 24 hours. All incubations were performed in the presence of 1 µg/ml doxycycline to ensure TAM67 expression in the clones.

The percentage of cells in G1, S and G2/M phases of the cell cycle was quantified using Flowjo analysis software which fits the data to the Watson Pragmatic mathematical model (Watson *et al*., 1987). The results show that the largest proportion of the cells were in the G1 phase and this proportion showed a marginal decrease in CaSki-TAM67 Clone 5 and in CaSki-TAM67 Clone 7 cells (Figure 4.2.B and C) compared to the CaSki parental cells (Figure 4.2.A). Similarly, a marginal decrease in cells in the S phase was also observed, in the TAM67 expressing CaSki clones compared to the control cells. However, a significant increase in the G2/M population was observed in both CaSki-TAM67 Clone 5 and CaSki-TAM67 Clone 7 cells. This two-fold increase represented an approximate doubling of cells

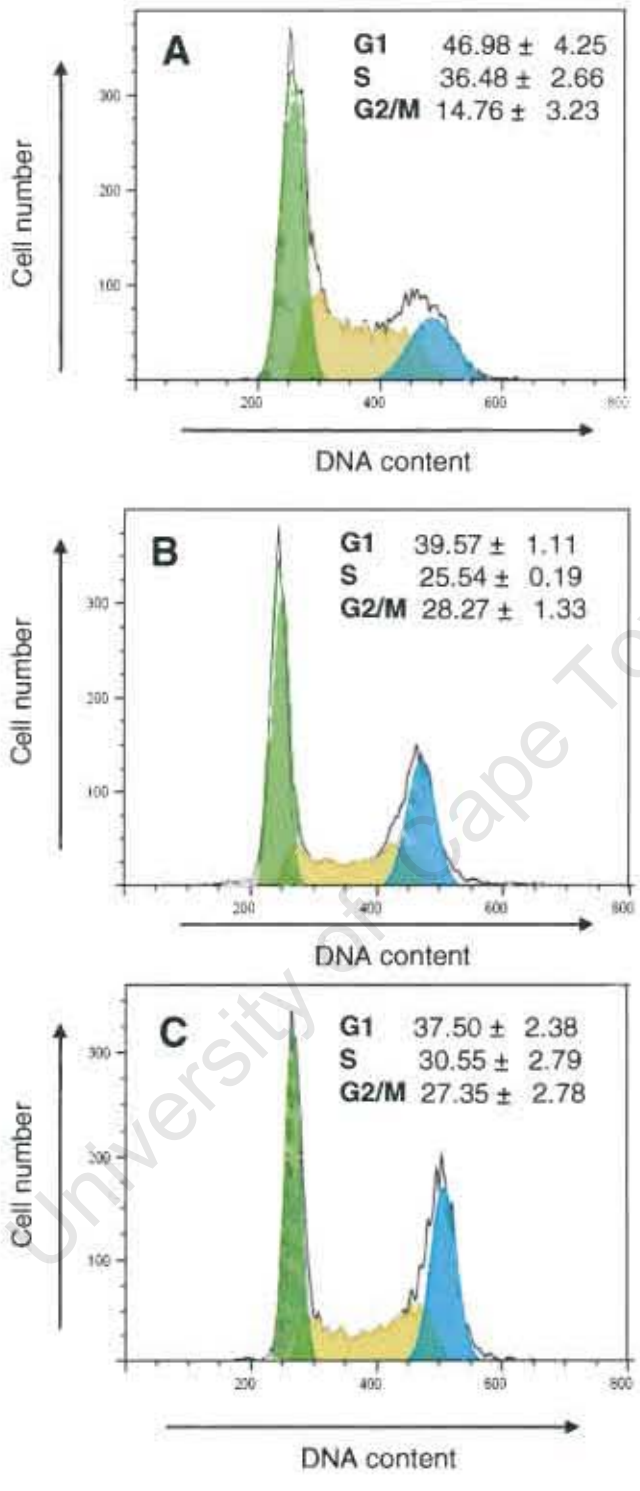


Figure 4.2. Inhibition of AP-1 results in altered cell cycle profile. Representative DNA histogram profiles obtained by Propidium Iodide fluorescence assessed by FACS flow cytometry of (A) CaSki and (B) CaSki-TAM67 clones 5 and (C) CaSki-TAM67 clone 7 grown in the presence of doxycycline. Quantification of the percentages of cells in G1 phase, S phase and G2/M phase of the cell cycle was performed by Flowjo analysis software. Values indicate the mean ±SE of the experiment performed in triplicate. *pvalue ≤ 0.05. Experiments were performed in triplicate at least two times.

in this phase of the cell cycle from 14.76 ± 3.23% in CaSki cells to 28.27 ± 1.33% and 27.35 ± 2.78% in CaSki-TAM67 Clones 5 and 7 respectively.

No significant sub G1 peak, indicative of cell death, was observed and aggregates and debris were not used in the analysis. The cell cycle response seen in the CaSki-TAM67 stable clones having doxycycline-inducible TAM67 expression suggested that fewer cells were progressing through the cell cycle possibly due to a cell cycle block initiated by TAM67 at the G2/M checkpoint.

4.2.2. The effect of TAM67 on $p21^{CIP1}$ expression

4.2.2.1. Altered $p21^{CIP1}$ expression in TAM67 expressing CaSki cells detected by Western blot analysis

To determine a possible mechanism for the changes in cell biology and cell cycle progression by inhibition of AP-1 by TAM67, the effects of TAM67 on the cell cycle inhibitory proteins $p21^{CIP1}$ and p53 were investigated.

Western blot analysis for $p21^{CIP1}$ expression in cells incubated in the absence or presence doxycycline for 48 hours showed an increase in $p21^{CIP1}$ levels in TAM67 expressing Clones 5 and Clone 7 (Figure 4.3.A). Quantification of $p21^{CIP1}$ expression relative to β-tubulin levels is shown in Figure 4.3.B. Similarly, higher $p21^{CIP1}$ levels were shown in TAM67 expressing pools of cells and an additional CaSki-TAM67 Clone, Clone 4 (Figure 4.3.C). These results show the presence of TAM67 expression and the subsequent decrease in endogenous Jun levels associates with an increase in $p21^{CIP1}$ protein.

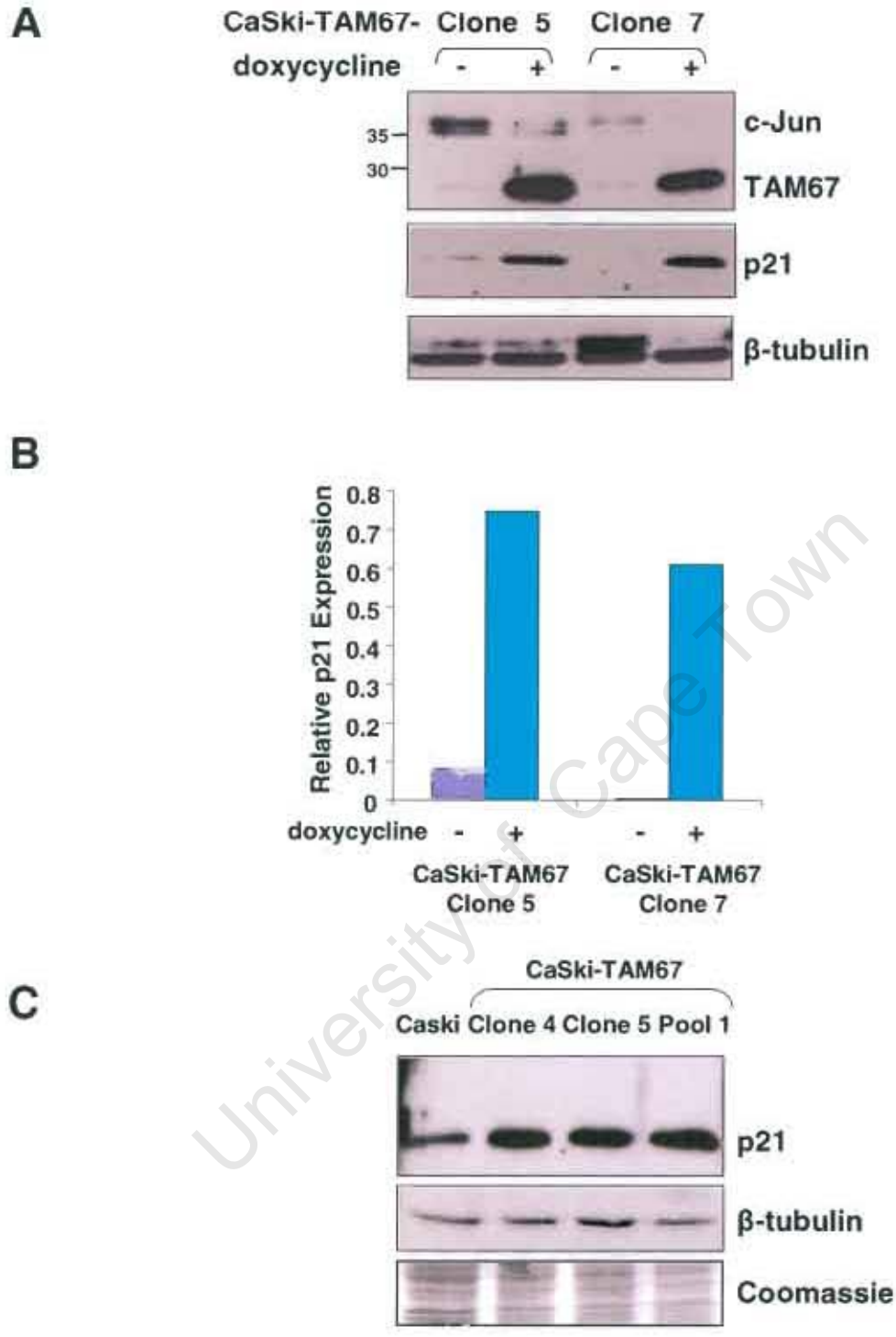


Figure 4.3. $p21^{CIP1}$ expression levels in CaSki TAM67 expressing cells. Cells were grown in the absence or presence of 1 µg/ml doxycycline. Fifty µg of proteins were subjected to Western blot analysis with an anti-$p21^{CIP1}$ specific antibody. The blots were stripped and reprobed with β-tubulin as a loading control. (A). $p21^{CIP1}$ expression in CaSki TAM67 Clone 5 and Clone 7. (B). $p21^{CIP1}$ expression levels quantified by densitometric scanning and as expressed as arbitrary densitometric units relative to β-tubulin levels. (C). $p21^{CIP1}$ expression in CaSki, and CaSki-TAM67 expressing pools and Clone 4 and Clone 5 grown in the presence of doxycycline. Coomassie staining of the gel after transfer is also shown.

4.2.2.2. Altered $p21^{CIP1}$ expression levels in TAM67 expressing CaSki cells detected by Immunofluorescence

Additional experiments were performed to confirm that inhibition of AP-1 activity resulted in an increase in $p21^{CIP1}$ expression. CaSki and TAM67 expressing cells were incubated in the presence of doxycycline for 48 hours and immunofluorescence for $p21^{CIP1}$ was performed. Standard fluorescent microscope images of $p21^{CIP1}$ levels revealed higher $p21^{CIP1}$ in a TAM67 expressing clone compared to the CaSki control cells (Figure 4.4). The experiment was repeated and viewed using confocal microscopy in order to obtain images of better resolution. These experiments confirmed higher expression of $p21^{CIP1}$ in TAM67 expressing CaSki cells. In addition, the confocal images showed the altered morphology in TAM67 expressing cells (flattened and larger cells as previously determined) (Figure. 4.5).

Taken together Western blot and immunofluorescence analysis of $p21^{CIP1}$ suggest that the inhibition of AP-1 activity in cervical cancer cells alters the expression of the cell regulatory $p21^{CIP1}$ protein. Therefore it can be deduced, that high expression of AP-1 in cervical cancer may likely result in the suppression of cell cycle inhibitory proteins such as $p21^{CIP1}$ causing the unchecked growth associated with cancer. When blocking AP-1 the inhibitory effect on $p21^{CIP1}$ is released and this causes a decrease in cell growth through inhibition of cell cycle progression.

4.2.3 The effect of TAM67 on p53 expression

$p21^{CIP1}$ expression has been reported to be regulated by both p53-dependent and p53-independent mechanisms. Therefore to determine whether p53 had any role in the increase of $p21^{CIP1}$ observed in the CaSki-TAM67 cells, its expression in CaSki parental and TAM67 expressing cells was analysed. The CaSki cervical cancer cell line contains wild type p53, however in HPV-positive cervical cancer cells, p53 is rapidly degraded and hence low levels

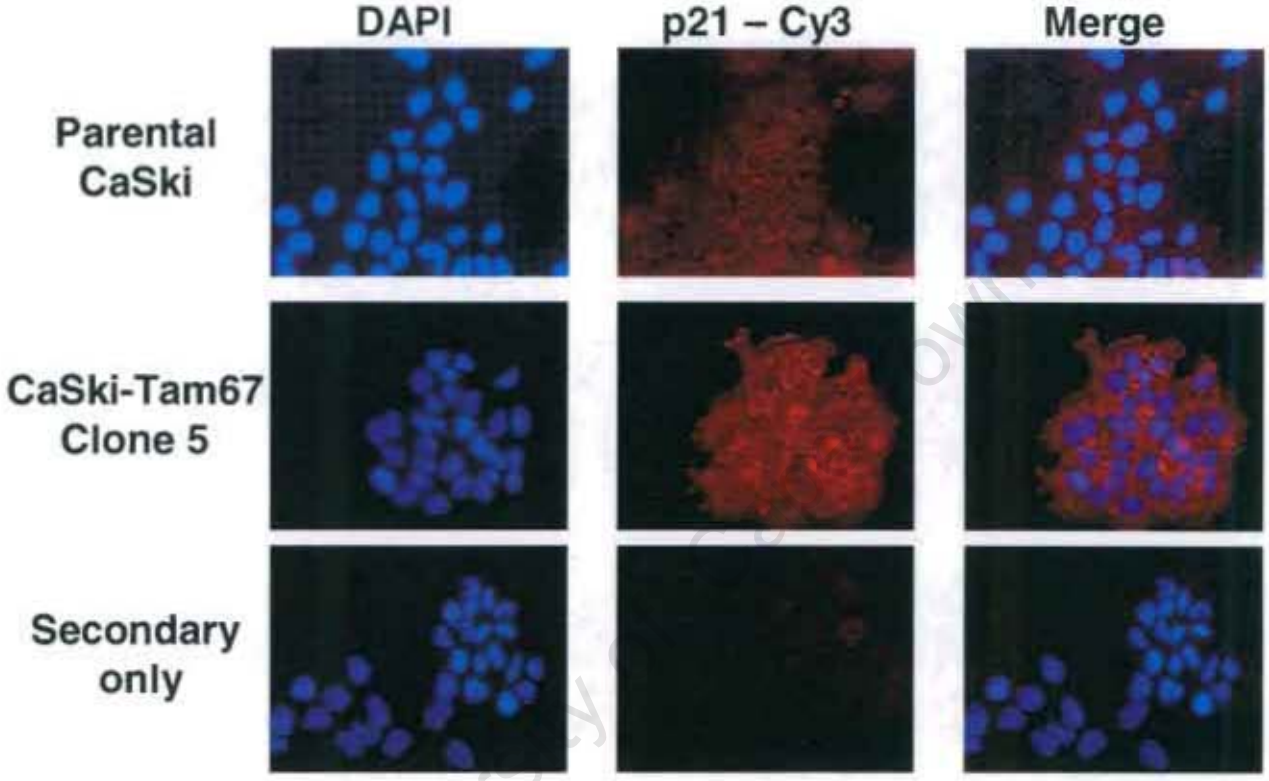


Figure 4.4. $p21^{CIP1}$ immunofluorescence in CaSki and CaSki-TAM67 expressing cells using Fluorescent microscopy. Immunofluorescence showing $p21^{CIP1}$ expression in control CaSki cells and TAM67 expressing CaSki-TAM67 clone 5 cells grown in the presence of doxycycline for 48 hours. Fixed cells on coverslips were incubated with primary rabbit anti-$p21^{CIP1}$ specific antibody followed by incubation with a secondary Cy3 conjugated anti-rabbit antibody. Cells were incubated with DAPI to visualize the nuclei and fluorescent images were captured using the Axiovision Fluorescence microsope (400X magnification). To control for non specific antibody interaction, a negative control with secondary antibody only was included.

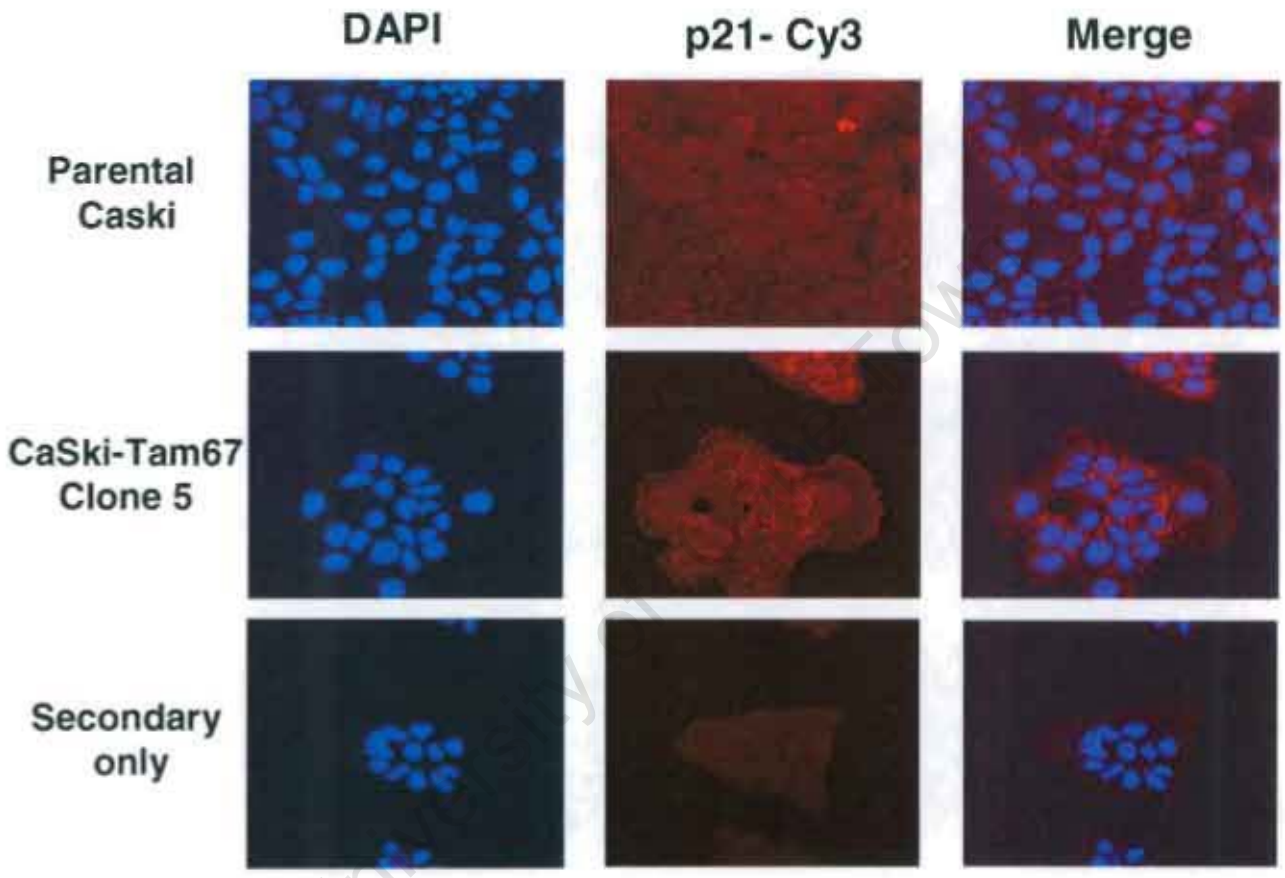


Figure 4.5. $p21^{CIP1}$ immunofluorescence in CaSki an CaSki-TAM67 expressing cells viewed under the Confocal Fluorescent microscope. Confocal Immunofluorescence images showing $p21^{CIP1}$ expression in CaSki-TAM67 clone 5 cells grown in doxycycline for 48 hours. Fixed cells were incubated with antibody against $p21^{CIP1}$ as described in Fig. 4.4. DAPI was used to visualize the nuclei and images were captured using the LSM Confocal Fluorescence microsope under 400X magnification. A negative control with secondary antibody only was included to control for non specific antibody interaction.

are detected in these cells (Scheffner *et al.*, 1991; Tommasino *et al.*, 2003). Thus in order to determine its expression levels by western blot analysis, a larger amount of protein (100 µg) was analysed compared to the 30 µg used in other western blot analyses.

Western blot and immunofluorescence analysis for p53 revealed no change in p53 expression levels when comparing CaSki cells with the two TAM67 expressing clones (Fig. 4.6.A and 4.6.B). These results suggest that the increase in $p21^{CIP1}$ protein in TAM67 expressing cells levels is likely to occur in a p53-independent manner.

4.2.4. The effect of TAM67 on p21 promoter activity

These results suggested that p21 regulation by inhibition of AP-1 with TAM67 occurs in a p53-independent manner proposing a possible direct regulation of the p21 promoter by AP-1 in cervical cancer cells which was next investigated. A -2500/+50 p21 promoter construct spanning the -2500 to +50 region fused to a luciferase gene (-2500/+50 p21-Luc) was used in both transiently transfected TAM67 CaSki cells and within doxycycline-inducible TAM67 clones. Co-transfection of the pCMV-TAM67 expression vector with the -2500/+50 p21-Luc in CaSki cells caused a significant decrease in the p21 promoter activity compared to the controls (Figure 4.7A). The empty pCMV expression vector was co-transfected with the 2500/+50 p21-Luc promoter construct as a control to discount any inhibitory effects due to the vector alone or higher DNA amounts, and no significant decrease in p21 promoter activity was seen by the empty pCMV, thus attributing the activity measured to p21 promoter activity. The promoterless pGL3 was used as an additional control.

Similar results were obtained when the -2500/+50 p21-Luc promoter construct was transfected in the CaSki-TAM67 expressing clones, Clone 5 and Clone 7 (Figure 4.7.B).

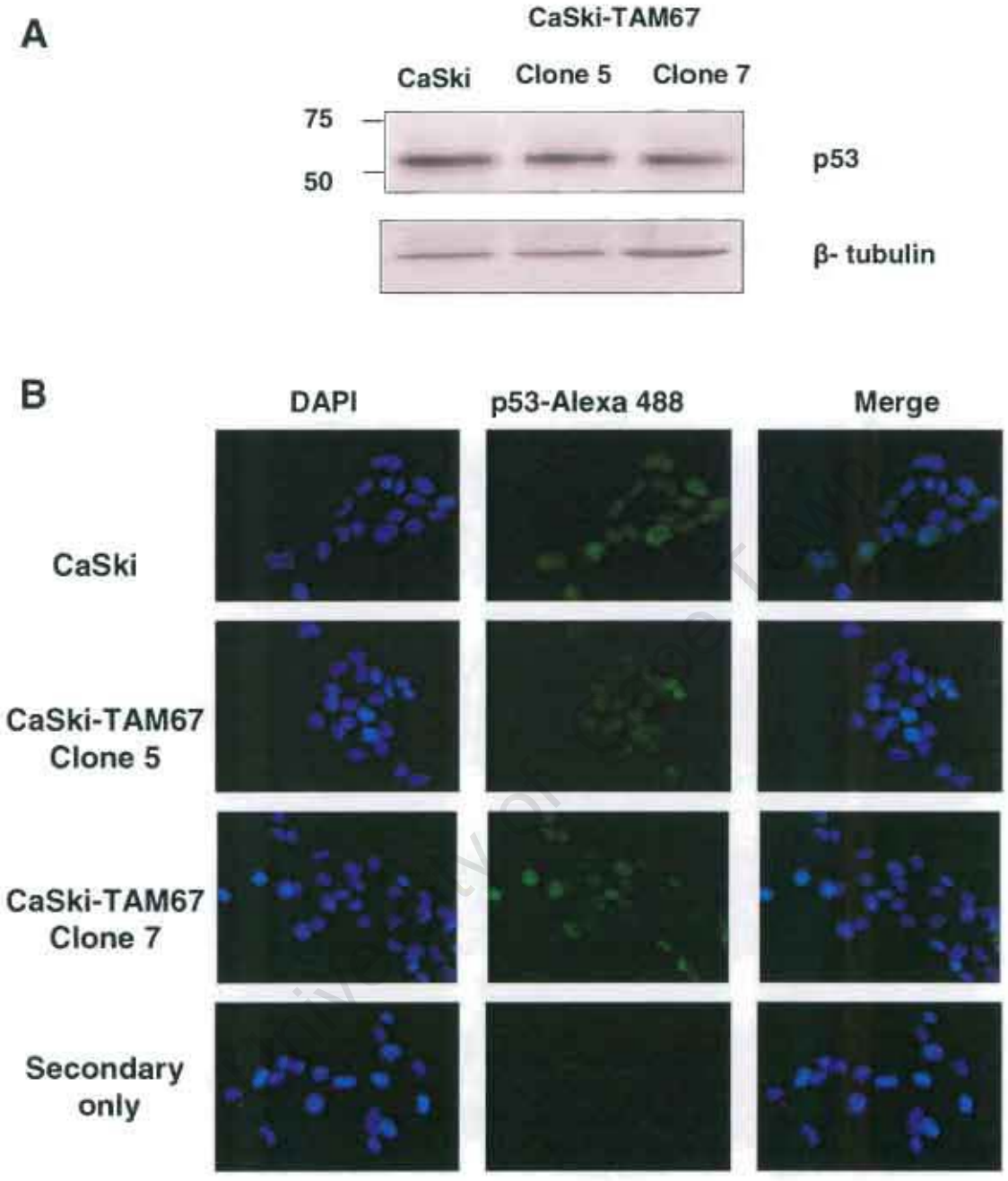


Figure 4.6. p53 levels in CaSki-TAM67 expressing cells. (A). p53 expression in CaSki and TAM67 expressing stable clones. Cells were grown in the presence of doxycline for 48 hours and 100 μg of proteins were resolved on 12% SDS-PAGE and subjected to western blot analysis with mouse anti-p53 specific antibody. Blots were stripped and reprobed with β-tubulin as a loading control. (B). Immunofluorescence showing p53 expression in CaSki and TAM67 expressing stable clones. Cells were grown in the presence doxycycline for 48 hours and fixed cells on coverslips were incubated with primary mouse anti-p53 specific antibody followed by incubation with an Alexa-488 conjugated anti-mouse secondary antibody. DAPI shows nuclear staining and images were captured on Axiovision Flourescence microsope (400X magnification).

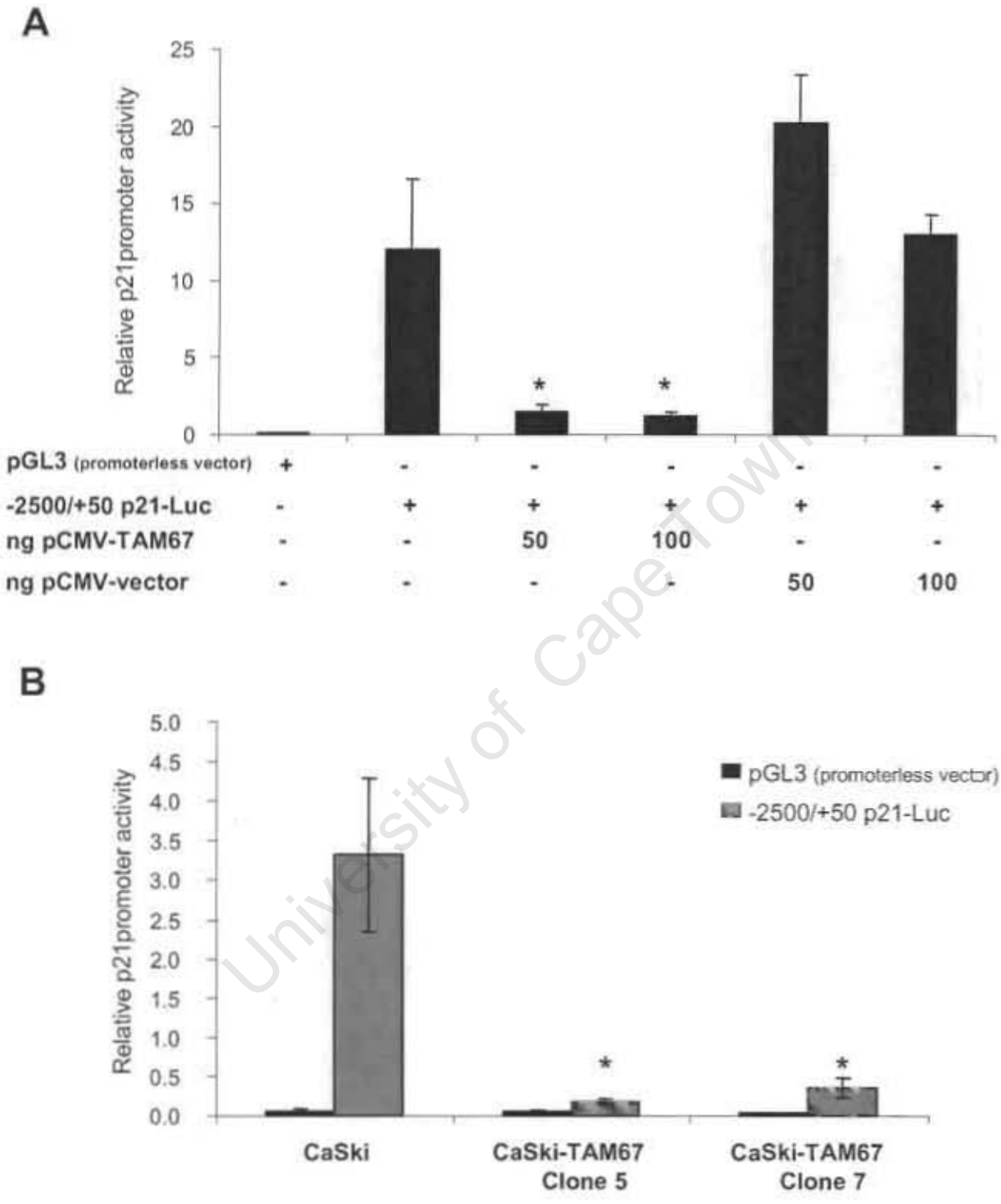


Figure 4.7. TAM67 affects p21 promoter activity in CaSki cells. (A). Transient co-transfection of -2500/+50 p21-Luc and pCMV-TAM67 in CaSki cells. (B). CaSki and CaSki-TAM67 expressing clones were incubated in the presence of doxycycline for 24 hours after transfection with -2500/+50 p21-Luc or promoterless pGL3 vector. pRL-TK-Renilla-Luc was used as a control for transfection effiency The results show the mean ±SE of the experiment in triplicate and the experiment was repeated at least three times. *p value ≤ 0.05.

showing that inhibition of AP-1 activity resulted in a decrease in p21 promoter activity. Based on our earlier findings showing an increase in endogenously expressed $p21^{CIP1}$ protein in response to inhibition of AP-1 by TAM67, these promoter results were contrary to what we had anticipated. As the promoter construct used contains only a portion of the p21 promoter, it was possible that sequences not included may be required for additional regulation by AP-1.

On further inspection of a larger region of the p21 promoter (-5000 to +50) using MatInspector, a software program that searches for transcription factor binding sites (Cartharius *et al.*, 2005), a number of potential AP-1 binding sites were identified that are not included in the -2500 to +50 region. A potential AP-1 binding site with 100% homology to the consensus AP-1 binding site (TGACTCA) at -4165 and additional AP-1-like sites were identified within the upstream region of the p21 promoter (Figure 4.8). The presence of these potential AP-1 binding sites is however not sufficient as an indicator of transcriptional function and additional experiments will have to be performed in the future to determine their role on promoter activity. Cloning of the larger p21 promoter construct (-5000 to +50) is currently ongoing; however the plasmid construction was not completed in time to include in this thesis. Therefore it anticipated that inhibition of AP-1 activity with TAM67 may result in the regulation of the p21 promoter possibly through the AP-1 binding site at position -4165

Studying the effects of TAM67 inhibition on AP-1 allowed the molecular mechanisms that are associated with the role of AP-1 in cervical cancer cells to be elucidated. The results from this study show that AP-1 is acting via a p53-independent repression on $p21^{CIP1}$ which suggests a more direct regulation of AP-1 on the p21 promoter possibly by the potential AP-1

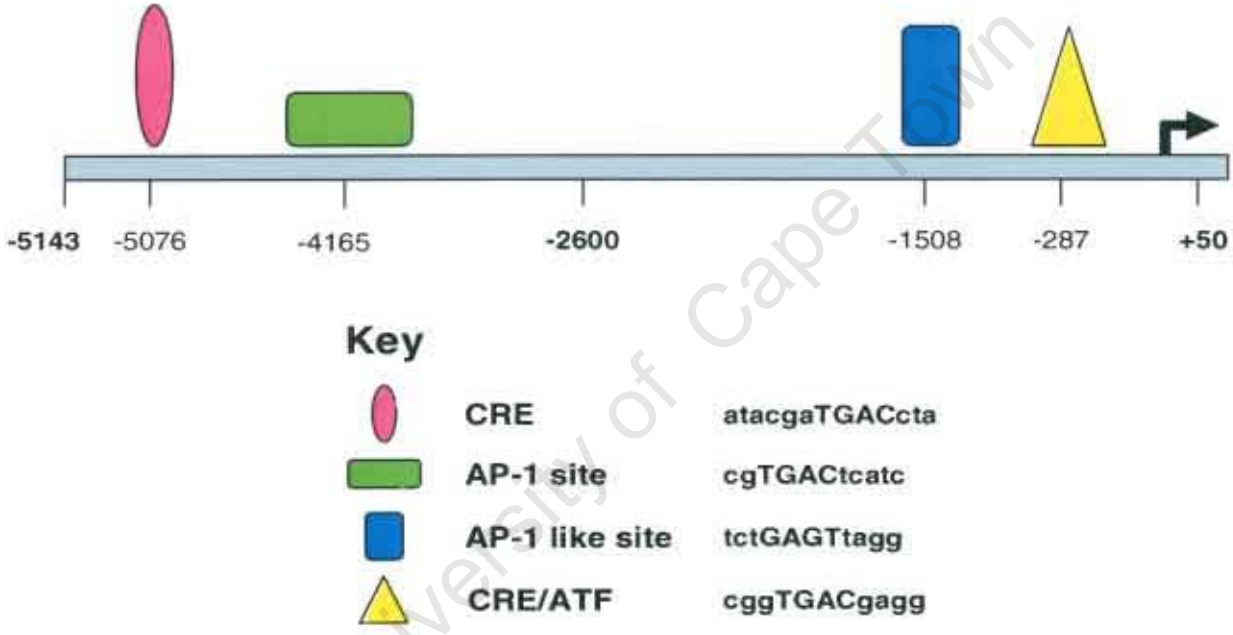


Figure 4.8. Diagram depicting potential AP-1 sites in the p21 promoter. A search by MatInspector software (Cartharius *et al.*, 2005), identified a number of potential AP-1 sites in the p21 promoter.

sites within the -5000/+50 promoter region. Future experiments may be able to elucidate whether this is in fact the mechanism through which AP-1 has a growth regulatory role in cervical cancer cells.

4.3 DISCUSSION

Unraveling the molecular mechanisms responsible for the changes caused by AP-1 inhibition in cervical cancer cells is important for understanding the biological processes governed by AP-1 in cervical cancer.

AP-1 has been implicated in cell cycle progression in many different cell types and has been shown to be involved in regulation at both the G1/S transition and G2/M transitions of the cell cycle (Chung *et al.*, 2002; Fan *et al.*, 2001; Schreiber *et al.*, 1999). Antibodies directed against all three Jun Family proteins have been shown to inhibit cell cycle progression in mouse fibroblasts (Kovary and Bravo, 1991). A closer look at the effect of TAM67 on the cell cycle of CaSki cervical cancer cells showed a two-fold increase in G2/M phase in CaSki-TAM67, suggesting that inhibition of AP-1 in cervical cancer cells slows them down at the G2/M checkpoint. This is an important checkpoint in the cell cycle and by implication AP-1 exerts a proliferative effect at this stage of the cell cycle in CaSki cervical cancer cells.

The key regulatory proteins in cell cycle progression are the cyclins/cyclin dependent kinases (Cdks) that are controlled by a number of Cdk inhibitors (Dash and El-Deiry, 2004; Stillman, 1996). A better understanding of AP-1 regulation in CaSki cell cycle was gained with a more detailed analysis of the underlying mechanisms. The universal Cdk inhibitor $p21^{CIP1}$ is required at both G1/S and G2/M checkpoints (Duli *et al.*, 1998). At the G1/S transition, it inhibits the Cdks responsible for the destabilisation of Rb/E2F complexes inhibiting cell proliferation and at the G2/M checkpoint $p21^{CIP1}$ associates with and inhibits the mitotic cyclin dependent kinases to promote cell regulation and cause G2 cell cycle arrest (Duli *et al.*, 1998; Niculescu *et al.*, 1998).

$p21^{CIP1}$ levels were found to be increased in TAM67 expressing cells, suggesting that TAM67 counteracts an AP-1 mediated repression of $p21^{CIP1}$ in cervical cancer cells. Although $p21^{CIP1}$ is considered an important downstream target of the cell cycle regulatory protein p53, the transcriptional regulation of $p21^{CIP1}$ expression by AP-1 can occur by both p53 dependent and p53 independent mechanisms (Chung *et al.*, 2002; Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). Another key factor that is involved in the proliferation in cervical cancer cell lines is the expression of the HPV E6 and E7 viral oncoproteins. The CaSki cells have the HPV16 genome in integrated in the host genome (Hietanen *et al.*, 2000). AP-1 has also been shown to be a key regulatory molecule in HPV gene expression (Soto *et al.*, 2000). Therefore, within the context of cervical cancer cells possible AP-1 repression of $p21^{CIP1}$ expression is summarized in Fig. 4.12. Briefly, (1) AP-1 positively regulates the expression of HPV E6 that inhibits p53 expression and hence $p21^{CIP1}$ expression, (2) A direct negative regulation of AP-1 on p53 expression and hence $p21^{CIP1}$ expression or (3) AP-1 directly inhibiting the p21 promoter.

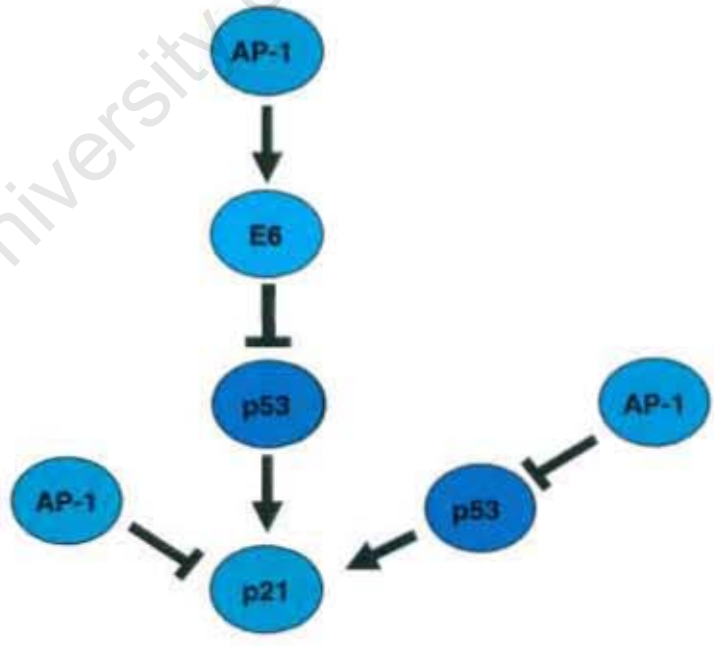


Figure 4.12. Possible mechanisms through which TAM67 may affect AP-1 activity and regulation of $p21^{CIP1}$ expression in cervical cancer cells. These mechanisms include two p53-dependent mechanisms and a third p53-independent mechanism of direct regulation of AP-1 on $p21^{CIP1}$ expression.

AP-1 has been reported to regulate both the activation and repression of p21 ^{CIP1} expression depending on the cellular context (Kardassis *et al.*, 1999; Wang *et al.*, 2000). A p53 dependent repressive mechanism on p21 ^{CIP1} expression in mouse fibroblasts showed that c-Jun was responsible for the negative regulation of p53 transcription via an AP-1 site in the p53 promoter as elevated levels of p53 and p21^{CIP1} were detected in the absence of c-Jun. Conversely, overexpression of c-Jun was shown to repress both p53 and p21^{CIP1}expression (Schreiber *et al.*, 1999). Also AP-1 has been shown to negatively regulate the transcriptional activity of p53 by blocking the association of p53 with the p21 promoter (Shaulian *et al.*, 2000). Another p53 dependent mechanism of AP-1 on p21^{CIP1} expression showed a repression of AP-1 on p21^{CIP1} at the G2/M phase. In vinblastine treated human, carcinoma cell lines the activation of AP-1 has been reported to result in a decrease in both p53 and p21^{CIP1} which was successfully reversed by TAM67 expression (Fan *et al.*, 2001). Unchanged p53 expression in response to AP-1 inhibition by TAM67 observed in this study suggests that the upregulation of p21^{CIP1} in the CaSki-TAM67 expressing cells is p53-independent. Although HPV positive cervical cancer cell lines like CaSki do not contain a dysfunctional mutant p53, it may be possible that the normal functioning of wild type p53 within this cell line is disrupted by its interaction with the viral E6 oncoprotein (Scheffner *et al.*, 1991).

p53-independent mechanisms describing the regulation of p21^{CIP1} by AP-1 interacting with the p21 promoter have also been determined in the literature (Chung *et al.*, 2002; Crowe *et al.*, 2000). These studies report that AP-1 causes activation of p21 ^{CIP1} in a p53-independent manner in human lung cancer cells lacking p53 (Chung *et al.*, 2002). The activation of AP-1 caused an increase in the expression of p21^{CIP1} and resulted in a G2/M growth arrest. This activation of p21^{CIP1} by AP-1 is reported to occur via the binding of AP-1 to a novel AP-1 like site (TGAGGAA) between nucleotides -2203/-2197 of the p21 promoter (Chung *et al.*, 2002).

Another p53-independent mechanism includes a less direct mechanism involving the interaction of c-Jun with the Sp-1 transcription factor at a Sp-1 site in the p21 promoter (Kardassis *et al.*, 1999; Wang *et al.*, 2000). The interaction with the Sp-1 transcription factor has been described to either induce or repress the expression of $p21^{CIP1}$ in different cell types (Wang 2000, Kardassis, 1999). AP-1 has been reported to repress $p21^{CIP1}$ expression directly via an AP-1 like site at position -1510 in the promoter (Crowe *et al.*, 2000). The AP-1 mediated repression of $p21^{CIP1}$ expression determined in CaSki cells in this study may be a result of a similar direct mechanism and thus TAM67 would be responsible for countering the repression directly on the p21 promoter.

These results show that inhibition of AP-1 by TAM67 caused an increase in endogenous $p21^{CIP1}$ suggesting that TAM67 may cause a similar increase in p21 promoter activity, however, -2500/+50 p21 promoter assays contradicted the findings. As the -2500/+50 p21 promoter construct represents only a portion of the p21 promoter, it is possible that this construct may not reflect the regulation of the endogenous promoter. It may be that the sequences essential for AP-1 mediated repression on the p21 promoter in cervical cancer cells occurs within the larger p21 promoter and possibly other repressor regions. This theory is supported by the identification of potential AP-1 binding sites in the p21 promoter revealing a consensus AP-1 binding site of TGACTCA at position -4165 within the -5000/+50 p21 promoter. Further investigations to fully elucidate the regulation of AP-1 of $p21^{CIP1}$ in cervical cancer cells are required.

Future experiments include cloning a larger p21 promoter region spanning -5000 to +50 and determining the effect of inhibiting AP-1 activity on p21 promoter regulation. Should this promoter construct show regulation by AP-1, the AP-1 consensus site TGACTCA, at position -4165 will be further investigated by mutational analysis. Chromatin Immuno Precipitation or

ChIP assays and Electrophoresis Mobility Shift Assay (EMSA) to determine whether AP-1 is binding directly to the p21 promoter at this site will also be explored.

The molecular mechanisms underlying AP-1 inhibition by TAM67 are complex and therefore require further investigation. In this study, the use of TAM67 to inhibit AP-1, allowed the molecular mechanisms to be investigated and revealed that AP-1 is involved in the repression of the cell cycle inhibitor $p21^{CIP1}$, suggesting an AP-1 regulatory mechanism associated with the biological processes mediated by AP-1 in cervical cancer cells.

CHAPTER 5

CONCLUSIONS

5.1 AP-1 and cancer

AP-1 overexpression has been associated with numerous human cancers including breast, endometrial, colon and skin cancer, where it is involved in numerous cellular processes (Bamberger *et al.*, 2001; Vleugel *et al.*, 2006; Wang *et al.*, 2002; Young *et al.*, 1999). In this study, levels of AP-1 component proteins, c-Jun, Jun B and Jun D were analysed in normal and cervical cancer patient tissue obtained from patients of Groote Schuur Hospital, South Africa. While a general trend towards higher expression of c-Jun was observed in the cancer group, there was significantly higher Jun B expression in cervical cancer patient group compared to normals, suggesting Jun B as an important component of the AP-1 complex in cervical cancer. Jun D expression levels were found to be similar in both normal and cervical cancer tissue. The association of high c-Jun and Jun B levels in cervical cancers suggests a possible role for these members of the AP-1 complex in cervical cancer.

Both c-Jun and Jun B are described as immediate response genes and thus the varying expression levels of these two proteins occurs as a result of numerous cellular signals (Ryseck and Bravo, 1991), whereas Jun D is ubiquitously expressed (Hirai *et al.*, 1989).

5.2 Biological significance of AP-1 inhibition to cervical cancer cells

AP-1 protein complexes are implicated in the regulation of cell proliferative signals on numerous cellular contexts. Previous studies suggest that AP-1 is also important for the promotion of growth of transformed cells as well as in cancer cells (Liu *et al.*, 2002; Neyns *et al.*, 1999; Rapp *et al.*, 1994). The constitutive overexpression of the inhibitor of AP-1,

TAM67, successfully inhibited proliferation of transformed rat embryo cells (Rapp *et al.*, 1994). The altered morphology of cells displaying a larger flatter cell size and the significant decrease in adherent cell proliferation of CaSki cervical cancer cells expressing TAM67 suggests that AP-1 is involved in the proliferation of cervical cancer cells. Similarly, AP-1 has been shown to promote the growth of ovarian cancer cell lines and breast cancer cells, where the inhibition of AP-1 activity by TAM67 was responsible for growth suppression (Liu *et al.*, 2002; Neyns *et al.*, 1999).

In this study, it was also shown that AP-1 is required for cervical cancer cells to grow in an anchorage-independent manner. As the ability of cells to grow in an anchorage-independent manner is a phenotypic trait of transformed and tumourigenic cells (Fukazawa *et al.*, 1995; Macpherson and Montagnier, 1964), these findings suggest a role for AP-1 in the tumourigenicity of cervical cancer. A study by Kielosto *et al.*, (2004), found that AP-1 is involved in anchorage-independent growth in NIH-3T3 fibroblasts transformed by ornithine decarboxylase and Ras. These authors showed that TAM67 was able to reverse the transformed phenotype as well as cause inhibition of cell proliferation. The ability of AP-1 to control the growth of cells in this manner has previously been described in rat fibroblasts and is found to be associated with the regulation of a number of AP-1 regulated genes (Katabami *et al.*, 2005; Kinoshita *et al.*, 2003; Leaner *et al.*, 2005).

Inhibiting individual AP-1 components, including c-Jun, Jun B and Jun D using siRNA was found to be specific to each Jun protein expression. Therefore the use of siRNA was found to be an effective approach to determine the contribution of each AP-1 component protein in the proliferation of cervical cancer cells. A combination of siRNA directed to c-Jun, Jun B and Jun D collectively, showed a similar inhibition on cell proliferation as that observed with

TAM67. siRNA directed against Jun B specifically resulted in a significant decrease in cervical cancer cell proliferation and thus suggested that the presence of Jun B in the AP-1 complexes found in cervical cancer cells was important for the proliferation of these cells. Deregulated expression and an altered Jun B structure have previously been observed in two cervical cancer cells, CC7T and HeLa cell lines (Choo *et al.*, 1995), supporting the findings of this study, that Jun B may be necessary for the growth of cervical cancer cells.

There are conflicting data regarding the role of Jun B in cellular proliferation (Castellazzi *et al.*, 1991; Leaner *et al.*, 2003; Mechta-Grigoriou *et al.*, 2001). There are some studies that show that c-Jun and Jun B work antagonistically, with c-Jun promoting proliferation and Jun B suppressing it (Jochum *et al.*, 2001; Mechta-Grigoriou *et al.*, 2001). Others demonstrate that c-Jun and Jun B co-operate in processes such as cellular transformation and proliferation and they show that AP-1 dimers composed of c-Jun and Jun B regulate transcriptional targets that may bring about cellular transformation in Rat1A fibroblasts (Leaner *et al.*, 2003). Jun B has also been shown to be effective in replacing c-Jun in cellular proliferation (Passegue *et al.*, 2002).

The potential for targeting both c-Jun and Jun B for anticancer cell therapy has been suggested by Gurzov *et al*, (2007). These authors show that injection of tumour cells with inactivated forms of both c-Jun and Jun B, prolonged the survival of mice and that the inhibition of these components of the AP-1 complex provides a promising approach in the treatment of cancers. In our study, we show that the inhibition of AP-1 complexes with both a dominant negative mutant, TAM67 and with siRNAs directed against individual Jun proteins affected the biology of cervical cancer cells implicating AP-1 as a key transcription factor.

5.3 Regulatory mechanisms of AP-1 in cervical cancer cells

AP-1 exerts its biological effects by regulating the transcription of its target genes but the exact mechanisms are poorly understood, however it is well established that AP-1 regulates cell cycle progression and proliferation in a cell type and growth specific manner (Angel and Karin, 1991; Eferl and Wagner, 2003).

The results indicate that AP-1 activity in cervical cancer cells is associated with the repression of endogenous $p21^{CIP1}$ expression that is p53-independent. This suggests that high levels of AP-1 in cervical cancer may reduce the cell growth inhibitory activity of $p21^{CIP1}$ as a cell cycle regulator. AP-1 may therefore be a contributing factor in cervical cancer, as inhibition of $p21^{CIP1}$ may result in the uncontrollable growth that is associated with cancer (Dash and El-Deiry, 2004; Kaufmann and Kaufman, 1993). The findings of this study show that disruption of AP-1 mediated repression on $p21^{CIP1}$ expression could be achieved by inhibiting AP-1 by TAM67. These results suggest that TAM67 prevents AP-1 inhibition of $p21^{CIP1}$expression, resulting in interference with cell cycle progression and the accompanying decrease in cell proliferation. This regulation was found to p53-independent thus implying a more direct regulation of AP-1 on the $p21^{CIP1}$ promoter. To determine if the AP-1 sites identified in the p21 promoter are transcriptionally important for the regulation of AP-1 on the p21 promoter remains to be elucidated in the future.

5.4 Concluding Statment

In summary, the relatively high levels of c-Jun and Jun B components of the AP-1 complex in cervical cancer patient tissue suggest a role for AP-1 in cervical cancer. Inhibition of AP-1 showed that AP-1 is biologically relevant in cell proliferation and that it may play a role in the tumourigenic phenotype of cervical cancer cells. Of the Jun family proteins, Jun B was found

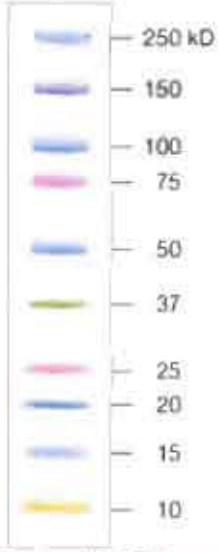
to be largely involved in cell proliferation. These present results suggest that inhibition of AP-1 activity acts via $p21^{CIP1}$ overexpression with an accompanying increase in cells in the G2/M phase of the cell cycle. Future studies will include understanding the manner through which AP-1 regulates $p21^{CIP1}$ expression in cervical cancer cells.

APPENDIX

APPENDIX A: PROTEIN MARKERS

Protein Molecular Weight Marker

Figure A.1. RPN800 Full-Range Rainbow Molecular Weight Markers (Amersham)
The ladder of proteins in the Full-Range™ Molecular Weight Markers on a 12% SDS-PAGE gel. The marker was used to estimate protein sizes during Western Blotting.



FigureA2. Biorad Kaleidoscope Marker
The ladder of Precision Plus Protein standard Biorad Kaleidoscope Marker run on a 4-20% Tris-HCL gel

APPENDIX B: SOLUTIONS

Cell maintenance solutions

Trypsinisation Solution (1L)

0.5 g	Trypsin
8 g	NaCl
1.45 g	$Na_2HPO_4.2H_20$
0.2 g	KCl
0.2 g	KH_2HPO_4
10 mM	EDTA pH 8

Made up to 1L with 1X PBS

Cell freeze media (50ml)

35 ml	DMEM media
10 ml	FBS (heat inactivated)
5 ml	DMSO

Heat inactivated FBS

Foetal Bovine serum heat inactivated at 56°C for 30min to inactivate and store at 20°C

Antibiotic solutions

Blastocyanin S Hydrogen Chloride (0.5µg/ml)

10 mg	Blastocyanin
2 mls	Sterile 1XPBS

Make 5 mg/ml 500 µl aliquots
Add 500 µl to 500 ml DMEM media

Doxycycline (2 mg/ml)

100 mg	Doxycycline
50 mls	dH_2O

Filter sterilize using 0.22µM syringe filter
Store aliquots at -20°C

Chloramphenicol 34 mg/ml

680 mg Chloramphenicol
20 ml 95% ethanol
Filter sterilize using 0.22µM syringe filter
Store in aliquots at -20°C
Use a working concentration of 12.5µg/ml

Ampicillicin 10mg/ml

100 mg ampicillin salt
10 ml sterile water
Filter sterilize using 0.22 µM syringe filter
Store in aliquots at -20°C
Use a working concentration of 100µg/ml

Protein Harvest solutions

RIPA buffer **200mls**

150 mM NaCl	1.752 g
10 mM Tris pH7	2 mls 1M Tris pH7
1% Triton X-100	2 mls 100% Triton X-100
1% Nadeoxycholate	2 g Nadeoxycholate
0.1% SDS	2 mls 10% SDS

Protein inhibitors

0.1M Na_2V0_4

0.1 M Phenylmethylsuphonyl fluoride (PMSF)

0.1742 g	PMSF
10 ml	100% Ethanol

Stored at -20°C

RIPA solubilization solution

200 µl	RIPA buffer
10X complete	Protease inhibitor (Roche)
4 µl	PMSF
2µl	0.1M Na_2VO_4

10X PBS

137 mM	NaCl
43 mM	$Na_2PO_4.7H_2O$ (pH 7.4)
14 mM	KH_2PO_4
27 mM	KCl

Autoclave

Western Blot solutions

4X Laemmli loading buffer

2.5 mls	1M Tris pH 6.8
3 mls	20% SDS
0.5 mls	0.1% Bromophenol blue
4 mls	Glycerol

Add 50 µl 10% β-mercaptoethanol to 0.5mls before use.

30% Acrylamide/Bisacrylamide (100 mls)

28 g	Acrylamide
1 g	Bisacrylamide

Warm dH_2O stored in a dark bottle at 4°C

1 M Tris pH 6.8

60.5 g	Tris base
300 ml	H_2O

pH with concentrated HCl to pH 6.8
Volume made up to 500ml

Autoclave

1 M Tris pH 8.8

60.5 g	Tris base
300 ml	H_2O

pH with concentrated HCl to pH 8.8,
Volume made up to 500 ml
Autoclave.

Gels

4% Stacking gel

ddH_2O	3.65 mls
1M Tris pH6.8	0.625 mls
30% Acrylamide/Bisacrylamide	0.65 mls
10% AMPS	50 µl
10% SDS	25 µl
TEMED	5 µll

Resolving gels	**12%**	**15%**
ddH_2O	2.15 mls	1.15 mls
1 M Tris pH 8.8	3.75 mls	3.75 mls
30% Acrylamide/bisacrylamide	4 mls	5 mls
10% SDS	50 µl	50 µl
10% AMPS	50 µl	50 µl
TEMED	5 µl	5 µl

10X Running Buffer (1L)

29 g	Tris
144 g	Glycine
10 g	SDS

Add dH_2O up to 1L

10X Transfer Buffer

30.3 g	Tris
140.4 g	Glycine

Add dH_2O to 1L

1X Transfer Buffer

100 ml	10X Transfer Buffer
200 ml	Methanol
700 ml	dH_2O

1X TBS-T (0.05%) Tween -20

50 mM	Tris pH 7.5
150 mM	NaCl
0.05%	Tween-20

Make up with dH_2O

Coomassie Blue Staining Solution (1L)

0.5 g	Coomassie Brillant Blue
500 ml	Methanol

100 ml	Acetic acid
400 ml	dH_2O

Destaining solution (1L)

50 ml	Methanol
70 ml	Acetic acid
880 ml	dH_2O

Non-Adherent cell proliferation solutions

1.5% methylcellulose

Autoclave 1.5 g in 100 ml bottle
Add 100 ml 10%FBS DMEM supplemented with penicillin and streptomycin
Stir overnight at 4°C

Polyheme (10X)

2.4g in 20 ml 95% ethanol
Rotate for 8 hours at 65°C
Make 1X in 95% ethanol
Filter sterilize

Cell Cycle Analysis solutions

RNase 6ml (50µg/ml)

300 µl	1 mg/ml RNase
5.7 ml	1X PBS

PIPES 0.1 M pH 6.8

3.014 g	PIPES
100 ml	dH_2O

pH to 6.8 to become clear

Propidium-Iodide solution

0.1 M	Triton-X-100
0.002 M	$MgCl_2$
0.1 M	NaCl
0.01 M	PIPES pH 6.8
0.01 M	Promidium Iodide

Mycoplasm Solutions

10% FBS /Penicillin and Streptomycin free media

45 ml	Penicillin and Steptomycin free media
5 ml	FBS

Hanks Balanced Salt Solution (without phenol red or sodium bicarbonate)

4.5 mM	KCl
0.3 mM	Na_2HPO_4
0.4 mM	KH_2PO_4
1.3 mM	$CaCl_2$

0.5 mM	$MgCl_2$
0.6 mM	$MgSO_4$
137 mM	NaCl
5.6 mM	D-glucose

Hoeschst No33258 Nuclear Stain (0.5µg/ml)

5 mg	Hoeschst No.33258
100 ml	Hanks Balanced Salt Solution

Store in dark covered bottle at 4°C.

Fixing solution

100 ml	Glacial acetic acid
300 ml	Methanol

Mounting fluid

22.2 ml	0.1 M Citric acid
27.8 ml	0.2 M $Na_2HPO_4 2H_2O$
50 ml	Glycerol

pH to 5.5
Store at 4°C

Immunofluorescence solutions

50mM NH_4Cl

0.265 g NH_4Cl
100 ml 1XPBS

4% paraformaldehyde in PBS

Prepare 16% PFA
16 g PFA to 80 ml dH2O
Cover with foil and stir for 1 hour on a heated stirer, not letting it exceed 60°C
Add few drops of 10 M NaOH, until the solution turns clear
Filter through 0.45 µM filter
Adjust pH to 7 with conc. HCl
Store in 2.5 ml aliquts at 20°C
Add 7.5 ml 1XPBS to make 4% PFA

0.5% Triton –X-100

500 µl Triton-X-100
100 ml 1XPBS

0.2% Gelatin

100 mg gelatin
10 ml 1XPBS

DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) (0.5mg/ml)

10 mg DAPI
20 ml PBS

Mowiol

2 g Mowiol

2 ml glycerol
4 ml ddH2O
Leave at RT O/N
8 ml 0.2 M Tris, pH8.5
Incubate at 50°C for 1 hour with occasional stirring
Store in 2 ml aliquots at -20°C
Add 2.5% anti-fading agent (N-propyl gallate) day before use
Centrifuge at 1200g for 5 mins to remove insolubles before mounting

Maxi preparation solutions

Luria Broth (1L)

10 g Tryptone
5 g Yeast Extract
10 g NaCl

Luria Agar (1L)

10 g Tryptone
5 g Yeast Extract
10 g NaCl
15 g Agar
Pour into plates

SOC medium

2.0 g	Tryptone
0.5 g	Yeast extract
1 ml	1M NaCl
0.25 ml	1M KCl
1 ml	2M Mg^{2+} stock, filter sterilised
1 ml	2M glucose, filter sterilized

Add tryptone, yeast extract, NaCl and KCl to 97 ml dH_2O. Stir to dissolve. Autoclave and cool to RT. Add 2 M Mg^{2+} stock and 2M glucose, each to a final concentration of 20 mm. Bring to100 ml with sterile dH_2O. Filter the complete medium through a 0.2 µm filter unit. The final pH should be 7.0.

2M Mg^{2+} stock

20.33 g $MgCl_2 \cdot 6H_2O$
24.65 g $MgSO_4 \cdot 7H_2O$
Add dH_2O to 100 ml. Filter sterilised.
0.1 M $CaCl_2$

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