

Lamina-associated polypeptide 2 (LAP2) expression patterns in transformed and cancer cells

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Table of Contents

Title	
Plagiarism Declaration	ii
Acknowledgements	iii
Table of contents	iv
Abbreviations	ix
Abstract	xii
Chapter 1: Introduction	1
1.1 Cervical cancer as a model system	1
1.1.1 Epidemiology of cervical cancer	1
1.1.2 Molecular mechanisms involved in cervical cancer development	3
1.1.3 Expression profiling in cervical cancer	6
1.2 Thymopoietin/Lamina-associated polypeptide 2 (LAP2)	8
1.2.1 Characterisation of Thymopoietin/LAP2	8
1.2.2 Functional significance of LAP2 α	10
1.2.3 Functional significance of LAP2 β	12
1.2.4 Focus of current research on LAP2 α and LAP2 β	14
1.3 The involvement of LAP2 in cancer	15
1.4 Significance	16
1.5 Aim	17
1.6 Objectives	17
Chapter 2: Materials and Methods	18
2.1 Cervical cancer patient material	18
2.2 Tissue cell culture	18
2.2.1 Cell lines	18
2.2.2 Tissue cell culture	20

2.2.3 Mycoplasma testing	21
2.3 Real-time RT-PCR analysis	21
2.3.1 RNA extraction	21
2.3.2 RNA quantification	21
2.3.3 RNA agarose gel electrophoresis	22
2.3.4 cDNA synthesis	22
2.3.5 Primers	23
2.3.6 Real-time RT-PCR	24
2.3.7 Agarose gel electrophoresis	25
2.4 Western blot analysis	25
2.4.1 Antibodies	25
2.4.2 Protein extraction	26
2.4.2.1 Protein extraction from cells grown under adherent conditions	26
2.4.2.2 Protein extraction from cells grown under anchorage-independent conditions	27
2.4.3 Protein quantification	27
2.4.4 SDS polyacrylamide gel electrophoresis	28
2.4.5 Immunoblotting	28
2.4.6 Immunodetection	29
2.4.7 Re-probing blots	29
2.4.8 Coomassie staining	29
2.4.9 Densitometric analysis of western blots	30
2.5 Immunofluorescence	30
2.5.1 Immunohistochemistry on archival patient tissue sections	30
2.5.2 Immunocytochemistry on cell lines	31
2.6 siRNA inhibition experiments	32
2.6.1 Transient siRNA transfection	32
2.6.2 Western blot analysis of siRNA transfection	33
2.7 Cell proliferation assays	33
2.7.1 Cell proliferation assay following siRNA transfection	33
2.7.2 Colony forming assay following siRNA transfection	34

2.8 Drug sensitivity	35
2.8.1 IC ₅₀ determination	35
2.8.2 Drug treatment following siRNA transfection	36
2.9 Cell cycle profile analysis	36
2.9.1 Anchorage-independent cell cycle analysis following siRNA transfection	36
2.10 Apoptosis assays	37
2.10.1 Caspase 3/7 activity assay	37
2.10.2 Poly (ADP-ribose) polymerase (PARP) cleavage assay	38
2.11 Cycloheximide protein stability assay	39
2.12 Statistical analysis	39
Chapter 3: LAP2 expression in cancer and transformed cells	41
3.1 Introduction	41
3.2 Results	45
3.2.1 LAP2 α expression in cervical cancer patient material	45
3.2.1.1 LAP2 α mRNA expression in normal versus cervical cancer patient specimens	45
3.2.1.2 LAP2 α protein expression in normal versus cervical cancer patient specimens	47
3.2.2 LAP2 expression in cultured cells	50
3.2.2.1 Western blot analysis of LAP2 expression	50
3.2.2.2 Immunocytochemical analysis of LAP2 α expression	55
3.3 Discussion	59
Chapter 4: Effect of LAP2 inhibition on cancer cell biology	62
4.1 Introduction	62
4.2 Results	65
4.2.1 LAP2 inhibition in cancer and transformed cell lines using siRNA molecules	65
4.2.2 Effects of LAP2 inhibition on cancer cell biology	67
4.2.2.1 Effects of LAP2 inhibition on cancer and transformed cells	

grown under adherent cell proliferation conditions	67
4.2.2.2 Effects of LAP2 inhibition on cell proliferation under various conditions of stress	71
(i) LAP2 inhibition and serum stress	71
(ii) Cell proliferation in response to LAP2 inhibition and chemotherapeutic drug treatment	72
(iii) Cell proliferation in response to LAP2 inhibition under conditions of anchorage-independent cell growth	74
4.2.2.3 Effects of LAP2 inhibition on cell cycle regulatory protein expression	79
4.2.2.4 Effects of LAP2 inhibition on cell cycle progression under anchorage-independent growth conditions	81
4.2.2.5 Effects of LAP2 inhibition on cell death through apoptosis in cells grown under anchorage-independent growth conditions	83
4.3 Discussion	86
Chapter 5: Effect of LAP2 inhibition on Rb expression levels	89
5.1 Introduction	89
5.2 Results	92
5.2.1 Effects of LAP2 inhibition on Rb protein expression	92
5.2.2 Effects of LAP2 inhibition on Rb mRNA expression	95
5.2.3 Effects of LAP2 inhibition on Rb protein stability	97
5.3 Discussion	100
Chapter 6: Conclusion	102
6.1 Expression of LAP2 in cancer	102
6.2 LAP2 inhibition and cancer cell biology	103
6.3 LAP2 inhibition and decreased Rb expression	105
6.4 Concluding statement	106
References	107

Bibliography	117
Appendix	118
Appendix A. Protein and DNA markers	118
Appendix B. Solutions	119
B.1 Tissue cell culture	119
B.2 Real-time RT-PCR	120
B.3 Western blot	121
B.4 Immunofluorescence	123
B.5 Cell proliferation assays	124
B.6 Colony forming assay	125
B.7 Cell cycle	125

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Abbreviations

BCA	Bicinchoninic Acid
bp	base pairs
BSA	bovine serum albumin
°C	celsius
cDNA	complementary DNA
CDK	cyclin dependent kinase
CHX	cycloheximide
CIN	cervical intraepithelial neoplasia
CP	<i>cis</i> -diaminedichloroplatinum
C _T	cycle threshold
Cyc	cyclophilin
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
dsDNA	double stranded DNA
E2F	elongation factor two
EDTA	ethylene diamine tetra acetic acid
5'FU	5'fluorouracil
FACS	fluorescent activated cell sorting

FCS	foetal calf serum
GusB	β -glucuronidase
HPV	human papilloma virus
hr	hour
IC ₅₀	50% inhibitory concentration
kb	kilobases
kD	kilodaltons
LAP2	Lamina-associated polypeptide two
LAP2 α	Lamina-associated polypeptide two alpha
LAP2 β	Lamina-associated polypeptide two beta
LEM	LAP2 Emerin Man1 family of inner nuclear proteins
M	molar
mg	milligram
ml	millilitre
MOPS	3-[N-morpholino] propanesulphonic acid
mRNA	messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
nm	nanometre
nM	nanomolar
OD	optical density
p	probability value
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase

PBS	phosphate-buffered saline
PI	propidium iodide
poly-HEMA	poly-2-hydroxyethyl methacrylate
Rb	retinoblastoma protein
pRb (807/811)	phospho-Rb (phosphorylated on serine residues 807 and 811)
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
siRNA	short interfering RNA
TBST	Tris-buffered saline with Tween 20
TMPO	thymopoietin
$t_{1/2}$	protein half-life
μg	microgram
μl	microlitre
μM	micromolar
V	volts

Abstract

The Lamina-associated polypeptide two (LAP2) proteins comprising three human isoforms, LAP2 α , LAP2 β and LAP2 γ have been shown to provide a structural framework in the nucleus and to facilitate nuclear assembly and disassembly during the cell cycle. Expression profiling studies, using microarrays, identified elevated levels of LAP2 α in cervical cancer patient material compared to normal. Altered expression of LAP2 may thus have significance in the development of certain cancers. The aim of this project was thus to independently confirm the up-regulation of LAP2 α in cancer material and to determine the effect of inhibiting its expression on the biology of cancer and transformed cells. LAP2 α mRNA and protein expression was shown to be elevated in cervical cancer tissue compared to normal cervical tissue by Real-time RT-PCR and immunohistochemical analysis respectively. Interestingly, LAP2 (both the LAP2 α and LAP2 β isoforms) was shown to be overexpressed in cervical cancer cell lines compared to a normal primary cervical epithelial cell line. Higher LAP2 expression appears to associate with cellular transformation as increased expression was observed in transformed human fibroblast cells compared to normal fibroblasts. LAP2 expression was also elevated in oesophageal cancer cell lines compared to normal suggesting that the overexpression of LAP2 associates with multiple cancer types. In order to determine the role of LAP2 in cancer cell biology, its expression was inhibited using specific siRNA molecules. Inhibition of LAP2 did not have an effect on adherent cell proliferation; however under anchorage-independent growth conditions a significant decrease in cell proliferation and colony formation was observed in LAP2 knockdown cells. This was accompanied by a decrease in cyclin D1 levels and an increase in p16 levels in LAP2 siRNA transfected cells. Our results did not conclusively show

that this decrease in proliferation was as a result of an alteration in the cell cycle profile or due to an increase in apoptosis. In addition, inhibition of LAP2 expression resulted in a decrease in Rb protein expression. It is proposed that LAP2 plays a role in stabilizing the Rb protein, as inhibition of LAP2 expression did not affect Rb mRNA levels but substantially reduced the protein half-life. In summary, increased LAP2 expression associates with transformed and cancer cells and suggests potential for use as a cancer biomarker. Its potential as an anti-cancer therapeutic, however requires further investigation.

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Chapter 1: Introduction

1.1 Cervical cancer as a model system

1.1.1 Epidemiology of cervical cancer

Cervical cancer is the second most common form of cancer in women worldwide (Kamangar *et al.*, 2005), and within the South African population (Mqoqi *et al.*, 2004). The incidence rate is approximately two-fold greater in developing countries, such as South Africa, compared to more developed countries (Kamangar *et al.*, 2005). As such, cervical cancer has a 0.59 mortality to incidence rate (MR:IR) worldwide with a staggering 0.79 MR:IR in Africa compared to 0.30 in North America (Kamangar *et al.*, 2005). The high incidence of the disease in developing countries is highlighted in Fig. 1.1.

The age standardized incidence rate (ASR) of cervical cancer in South Africa, in 1999, was 38/100 000 (Mqoqi *et al.*, 2004), where there was a considerable difference in the incidence rates between different groups within the population. Black South African females had an ASR of 34/100 000 of developing cervical cancer, compared to white females with an ASR of 12/100 000 (Mqoqi *et al.*, 2004). Collectively, South African women have a 1:31 lifetime risk of developing cervical cancer according to the most recent 1999 National Cancer Registry report.

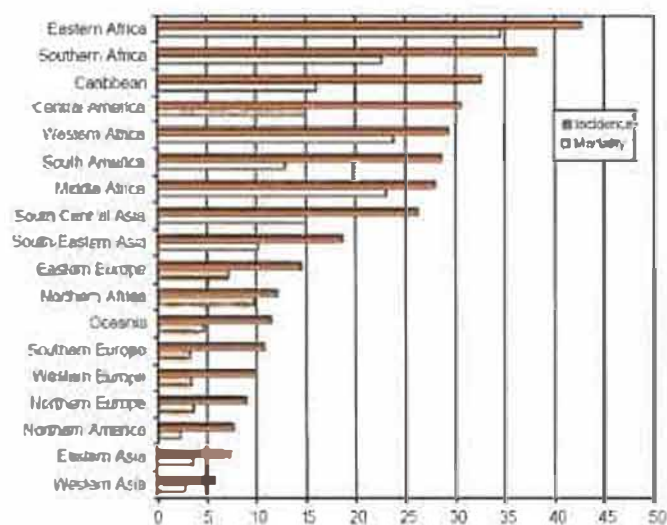


Fig. 1.1: Age-standardized incidence and mortality associated with cervical cancer worldwide per 100 000 women. The Southern African region has the second highest incidence of the disease globally and a correspondingly high mortality rate. (Sankaranarayanan and Ferlay, 2006).

Papanicolaou (Pap) smears are routinely used to assess cytological abnormalities from cervical swabs, and are the principle screening method used for preventing onset of this disease. However, there are insufficient cervical screening programs in developing countries, and hence late diagnosis is thought to be the major contributing factor as to why the mortality rate is so high in less developed countries.

This histological based Pap test, while proven to be highly effective in reducing the incidence and mortality of the disease, detects morphological changes and thus often has a high rate of false positive and false negative results due to inadequate specificity or sensitivity of the test (Nanda *et al.*, 2000). Liao *et al.* (1994) have highlighted a need for the identification of diagnostic biomarkers to supplement cytological tests to help address some of these problems.

1.1.2 Molecular mechanisms involved in cervical cancer development

Between 90 and 100% of cervical cancer cases are caused by infection with the human papilloma virus (HPV) and 70% of these cases are as a result of infection with either the HPV18 or HPV16 types (Bosch *et al.*, 2002). The HPV virus infects and replicates within the basal epithelial cells of the cervix. The infection is most often cleared by the host resulting in HPV type specific immunity, if not, the infection can persist resulting in integration of the viral genome into the host DNA, which can ultimately lead to carcinogenesis (Fig. 1.2). The important discovery that linked HPV infection to the development of cervical cancer was made by Harald zur Hausen who shared the Nobel Prize for Physiology or Medicine in 2008.

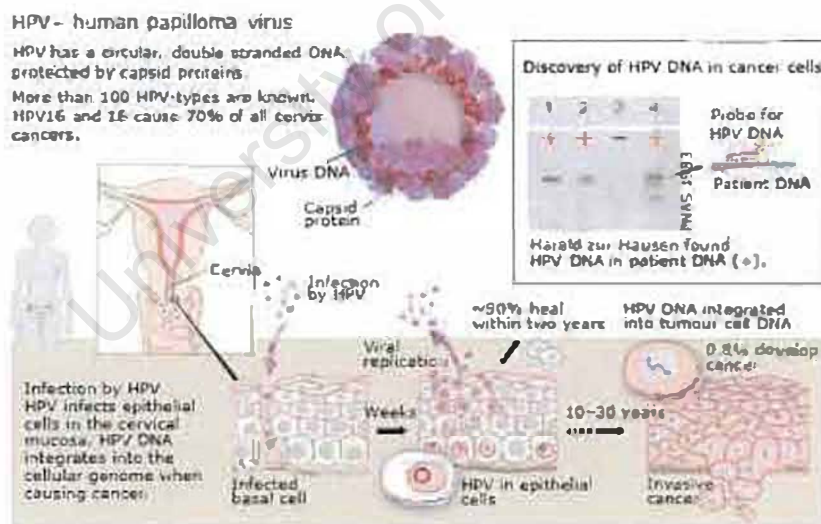


Fig. 1.2 HPV infection and the development of cervical cancer. (The Nobel Committee for Physiology or Medicine 2008 / Illustration by Annika Röhl).

At the molecular level it is accepted that cancer develops in multiple stages through the alteration of expression, or activity, of multiple protein encoding genes (Farber, 1984). In the case of cervical cancer, this is triggered by high levels of viral encoded early oncoproteins, E6 and E7, which can bind to, and inactivate, the growth regulatory proteins, p53 and retinoblastoma (Rb) respectively (reviewed in Bosch *et al.*, 2002). The presence of the HPV virus, which affects the activity of tumour suppressor proteins, and hence other cell cycle regulatory genes, as well as mutations in other important cell cycle genes, leads to cellular transformation which can ultimately result in the development of cervical cancer. However, in line with the multistep-multigene hypothesis, there are additional cofactors that are necessary for viral-induced carcinogenesis; including environmental factors such as smoking and diet, as well as host-related factors such as susceptibility to specific genetic mutations (Tjalma *et al.*, 2005).

Genetic mutations can thus result in the transformation of normal cells into cancer cells. These cancer cells are characterized by a number of properties described by Hanahan and Weinberg (2000). These include the ability to replicate indefinitely, the loss of response to tumour suppressor proteins, as well as the self-induction of pro-growth signals, which allows cancer cells to proliferate in an uncontrolled manner. This is facilitated by the fact that cancer cells are able to avoid programmed cell death (apoptosis), can metastasize from the primary tumour site to other regions of the body, and can generate their own blood supply (Hanahan and Weinberg, 2000).

These characteristics of individual cancer cells manifest themselves at a histological level, where cervical cancer can be observed to progress through several detectable stages. Cervical intraepithelial neoplasia stage I (CIN I), or mild dysplasia, is defined by the presence of abnormal, enlarged nuclei in cells in the lower epithelial layer, while the middle and upper sections still contain 'normal' cells undergoing differentiation. CIN II is characterized by the presence of these abnormal cells in the lower and middle sections of the epithelium, while CIN III represents a state where there are few undifferentiated cells, and irregular cells are present throughout the epithelium, including the upper layer (Buckley *et al.*, 1982). The final step in cancer development is the progression to invasive squamous cell carcinoma (zur Hausen, 2000). This sequence of pathological changes is associated with alterations in gene expression which are essential to the development of cancer.

Two prophylactic vaccines preventing HPV infection, and thus the development of cervical cancer, have been developed, and are currently available on the market. The first, Gardasil®, developed by Merck protects against four HPV types (HPV6, 11, 16, 18) (Sharma and Sharma, 2007) while the GlaxoSmithKline developed, Cervarix® vaccine, is bivalent against HPV16 and 18 (Kyriogou and Shafi, 2008). Consequently, neither of these vaccines protects against all HPV types and their effectiveness in protecting against the disease in the long term is still unknown. They are both expensive, at about \$100 per course, and are thus not viable solutions for use in developing countries where prevention is needed most. In addition, the vaccines are only a preventative measure and have no benefit to people who have already contracted the disease.

1.1.3 Expression profiling in cervical cancer

In order for the alterations in gene expression in the cancer state to be studied, expression profiling in matched normal and tumour tissue can be performed. A study in our laboratory aimed at identifying gene targets that associate with cervical cancer using cDNA microarray analysis, and a cohort of cervical cancer patient tissue samples, has quantitatively shown that there are a wide range of genes that are differentially expressed in cancer cells compared to normal cells (Table 1) (van der Watt *et al.*, unpublished data).

Many of the genes identified are known to be involved in important cellular processes often disrupted in cancer (such as cell cycle, cell division, signalling, metabolism and cell adhesion) and hence may serve as potential markers of the disease and pose as attractive therapeutic targets. In this study, the potential of Lamina-associated polypeptide 2 (LAP2)/thymopoietin (TMPO) as a marker and therapeutic target, will be investigated in transformed and cancer cells. LAP2 was selected based on the following criteria: (1) a highly significant three-fold change in expression in cancer compared to normal tissue, which has been observed in two independent data sets [(van der Watt *et al.*, 2009) and (Rosty *et al.*, 2005)]. The observed overexpression of LAP2 in cervical cancer in studies performed in our laboratory is in agreement with the findings of a similar study conducted by Rosty *et al.* (2005). They identified a “cervical cancer proliferation cluster” of genes (one of which was LAP2) the expression of which correlates with that of HPV E6/E7 mRNA (Rosty *et al.*, 2005). (2) An additional criterion was based on the important

role that LAP2 plays in cell dynamics, nuclear structure and regulation of transcriptional activity as discussed below.

Table 1. Representative genes differentially expressed in cervical cancer tissue compared to normal cervix

Gene	Description	fold change	p-Value
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	4	7.8E-09
CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)	4	2.2E-06
MTHFD2	Methylenetetrahydrofolate dehydrogenase 2	3	2.8E-04
RAN	RAN, member RAS oncogene family	3	2.1E-06
SNRPB	Small nuclear ribonucleoprotein polypeptides B and B1	3	5.8E-06
HMGF	Hepato-ma-derived growth factor (high-mobility group protein 1-like)	3	5.5E-05
HSP60	heat shock 60kD protein 1 (chaperonin)	3	8.7E-05
SLC25A5	Solute carrier family 25 (mitochondrial carrier)	3	1.4E-04
PCNA	Proliferating cell nuclear antigen	3	8.4E-07
STMN1	Stathmin 1/oncoprotein 18	3	7.5E-07
TMPO	Thymopoletin/LAP2	3	2.9E-04
TPM3	Tropomyosin 3	3	2.4E-09
THBS2	Thrombospondin 2	2	4.7E-05
ITGB1	Integrin, beta 1	2	4.0E-04
HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1	2	6.5E-05
	protocadherin alpha 9	2	3.7E-07
	Transcribed locus	2	4.8E-04
PSMD11	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	2	2.4E-05
XPO1	Exportin 1 (CRM1 homolog, yeast)	2	4.5E-04
TMSB10	Thymosin, beta 10	2	3.9E-07
KPNB1	Karyopherin (importin) beta 1	2	7.5E-05

Genes two fold upregulated in cancer specimens compared to normal. $p < 0.0005$

Table 1: Genes differentially expressed in cervical cancer tissue compared to normal tissue (van der Watt *et al.*, 2009 and unpublished data).

1.2 Thymopoietin/Lamina-associated polypeptide 2 (LAP2)

1.2.1 Characterisation of Thymopoietin/LAP2

LAP2 was originally identified as a 49 amino acid protein called thymopoietin (Basch and Goldstein, 1974). Thymopoietin is in fact, a proteolytic cleavage product of the N-terminus of the LAP2 protein which was generated during the isolation and characterisation of the protein from bovine thymic tissue (Harris *et al.*, 1995) and shown to be involved in thymocyte differentiation (Basch and Goldstein, 1974). It was later determined that three different isoforms of human thymopoietin/LAP2 exist; namely: α , β , and γ , which arise from alternative splicing of the mRNA transcript (Harris *et al.*, 1994). These isoforms were subsequently shown to be expressed in a variety of tissues, suggesting that they may have functions other than thymocyte differentiation (Harris *et al.*, 1994).

The human LAP2 isoforms (LAP2 α , LAP2 β , LAP2 γ) are encoded by a single LAP2 gene and are alternatively spliced from eight exons to produce proteins of differing molecular weights; 75 kD, 51 kD and 39 kD for LAP2 α , LAP2 β and LAP2 γ respectively (Harris *et al.*, 1994). The three isoforms share a common 187 amino acid N-terminal region, of which the first 49 amino acids correspond to the originally identified thymopoietin protein. The alpha isoform contains a unique 506 amino acid C-terminal tail, while the beta and gamma isoforms differ only by a 109 amino acid beta specific domain (Harris *et al.*, 1994). The alpha isoform of LAP2 is non-membrane bound and is

essentially nucleoplasmic; localising to the interior of the nucleus, unlike its beta and gamma counterparts which contain a transmembrane domain and are thus anchored in the inner nuclear membrane (Dechat *et al.*, 1998) (Fig. 1.3).

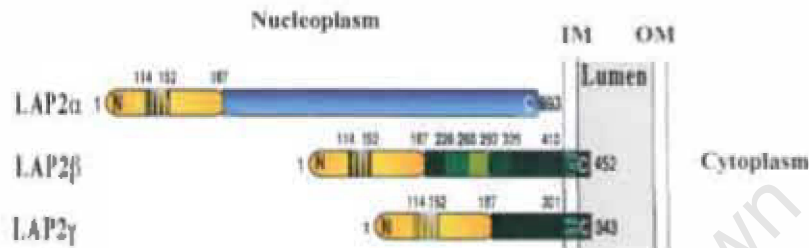


Fig. 1.3: Schematic representation of the human LAP2 alpha, beta and gamma isoforms. Numbers indicate amino acid positions, IM; inner nuclear membrane, OM; outer nuclear membrane. (Adapted from Dechat *et al.*, 2000b).

Differences in relative expression of these isoforms appear to be tissue specific (Ishijima *et al.*, 1996). The discovery of seven mRNA transcripts from the homologous mouse lamina-associated polypeptide 2, suggests the existence of more than the three originally discovered human forms (Berger *et al.*, 1996). While the LAP2 β isoform is conserved across several phyla, LAP2 α seems to be found exclusively in mammals (Prüfert *et al.*, 2004).

In addition to the homology evident between the LAP2 isoforms, protein sequence analysis has revealed a conserved 40 amino acid domain within the N-terminus of the LAP2, emerin and MAN1 inner nuclear proteins and they have thus been clustered together to form the LEM domain protein family (Lin *et al.*, 2000).

The alpha and beta isoforms have been well characterised and are described briefly below. However, not much is known about the gamma isoform other than the fact that it is structurally similar to the LAP2 β isoform (Dechat *et al.*, 2000b).

1.2.2 Functional significance of LAP2 α

cDNA microarray analysis revealed the LAP2 α isoform to be up-regulated in cervical cancer tissue. LAP2 α was initially reported to play a role in processes related to nuclear structure, and in nuclear re-assembly following mitosis (Dechat *et al.*, 1998).

The LAP2 proteins have been shown to interact with a variety of proteins, including the lamins, which are important in preserving nuclear structure, as they maintain an association between the nuclear membrane and chromatin via interactions with various proteins such as LAP2 α (reviewed in Gruenbaum *et al.*, 2000). LAP2 α interacts with lamin A and lamin C which are different splice variants encoded by the lamin A gene (reviewed in Gruenbaum *et al.*, 2000). The interaction between LAP2 α and lamin A occurs via the unique C-terminus of the LAP2 α isoform as shown by *in vitro* binding as well as *in vivo* co-localisation studies (Dechat *et al.*, 2000a).

In addition to binding to A- and C-type lamins, LAP2 α 's interaction with chromosomes has also been shown. Vlcek *et al.* (1999) revealed a ~350 amino acid region (nuclear targeting domain) in the C-terminal tail of the alpha isoform that is necessary for association with mitotic chromosomes. In addition to binding chromosomes directly,

LAP2 α also interacts with the DNA-crosslinking protein, barrier-to-autointegration factor (BAF), which together have been shown to co-localise to chromatin core structures (Dechat *et al.*, 2004).

The breakdown and assembly of the nuclear lamina network is essential for cell cycle progression (reviewed in Gruenbaum *et al.*, 2000). It has been shown that LAP2 α plays a role in nuclear assembly following mitosis (Dechat *et al.*, 1998). LAP2 α 's involvement in the cell cycle is demonstrated partly by the fact that LAP2 α displays differential localisation (cytoplasmic or nucleoplasmic) during the process of mitosis and this corresponds to alterations in the phosphorylation status of the protein (Dechat *et al.*, 1998). Three cyclin-dependent kinase sites have been identified within the C-terminal region of LAP2 α , which are phosphorylated upon entry into mitosis (Gajewski *et al.*, 2004). In addition, LAP2 α has recently been shown to affect the targeting of A- and C-type lamins to the nuclear interior, and it has been suggested that this may also affect cell cycle progression (Naetar and Foisner, 2009). Conversely, nuclear disassembly and chromatin re-organisation are also important events in the process of apoptosis. LAP2 α has been shown to be cleaved within its chromosome binding domain by caspases during apoptosis, thereby preventing its association with chromatin, and allowing disruption of nuclear structure (Gotzmann *et al.*, 2000).

It has also been proposed that LAP2 α has an additional function in regulating transcriptional activity. The C-termini of LAP2 α and lamin A/C have been shown to interact with the B and C pockets of the retinoblastoma protein (Rb) (Markiewicz *et al.*,

2002). Hypophosphorylated Rb is anchored in the nucleus and binds to the E2F transcription factor thereby preventing activation of S-phase specific genes (Mittnacht and Weinberg, 1991). Upon phosphorylation of Rb, it is released from the nucleus resulting in de-repression of E2F, allowing the transcription of E2F target genes. LAP2 α has been shown to regulate E2F activity by sequestering hypophosphorylated Rb, resulting in maintenance of E2F inactivity (Dorner *et al.*, 2006). High levels of LAP2 α have also been shown to be important in adipocyte differentiation and it has been proposed that the LAP2 α -Rb complex helps to maintain a balance between the processes of differentiation and proliferation (Dorner *et al.*, 2006). LAP2 α 's interaction with Rb is further supported by studies illustrating that LAP2 α is essential for controlling the localisation and phosphorylation state of Rb and that mislocalisation of these proteins halts cell proliferation (Pekovic *et al.*, 2007).

1.2.3 Functional significance of LAP2 β

Unlike the LAP2 alpha isoform, the LAP2 beta isoform contains a transmembrane domain and is thus anchored in the inner nuclear membrane forming part of the nuclear envelope. A region within the C-terminal tail of LAP2 β is important for targeting this isoform to the nuclear envelope (Furukawa *et al.*, 1995). It is from this position that LAP2 β interacts with B-type lamins present in the nuclear lamina (Foisner and Gerace, 1993). Like LAP2 α , LAP2 β interacts with the BAF nuclear protein which mediates the association between LAP2 β and chromosomes in the late stages of mitosis (Furukawa,

1999). A solution structure of LAP2 β showed that the LEM and LEM-like domains within the N-terminus are important for binding DNA and BAF (Cai *et al.*, 2001).

The importance of the LAP2-lamin interaction in mediating nuclear assembly in a phosphorylation dependent manner was first indicated by Foisner and Gerace (1993). This has subsequently been validated by Gant *et al.* (1999) who showed LAP2 β to mediate chromatin-nuclear membrane attachment and to play a role in lamina assembly. LAP2 β , together with other inner nuclear membrane proteins, have recently been shown to be involved more specifically in the regulation of nuclear envelope formation following mitosis (Anderson *et al.*, 2009). Yang *et al.* (1997) have also shown LAP2 β to be involved in nuclear lamina growth in the G₁ phase of the cell cycle, thereby implicating its involvement in cell cycle progression.

In addition to its structural role described above, LAP2 β has been proposed to perform other regulatory functions within cells. It has been shown to associate with the HA95 chromatin associated protein, and this interaction has been shown to be important in the initiation of DNA replication (Martins *et al.*, 2003). More recently, LAP2 β has been implicated in regulating transcriptional activity. This has been shown to be as a result of interactions with the germ cell-less (GCL) protein, where together these proteins decrease the activity of the DP-E2F complex, resulting in greater transcriptional repression than that exerted by Rb (Nili *et al.*, 2001). In addition, LAP2 β has been shown to interact with histone deacetylase 3 (HDAC3) inducing the deacetylation of histone H4, and has been

shown to repress the transcriptional activity of the p53 and NF- κ B transcription factors (Somech *et al.*, 2005).

1.2.4 Focus of current research on LAP2 α and LAP2 β

It is evident that both LAP2 α and LAP2 β interact with a variety of nuclear proteins forming complexes that are important in maintaining the architecture of the nuclear lamina during the cell cycle and regulating transcriptional activity (Fig. 1.4); however the precise mechanism of how this might occur is still largely unknown.

The majority of research on LAP2 is focused on the role it plays, together with lamins, in laminopathic diseases. This is a group of heritable disorders which are as a result of mutations in A-type lamins and other nuclear proteins as reviewed in (Broers *et al.*, 2004). These include Emery-Dreifuss Muscular Dystrophy, dilated cardiomyopathy (in which the LAP2 gene has been shown to contain a mutation (Taylor *et al.*, 2005)) and Hutchinson-Gilford progeria. There is however, very little known about LAP2's involvement in cancer.

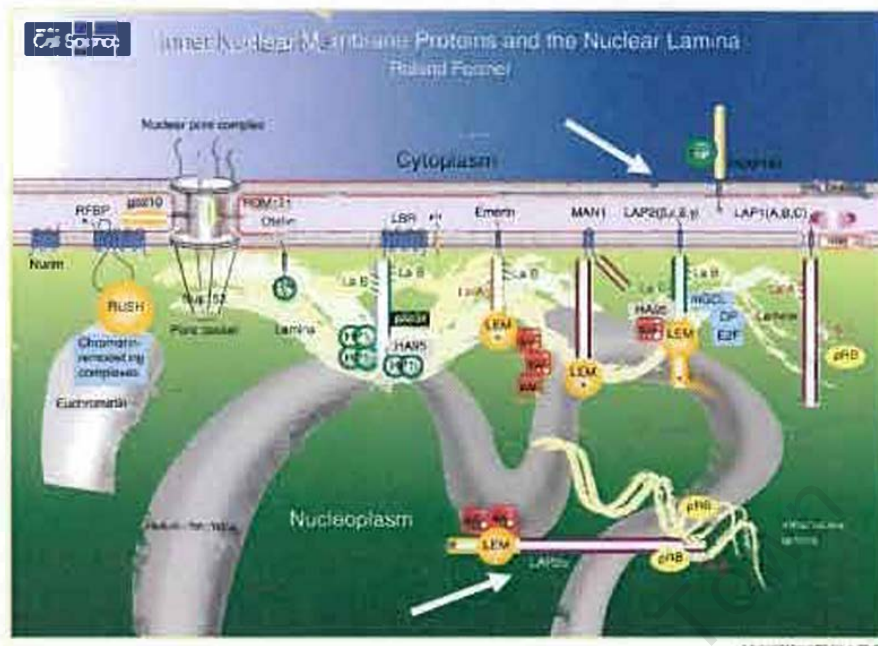


Fig. 1.4. Schematic representation of the mammalian nuclear lamina and inner nuclear membrane proteins. LAP2 α is indicated by the lower arrow and is shown with its various interaction partners. LAP2 α displays a nucleoplasmic localisation and can bind to chromosomes via the barrier-to-autointegration factor (BAF) via its LEM domain within its N-terminus as well as Lamin A/C and the retinoblastoma protein (Rb) via its unique C-terminal tail. LAP2 β is indicated by the upper arrow where its association with the inner nuclear membrane and lamin B within the nuclear lamina is shown. Its association with DNA via BAF and HA95 and the germ cell-less (GCL) transcriptional regulator is also highlighted. As this diagram is representative of the mouse nucleus, the LAP2 ϵ and LAP2 δ homologues are also included (Foisner *et al.*, 2001).

1.3 The involvement of LAP2 in cancer

Expression profiling using microarrays, performed in our laboratory has indicated that cervical cancer patient material displays elevated levels of the alpha isoform of LAP2 (van der Wan *et al.*, unpublished data). LAP2 expression has also been shown to be associated with cancer in various other large-scale genome-wide analyses [Pomeroy *et*

al., 2002), (Yokota *et al.*, 2004), (Agrawal *et al.*, 2002), (Welsch *et al.*, 2002), (LaTulippe *et al.*, 2002) and (Rosty *et al.*, 2005)]. There are, however limited reports on the validation of this up-regulation in cancer. One recently published paper observed LAP2 α mRNA overexpression in several primary tumours, namely stomach, breast, larynx, lung and colon, where its expression seemed to be regulated by the E2F transcription factor, the activity of which is often abrogated in cancer due to dysfunctional Rb (Parise *et al.*, 2006). Another study reported increased LAP2 β expression in neuroblastoma compared to normal nerve tissue (Weber *et al.*, 1999). Expression of the LAP2 proteins in other cancer types, as well as the potential role it plays in cancer development remains to be elucidated.

1.4 Significance

Cancer is a highly complex disease where multiple gene regulatory pathway alterations result in tumour cells that can far outgrow their normal counterparts. In cervical cancer as an example, HPV infection results in the altered regulation of numerous genes involved in important cellular processes, which ultimately leads to the development of the disease. An understanding of the changes in genes associated with the development of cancer may assist in early detection and treatment strategies. This project aims to characterise a possible link between LAP2 expression and cancer. In addition, the identification of genes that are functionally relevant to the biology of cancer cells could ultimately pose as potential therapeutic targets for the disease.

1.5 Aim

Previous studies in our laboratory which used gene expression array technologies, have suggested that LAP2 α levels are elevated in cervical cancer patient material compared to normal. In this study we aim to explore the differential expression patterns of LAP2 in normal and tumour patient tissue, as well as in cell lines grown in culture. The role of LAP2 in cancer cells will also be determined by inhibiting its expression in cells in culture. The potential for use of LAP2 as a biomarker as well as a potential therapeutic target will thus be investigated.

1.6 Objectives

1. To determine if LAP2 associates with cancer by,
 - (i) investigating expression of LAP2 in cervical cancer patient material compared to normal tissue and,
 - (ii) determining the expression of LAP2 in normal cells and transformed and cancer cells of different origins.

2. To determine the functional significance of LAP2 to cancer cell biology by inhibiting its expression in cells in culture.

3. To investigate the association between LAP2 and Rb expression levels.

Chapter 2: Materials and Methods

2.1. Cervical cancer patient material

All cervical patient material was previously collected from Groote Schuur Hospital, Cape Town, South Africa (Cancer Biology Group, Medical Biochemistry, UCT). Cancer tissue was collected from patients being treated for cervical dysplasia and carcinoma, while non-cancerous or 'normal' biopsy samples were collected from patients who were admitted for hysterectomies for reasons other than cervical abnormalities. A pathologist confirmed either normal or diseased status. All samples were obtained with patient consent and the study was approved by the Research Ethics Committee of the University of Cape Town (UCT) (REC REF:153/2004). All cervical cancer samples were identified as being HPV positive with 70% of samples infected with the HPV16 type (van der Watt *et al.*, 2009).

2.2 Tissue cell culture

2.2.1 Cell lines

For the determination of LAP2 expression in normal, transformed and cancerous cervical cells grown in culture, the following cell lines were used: a normal primary cervical epithelial cell culture, HCX, as well as its E6/E7 transformed counterpart, HCX-E6/E7, both of which were obtained from Dr. C. Barker, NIH, USA. Cervical cancer cell lines

CaSki (HPV16 positive), HeLa (HPV18 positive), ME-180 (HPV68 positive), MS751 (HPV18 & HPV45 positive), SiHa (HPV16 positive) and C33A (HPV negative, mutant p53) cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA).

Comparisons between LAP2's expression in normal and transformed fibroblasts were performed using the normal lung fibroblast cell line, WI38, as well as its SV40 transformed SVWI38 and gamma-irradiated CT-1 counterparts (Namba *et al.*, 1980). These cell lines, as well as the CCD1068SK normal breast skin fibroblast, were obtained from the ATCC. The FG₀ normal skin fibroblast was obtained from the Department of Medicine, UCT.

In order to determine the expression of LAP2 in an alternative cancer type, normal and cancerous oesophageal cell lines were also used. Normal hTERT-immortalized human oesophageal keratinocytes, EPC2-hTERT, were a gift from Prof. A. K. Rustgi (University of Pennsylvania, Philadelphia, USA) and were used to compare expression in the WHCO1 and WHCO5 South African oesophageal cancer cells lines (Veale and Thornley, 1989) and the Japanese-derived KYSE450 and KYSE520 oesophageal cancer cell lines (Shimada *et al.*, 1992).

2.2.2. Tissue cell culture

All non-primary cell lines were maintained at 60-80% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Gibco, Paisley, Scotland) and 100 units/ml penicillin and 100 µg/ml streptomycin (P/S) at 37°C in a 5% CO₂ incubator. Cells were sub-cultured with a 0.05% Trypsin-EDTA solution and neutralized with DMEM.

The HCX, HCX-E6/E7 and EPC2 primary cells were maintained in Keratinocyte Serum Free Medium (KSFM) (Invitrogen, Carlsbad, CA, USA) supplemented with Bovine Pituitary Extract (50 µg/ml for EPC2, HCX and HCX-E6/E7) and human epidermal growth factor (1 ng/ml for EPC2 and 26 ng/ml for HCX and HCX-E6/E7) and 100 units/ml P/S. The HCX-E6/E7 cell line was grown in the presence of 50 µg/ml of the selection antibiotic, G418. Cells were grown to 80% confluency, sub-cultured with Trypsin-EDTA and neutralized with soybean trypsin inhibitor. The cells were pelleted by centrifugation and re-suspended in supplemented KSFM medium before being re-seeded.

After sub-culturing, excess cells were re-suspended in cell freezing medium and subjected to slow freezing, to prevent the formation of ice crystals. Cells were stored at -80°C for two weeks prior to long term storage in liquid nitrogen.

2.2.3 Mycoplasma testing

To ensure that cell lines were not contaminated with Mycoplasma, a microbial contaminant invisible to the naked eye, routine testing was performed 2-3 times a year. Cells were cultured in P/S-free DMEM for four days before being plated onto coverslips and incubated for a further 24 hours. Cells were fixed and stained with the Hoechst fluorescent DNA-binding stain before being mounted and visualised on the Zeiss Axiovert 200 Fluorescent microscope (Carl Zeiss, Jena, Germany). The absence of speckling in the cytoplasm between stained nuclei indicates Mycoplasma-free cell cultures.

2.3 Real-time RT-PCR analysis

2.3.1 RNA extraction

RNA was harvested from cell cultures at 80% confluence with QIAzol (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was re-suspended in diethylpyrocarbonate (DEPC)-treated dH₂O.

2.3.2 RNA quantification

A 1:250 dilution of the RNA was made and its absorbance at 260 nm determined on a Beckman DU-650 UV spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

The ratio of the absorbance at 260 nm to the absorbance at 280 nm was determined as an indication of RNA purity. The RNA concentration was calculated using Beer Lambert's Law which states that the absorbance of nucleic acids at 260 nm (A) is equal to the product of the concentration ($\mu\text{g}/\mu\text{l}$) of nucleic acid (c), the length of the optical path (l) and the molar extinction coefficient (ϵ). One A_{260} unit of single stranded RNA is equal to 40 $\mu\text{g}/\text{ml}$ H_2O and the optical path length is equal to 1cm.

2.3.3 RNA agarose gel electrophoresis

One microgram of RNA was electrophoresed on a 1.5% formaldehyde-agarose gel, containing 0.5 $\mu\text{g}/\mu\text{l}$ ethidium bromide, to determine the integrity of the extracted RNA. RNA was suspended in RNA loading buffer and allowed to separate on the gel for approximately 20 minutes at 65V. Only RNA of a high quality was used in subsequent experiments i.e. RNA with a 260 nm: 280 nm ratio of 1.8-2, a defined 2:1 ratio of 28S:18S ribosomal RNA subunits and an absence of smearing, indicative of degradation.

2.3.4 cDNA synthesis

The protocol for reverse transcribing RNA into cDNA was based on that of van Gelder *et al.* (1990). 500 ng of oligoT7-(dT)₂₀ primer (Promega, Madison, WI, USA) was hybridised to 2 μg of RNA by incubation at 70°C for 10 minutes. A mixture containing 0.5 mM dNTPs, 5X first strand buffer, 1.5 mM MgCl_2 , 1 μl of ImProm-II reverse transcriptase (Promega) and 40 units of RNasin RNase inhibitor (Promega) was added to

each mixture to yield a final volume of 20 μ l. The mixture was incubated at 42°C for two hours. The reverse transcriptase was inactivated by incubating the mixture at 70°C for 10 minutes. DEPC-treated water was added to yield a final concentration of 0.1 μ g/ μ l cDNA in a 30 μ l volume. Excess cDNA was aliquoted and stored at -80°C.

2.3.5 Primers

All primers were designed using Primer-Blast (NCBI) (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and their suitability tested using Primer Express Software (Applied Biosystems, Foster City, CA, USA). mRNA sequences were obtained from BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and aligned with primers using Multalin (Corpet, 1998). Where possible, primers were designed across adjacent exons in order to prevent the amplification of any contaminating genomic DNA. Cyclophilin (Cyc) and β -glucuronidase (GusB) were used as normaliser genes. Primer sequences and annealing temperatures are indicated in Table 2.1. The molarity of all primers was calculated using Beer Lambert's Law. Primers were stored as 20 μ M stocks.

Target	Forward primer	Reverse primer	T _a (°C)
Cyc	TGA GAC AGC AGA TAG AGC CAA GC	TCC CTG CCA ATT TGA CAT CTT C	60
Gus β	CTC ATT TGG AAT TTT GCC GAT T	CCG AGT GAA GAT CCC CTT TTTA	55
LAP2 α	GCA GGC AGA CAT TAG TCA AGC	CGA CCT ACA GTG GCA TTT CC	60
Rb	CAC GAA TGC AAA AGC AGA AA	GCC ATA AAC AGA ACC TGG GA	60

Table 2.1: Primer sequences and corresponding annealing temperatures (T_a) used in Real-time RT-PCR analysis. Primers are shown in a 5'-3' direction.

2.3.6 Real-time RT-PCR

Real-time RT-PCR is a quantitative technique for measuring the mRNA levels of a gene of interest, relative to an internal control or housekeeping gene (Houghton and Cockerill, 2006). Real-time RT-PCR was performed on triplicate samples with 2 μ l of cDNA (\sim 0.2 μ g), or dH₂O as a negative control, and 10 μ M gene specific forward and reverse primers. A two-step PCR reaction was followed using the KAPA SYBR qPCR Master Mix (KAPA Biosystems, Cape Town, South Africa) using the StepOne Real-Time PCR machine (Applied Biosystems). The reaction conditions were an initial denaturation step at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds with a single 40 second annealing/elongation step at the annealing temperature specific to the primer set. The SYBR Green I dye intercalates with dsDNA and emits fluorescence relative to the degree of intercalation. Amplification is monitored at each cycle in the PCR reaction where the cycle threshold (C_T) represents a value at which the fluorescence is greater than a chosen threshold distinguishable from background fluorescence (Houghton and Cockerill, 2006). A melt curve for the PCR products was generated using temperatures from 60-95°C to ensure a single product was amplified.

C_T values were calculated in the linear range of the amplification curves generated using StepOne Version2.0 software (Applied Biosystems). The $\Delta\Delta C_T$ method was used to calculate the level of target mRNA expression relative to that of the average of two known housekeeping genes, cyclophilin and GusB (Livak and Schmittgen, 2001).

2.3.7 Agarose gel electrophoresis

PCR products were separated via electrophoresis on a 2% agarose gel and visualised by ethidium bromide staining. This was to ensure that a single, specific PCR product was amplified and to ensure that there was no contamination or primer dimer formation. Samples were suspended in 6X loading dye (Fermentas Life Sciences, Burlington, Ontario, Canada) and electrophoresed alongside a 50 bp DNA ladder (50 bp GeneRuler, Fermentas) at 65V for approximately 1 hour.

2.4 Western Blot analysis

2.4.1 Antibodies

All primary antibodies and incubation conditions used are shown in Table 2.2. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling (Beverly, MA, USA) or Abcam (Cambridge, UK).

Antibody	Type	Dilution	Diluent	Company	Catalogue #
β-tubulin	polyclonal	1:1000	TBST	Santa Cruz	sc-9104
CDK4	polyclonal	1:2000	2.5% BSA	Santa Cruz	sc-601
cyclin D1	monoclonal	1:1000	TBST	Santa Cruz	sc-246
LAP2	polyclonal	1:1000	5% milk	Santa Cruz	sc-28541
LAP2α	polyclonal	1:1000	5% milk	Abcam	ab5162
p16	polyclonal	1:500	0.5% milk	Santa Cruz	sc-759
p21	polyclonal	1:250	0.5% milk	Santa Cruz	sc-756
p27	polyclonal	1:500	5% milk	Santa Cruz	sc-528
PARP	polyclonal	1:1000	7% milk	Santa Cruz	sc-7150
Rb	monoclonal	1:500	5% milk	Cell Signaling	9309
pRb (807/811)	polyclonal	1:500	5% BSA	Cell Signaling	9308

Table 2.2: Primary antibodies used in western blot analysis. (CDK4; cyclin dependent kinase 4, PARP; Poly (ADP-ribose) Polymerase, Rb; retinoblastoma protein, pRb(807/811); phosphorylated Rb on serine residues 807 and 811, TBST; Tris-buffered saline with Tween20, BSA; bovine serum albumin, milk; fat-free milk powder).

Goat anti-rabbit IgG-HRP-conjugated secondary antibody (170-6515) (Bio-Rad, Richmond, CA, USA) was used at a 1:5000 dilution in 5% milk and goat anti-mouse secondary antibody (170-6516) (Bio-Rad) was used at a 1:3000 dilution in TBST.

2.4.2 Protein extraction

2.4.2.1 Protein extraction from cells grown under adherent conditions

Cells were grown to 80% confluency in 35 mm or 60 mm cell culture dishes and protein lysates harvested in radioimmunoprecipitation assay buffer (RIPA) with a 1X complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mM Na₃VO₄ phosphatase

inhibitor. Cell lysates were sonicated for 30 seconds, centrifuged to remove cell debris, and the supernatant containing protein stored at -80°C before use.

2.4.2.2 Protein extraction from cells grown under anchorage-independent conditions

Cells were plated in 6-well plates coated with 1.5 ml poly-2-hydroxyethyl methacrylate (poly-HEMA) to prevent cell adhesion. For protein extraction, cells were pelleted from the growth medium by centrifugation at 1000 rpm for 5 minutes, re-suspended in protease inhibitor containing RIPA buffer, and processed as described above.

2.4.3 Protein quantification

Protein samples were quantitated using the Bicinchoninic Acid (BCA) assay (Pierce, Rockford, IL, USA) and the absorbance read at 595 nm on a BioTek EL800 (Bio-Tek Instruments, Winooski, Vermont, Canada) microplate reader using Gen5 software (Bio-Tek). The assay is based on the principle that proteins react with copper II to produce copper I which reacts with the BCA reagents to form a purple colour at 595 nm (Smith, 1985). A bovine serum albumin (BSA) dilution series was prepared to produce a standard curve which was used to determine protein concentrations of the unknown samples.

2.4.4 SDS polyacrylamide gel electrophoresis

Protein samples at a concentration of 30 µg were suspended in 2X Laemmli buffer and denatured at 95°C for 2 minutes prior to loading to ensure that the proteins were in their native conformation. Proteins were electrophoresed on a 4% stacking gel and separated on either a 8% or 10% resolving sodium dodecyl-sulphate (SDS) polyacrylamide gel. The Precision Plus Protein Kaleidoscope Standard (Bio-Rad) was loaded to determine the sizes of the resolved proteins. A voltage of 200V was applied for 1 hour.

2.4.5 Immunoblotting

Proteins were transferred to a Hybond™-ECL™ nitrocellulose membrane (Amersham Life Sciences, Amersham, UK) at 4°C at 100V for 70 minutes.

The membrane was blocked in 5% fat-free milk powder in TBST for 1 hour at room temperature with rotation to prevent non-specific binding of the antibody to the membrane. The membrane was incubated with primary antibody, overnight with rotation at 4°C.

The membrane was washed three times with TBST for 10 minutes each to remove unbound antibody and incubated with secondary antibody for one hour at room temperature with rotation. The membrane was washed a further three times for 10 minutes each with TBST.

2.4.6 Immunodetection

The specific protein bands were detected by enhanced chemiluminescence. This involved the use of the LumiGLO/LumiGLO Reserve chemiluminescent substrate system (KPL Inc, Gaithersburg, ML, USA) according to the manufacturer's instructions. X-ray films (AGFA CU-BP Medical X-Ray film, Mortsel, Belgium) were exposed to the membrane, with the chemiluminescent substrate, for various amounts of time depending on the antibody used. X-ray films were developed until the bands could be clearly visualised, before being added to a fixative agent.

2.4.7 Re-probing blots

Blots were stripped of primary antibody by incubation in 1 M glycine at pH 2.5 for 15 minutes. The mix was neutralized with 1 M Tris-HCl at pH 8.0 after which the membranes were incubated in blocking solution for 30 minutes prior to incubation with primary antibody. Blots were subsequently processed as previously described.

2.4.8 Coomassie staining

After transfer, the polyacrylamide gels were stained with Coomassie Blue staining solution for one hour. A destaining solution was added until the protein bands became visible above the background. This technique was used as an additional indication of sample loading.

2.4.9 Densitometric analysis of western blots

Following autoradiographic detection of western blots, the band intensity of proteins of interest (representative of protein expression) was determined by densitometry using a Chem Genius Densitometer and GeneSnap and GeneTools software (BioImaging Systems, Syngene, Frederick, MD, USA). Protein expression was determined relative to that of β -tubulin.

2.5 Immunofluorescence

2.5.1 Immunohistochemistry on archival patient tissue sections

Tissue sections of ~2-5 μm from paraffin-embedded sections of 16 cervical carcinomas and 9 normal cervical biopsies were used in immunohistochemical analysis. The sections were heat-fixed for 10 minutes prior to being re-hydrated and de-waxed in decreasing concentrations of xylol and ethanol. Antigen Retrieval was performed by pressure-cooking the slides in boiling EDTA pH 8.0 for 2 minutes. Slides were subsequently washed in phosphate-buffered saline with Tween20 (PBS-T) for 5 minutes and blocked in 0.2% gelatin in PBS-T for 30 minutes before being washed again in PBS-T and dried. LAP2 α specific antibody (5162) [Abcam, Cambridge, UK] was added at a 1:100 dilution in 0.2% gelatin and 100 μl of the mix was added to slides and allowed to incubate for 1 hour in a humidified chamber at room temperature. Slides were washed in PBS-T and incubated in 0.3% Sudan Black for 10 minutes to prevent auto-fluorescence. Slides were

washed a further two times with PBS-T for 5 minutes and dried before 100 μ l of secondary antibody mix was added to each slide. A 1:300 dilution of Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was made in the 0.2% gelatin blocking solution, added to slides and allowed to incubate for 45 minutes in a humidified chamber at room temperature. Slides were washed in PBS-T and incubated with 500 μ l of 100 ng/ml of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS-T for 5 minutes. Slides were washed again in PBS-T and dipped in dH₂O before being mounted in Mowiol 4-88 (Calbiochem, CA, USA). Slides were visualised on a Zeiss Axiovert 200 Fluorescent microscope (Carl Zeiss, Jena, Germany) using a Zeiss AxioCam camera and images viewed on Axiovision 4.6 software (Axiovision, Gottingen, Germany). Fluorescent intensity was quantitated using the densitometric mean of pixel intensity on the Axiovision software. Three fields of view were captured per slide, and two regions within each view were selected for quantitation.

2.5.2 Immunocytochemistry on cell lines

One hundred thousand cells were plated on ethanol-flamed coverslips in 35 mm dishes and allowed to proliferate until 60-70% confluence. The cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were subjected to three five-minute washes in PBS before being permeabilised with the addition of 0.5% Triton X-100 in PBS for 5 minutes. The cells were washed again in PBS before being quenched with 50 mM NH₄Cl in PBS for 5 minutes. The cells were blocked in 0.2% gelatin in PBS for 30 minutes. Primary antibody was added at a 1:100 dilution

for LAP2 α (5162) [Abcam] or Rb (9309) [Cell Signaling Technology, Beverly, MA, USA] in blocking solution for 45 minutes at room temperature in a humidified chamber. Cells were washed 3 times for 5 minutes with PBS. Fluorescently-labelled secondary antibody, Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch) at a 1:300 dilution, or Alexa488-conjugated goat anti-mouse (Invitrogen) at a 1:150 dilution in 0.2% gelatin blocking solution, was added for 45 minutes at room temperature in the dark. Cells were washed for 5 minutes with PBS and then incubated with 500 μ l 100 ng/ml DAPI in PBS for 5 minutes. After a 5 minute wash in PBS the cells were mounted onto slides in Mowiol 4-88 before being visualised. For each condition, the fluorescent signal of ten cells per field of view and ten fields of view per slide were quantitated.

2.6 siRNA inhibition experiments

2.6.1 Transient siRNA transfection

The expression of LAP2 was inhibited using siRNA specifically targeted to the mRNA sequence of LAP2 in order for the effects on cell biology to be determined. Cells were transfected with 20 nM of either LAP2 siRNA (h) (SC-43386) or a scrambled siRNA sequence Control siRNA-A (SC-37007) to eliminate the possibility of non-specific effects.

2.6.2 Western blot analysis of siRNA transfection

Approximately 100 000 cells were plated in 35 mm dishes and transfected with siRNA 24 hours later using TransFectin Lipid Reagent (Bio-Rad). Cells at a confluency of 60% were transfected with a siRNA master mix containing a 1:3 ratio of Transfectin:siRNA. Per reaction this translates to, the addition of 50 μ l of serum-free P/S-free media to an eppendorf to which 0.625 μ l of TransFectin was added and the mixture incubated for 5 minutes at room temperature. 20 nM i.e. 2 μ l of a 10 μ M stock of siRNA, was subsequently added to the mix, suspended by gentle tapping and allowed to stand at room temperature for 15-20 minutes. The media in the culture dishes was changed to 1 ml P/S-free 10% FCS DMEM and the 52 μ l Transfection mix was added dropwise to the cells. Cells were incubated with the transfection mix for 5-6 hours after which the medium was changed to 10% FCS DMEM containing 100 units/ml P/S.

2.7 Cell proliferation assays

2.7.1 Cell proliferation assay following siRNA transfection

The 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldridge, St Louis, MO, USA) colourimetric assay was used as a measure of cellular proliferation. The assay takes advantage of the fact that after incubation of cells with the MTT reagent, the yellow tetrazolium salt in the reagent is reduced to purple formazan crystals in the mitochondria of living cells. Briefly, 250 000 cells were plated in 60 mm

dishes and transfected with 20 nM control or LAP2 siRNA 24 hours following plating. Cells were subsequently trypsinised and 2500 cells were re-plated per well of a 96-well plate. 10 µl of MTT reagent (Sigma) was added to each well, including media-only containing wells, at the appropriate time points after transfection with siRNA. Four hours later 100 µl of solubilisation reagent was added to each well and incubated overnight to dissolve the formazan crystals. The absorbance, which is representative of the number of viable cells, was subsequently measured at 595 nm.

For cell proliferation assays at different serum concentrations, cells were plated in 2, 4 or 10% FCS in DMEM and the MTT assay performed as described above. For MTT analysis following serum starvation, cells were plated in DMEM containing no FCS for 24 hours prior to being plated in 10% FCS DMEM for the duration of the assay.

2.7.2 Colony forming assay following siRNA transfection

For anchorage-independent cell proliferation assays, cells were suspended in DMEM containing 1% methylcellulose which resulted in the formation of cell colonies which were quantitated as described by Fukazawa *et al.* (1995). Two hundred and fifty thousand cells were plated in 60 mm dishes and transfected with 20 nM control or LAP2 siRNA as described previously. Twenty thousand cells were subsequently re-suspended in 100 µl 1% methylcellulose (Sigma) in 10% FCS-containing DMEM and plated in quadruplicate per well of a 96-well plate. Each well had been coated with 100 µl of poly-(HEMA) (Sigma) 16 hours previously to prevent cell adhesion. Colonies were monitored for eight

days post transfection at which stage they were stained with 1 mg/ml *p*-iodo-nitrotetrazolium violet (Sigma) overnight at 37°C. Colonies were photographed by brightfield microscopy at 400x magnification using the Moticam 2500 camera (Motic, British Columbia, Canada) on a Zeiss Telaval microscope.

2.8 Drug sensitivity

2.8.1 IC₅₀ determination

The IC₅₀ for 5'Fluorouracil (5'FU) was determined in CaSki cells using a range of concentrations (0, 0.1, 1, 10, 25, 50, 100 and 200 µM). Ten thousand cells were plated in quadruplicate in 90 µl in 96-well plates and treated with 10 µl drug diluted in DMEM and dimethyl sulphoxide (DMSO) (Sigma) to give a final DMSO concentration of 0.2%. Cells were incubated for 48 hours, after which MTT and solubilisation solution was added to determine cell viability. Data was analysed in GraphPad Prism to determine the IC₅₀ of the drug. The IC₅₀ of 5'FU in CaSki was determined to be ~1 µM which was used in all further drug sensitivity experiments.

For cisplatin (CP) in CaSki, the optimum concentration resulting in ~50% cell death was experimentally determined to be ~86 nM.

2.8.2 Drug treatment following siRNA transfection

Two hundred and fifty thousand cells were plated in 60 mm dishes before being transfected with 20 nM control or LAP2 siRNA as described previously. Ten thousand cells were subsequently plated in quadruplicate in 96-well plates and treated with 1 μ M 5'FU or 86 nM CP. Cell proliferation was measured 48 and 96 hours post drug treatment using the MTT cell proliferation assay as previously described.

2.9 Cell cycle profile analysis

2.9.1 Anchorage-independent cell cycle analysis following siRNA transfection

The cell cycle profile of cells was determined following staining with propidium iodide which allows DNA distribution histograms to be plotted on the basis of the DNA content of a cell where n = the number of chromosomes in a non-dividing cell and $2n$ = the number of chromosomes following DNA synthesis (Krishan, 1975). Three hundred thousand cells were plated in 60 mm dishes and transfected with 20 nM control or LAP2 siRNA as previously described. Cells were re-plated on poly-HEMA-coated 6-well plates 24 hours post siRNA transfection. Wells were coated with 1.5 ml poly-HEMA 16 hours prior to plating. Cells were harvested four and eight days post transfection. Cell-containing medium, together with two PBS washes, was collected in 12 ml tubes. The cells were pelleted by centrifugation, re-suspended in DMEM and counted. The cells were fixed by the addition of 100% ice-cold ethanol and incubated at -20°C overnight.

Cells were removed from the ethanol by centrifugation at 1000 rpm for 5 minutes. The cell pellet was washed twice with PBS. 0.5×10^6 cells in PBS were aliquoted into eppendorfs and centrifuged at 1000 rpm for 5 minutes. RNase A (Fermentas) diluted in PBS was added to yield a final concentration of 50 $\mu\text{g/ml}$ in a volume of 200 μl . The cell mix was incubated with the RNase A solution at 37°C for 15 minutes. Twenty minutes prior to FACS analysis a 0.5 ml 0.01 M propidium iodide (PI) staining solution was added to each cell sample. 50 000 events were analysed using a FACSCalibur (Becton Dickinson BioSciences, San Jose, CA, USA) using CellQuest software to generate the cell cycle profile. The data was fitted to cell cycle profile models using ModFitLT 2.0 (Verity House Software, Topsham, ME, USA) and FlowJo 6.0 software (Tree Star Inc., Ashford, OR, USA). The Dean-Jett-Fox model was used to determine the cell distribution [(Dean and Jett, 1974) and (Fox, 1980)].

2.10 Apoptosis cell death assays

2.10.1 Caspase 3/7 activity assay

Caspases are known mediators of the apoptotic pathway and thus the activity of these enzymes can be determined using a luminogenic substrate where the luminescence produced is proportional to caspase activity and thus apoptosis (reviewed in Nicholson and Thornberry, 1997). Two hundred and fifty thousand CaSki cells were plated in 60mm dishes and transfected with 20 nM control or LAP2 siRNA as previously described. Cells were incubated for 24 hours after which 20 000 of the transfected cells were plated in

quadruplicate per well of poly-HEMA-coated 96-well plates. Seven days post siRNA transfection, cells were harvested from the medium, pelleted following centrifugation and re-suspended in 80 μ l cell culture medium. Twenty microlitres of this mix was centrifuged and re-suspended in RIPA, and a BCA quantitation performed to determine protein concentration. Sixty microlitres of the Caspase-Glo® 3/7 assay buffer (Promega) was added to the remaining 60 μ l cell mix and incubated at room temperature for 1 hour. Samples were transferred to white plates and luminescence quantified on a GloMax 96 Microplate Luminometer System (Promega).

2.10.2 Poly (ADP-ribose) polymerase (PARP) cleavage assay

PARP is a known *in vivo* caspase substrate (Nicholson *et al.*, 1995), and thus cleavage of this protein into two detectable fragments is representative of cells undergoing apoptosis. Two hundred and fifty thousand CaSki cells were plated in 60 mm dishes and transfected with 20 nM control or LAP2 siRNA as previously described. Cells were incubated for 24 hours after which the cells were trypsinised and plated on poly-HEMA-coated 6-well plates. Seven days post siRNA transfection cells were harvested from the medium, pelleted following centrifugation and re-suspended in 50 μ l RIPA. Lysates were quantitated and subjected to western blot analysis as described previously. The presence of a smaller 85 kD band, in addition to the 116 kD full length PARP band, is an indication of apoptosis.

2.11 Cycloheximide protein stability assay

Cycloheximide (CHX) is a protein synthesis inhibitor and it can thus be used to measure protein degradation and protein half-life as a measure of protein stability. One hundred thousand CaSki cells were plated in 35 mm dishes and transfected with 20 nM control or LAP2 siRNA as described previously. Cells were incubated for 48 hours and then treated with 50 µg/ml CHX (Sigma). Protein was harvested at 0, 3, 6, and 12 hour time points following CHX treatment and Rb levels analysed by western blot analysis as previously described. Rb and β-tubulin expression was determined by densitometric analysis of the western blot band intensities using a Chem Genius Densitometer and GeneSnap and GeneTools software. Densitometric values for Rb and β-tubulin were calculated relative to Day 0 and log values plotted against time. A linear trendline was fitted to the data points. The half-life of Rb was calculated using the following equation: $t_{1/2} = \log 2/m$ where $m = (y-c)/x$ on a linear graph.

2.12 Statistical analysis

All experiments were performed in triplicate and repeated at least two times. Results are presented as the mean value ± standard deviation unless stated otherwise. The Student's *t*-test was applied to calculate statistically significant differences between samples. A two-tailed distribution was used and, either equal variance was used for matched treated-untreated samples, or unequal variance was used for unmatched samples. Statistical significance was defined using a type I error or p-value of 0.05 where the p-value is the

probability of rejecting the null hypothesis when it is assumed to be true. All calculations were performed in Microsoft Excel and GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

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Chapter 3: LAP2 expression in cancer and transformed cells

3.1 Introduction

Cancer research is primarily approached from three angles namely; the early detection of the disease, the prevention of its occurrence and treatment development for patients who have contracted the disease. The identification of biomarkers associated with the disease, has an important role to play throughout the process of cancer progression and has the potential for both diagnostic and prognostic value (Diagram 3.1).

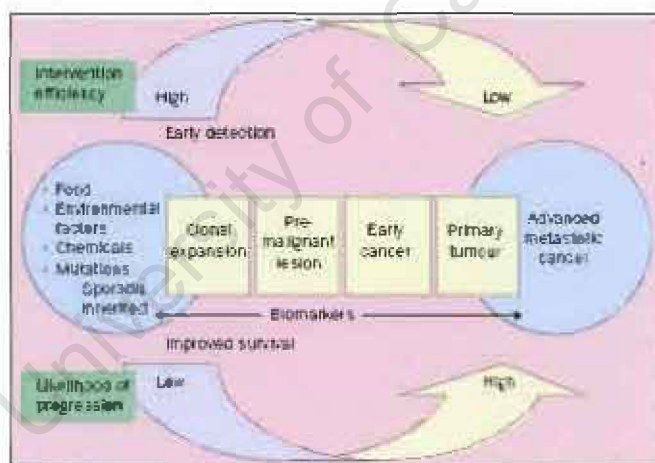


Diagram 3.1: Schematic representation of cancer development and the potential role of biomarkers in tracking the disease. (Srinivas *et al.*, 2001).

The identification of biomarkers in diseases such as cancer is now an established area of research. The National Cancer Institute (NIH, USA) defines biomarkers as biological molecules found in the body, either in body fluids, or in tissue sections, that are a sign of a normal or abnormal process, or of a condition or disease. Arguably, the most well

known example of a cancer biomarker is the Prostate Specific Antigen (PSA) identified in 1980 (Papsidero *et al.*, 1980). This protein has been shown to be released into circulation at high levels during early stages of prostate cancer development and to even greater, quantifiable levels in late stages of the disease, and has thus been used as a tumour biomarker for diagnosing prostate cancer.

Alterations in gene expression are known to be fundamental events in cancer progression, and form one of the processes responsible for an accumulation of biomarkers in the cancer state (Kulasingam and Diamandis, 2008). One of the main goals of our research is thus to determine whether proteins, which are shown to be overexpressed in cancer tissue compared to normal, can pose as potential biomarkers for identifying the disease condition. Expression profiling experiments, performed in our laboratory, identified a range of differentially expressed cDNA transcripts in cervical cancer tissue compared to normal tissue, one of which was recognised to encode the LAP2 α protein (van der Watt *et al.*, unpublished data). Similar genome-wide studies have also suggested an association between increased expression of the LAP2 gene and cancer. Expression profiling experiments have shown increased expression of the LAP2 gene in medullablastoma compared to normal cerebellum [(Pomeroy *et al.*, 2002), and (Yokota *et al.*, 2004)], colon cancer tissue compared to normal mucosa (Agrawal *et al.*, 2002), and the LAP2 gene has been shown to be regulated by the BRCA1 oncogene in breast cancer (Welsch *et al.*, 2002). LAP2 has also been implicated in cancer progression, as its expression was found to be elevated three-fold in metastatic prostate carcinomas compared to primary prostate carcinomas (LaTulippe *et al.*, 2002). LAP2 β has been shown to be highly expressed in

neuroblastoma cell lines and has been postulated to be a potential tumour marker (Weber *et al.*, 1999), while one study has shown overexpression of LAP2 α in larynx, lung, stomach, breast and colon primary tumours by quantitative RT-PCR (Parise *et al.*, 2006).

While the LAP2 alpha and beta isoforms differ in their C-termini and localisation, both isoforms are important in: maintaining nuclear architecture through interactions with nuclear lamins (Gruenbaum *et al.*, 2000), facilitating post-mitotic nuclear assembly, (Vlcek *et al.*, 1999) as well as regulating transcriptional activity through interactions with Rb (LAP2 α), HDAC (LAP2 β) and GCL (LAP2 β) [(Markiewicz *et al.*, 2002), (Nili *et al.*, 2001) and (Somech *et al.*, 2005)]. These proposed functions may be enhanced or abrogated in the cancer state as a result of LAP2 overexpression.

To our knowledge, no other studies have validated LAP2 overexpression in cervical cancer. Two approaches have thus been used to determine LAP2 expression in transformed and cancer cells. Firstly, biopsy samples from cervical cancer patient tissue material were assayed for LAP2 expression levels by Real-time RT-PCR analysis and immunohistochemical techniques on archival patient tissue sections respectively. Secondly, LAP2 protein levels were determined by western blot analysis in cultured cancer and normal cell lines.

In addition to investigating the expression of this gene in cervical cancer, its role in oesophageal cancer was also examined. Oesophageal cancer is the eighth most common cancer worldwide (Kamanagar *et al.*, 2005) and is the second most common cancer in

black South African males (Mqoqi *et al.*, 2004). Oesophageal cancer, like cervical cancer, is often only detected in the late stages of the disease and thus often results in a poor prognosis (reviewed in Tew *et al.*, 2005).

In order to determine whether LAP2 associates with cellular transformation, LAP2 expression in transformed cells in culture was also determined. Transformed cells are similar to cancer cells in that they display altered gene expression patterns and exhibit malignant properties, however they are not derived from *in vivo* cancers but are generated *in vitro* through the use of transforming agents such as viral oncoproteins and ultraviolet radiation. LAP2 expression in normal cell lines, which undergo a limited number of cell divisions due to their inability to produce telomerase and their susceptibility to contact inhibition, was compared to transformed and cancer cells that replicate indefinitely as they do not undergo contact inhibition but form foci on cell culture plates.

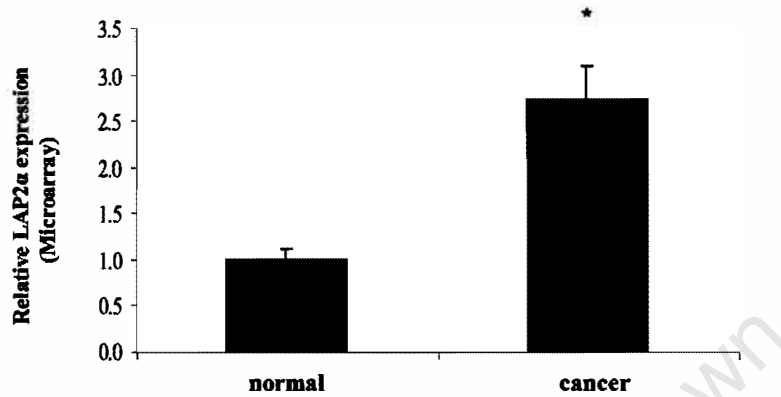
3.2 Results

3.2.1 LAP2 α expression in cervical cancer patient material

3.2.1.1 LAP2 α mRNA expression in normal versus cervical cancer patient specimens

Tissue biopsy samples were collected at Groote Schuur Hospital from patients with cervical cancer, as well as from patients who have had hysterectomies for reasons other than cervical dysplasia, hereafter referred to as 'normal patients' (Cancer Biology Group, Medical Biochemistry, UCT). RNA was extracted, complementary cDNA synthesized, and expression profiling using cDNA microarrays performed to determine the differential expression of genes in cancer compared to normal (van der Watt *et al.*, 2009). One of the cDNA transcripts identified to have altered levels of expression in the cancer samples compared to the normal, encodes the LAP2 α protein (Fig. 3.1.A). Increased LAP2 α expression was confirmed by performing Real-time RT-PCR analysis on RNA biopsy samples using primers targeted to the unique C-terminal tail of the LAP2 α isoform. Sixteen cancers and nine normals were analysed for LAP2 α expression relative to the Cyclophilin and β -glucuronidase genes which are routinely used as internal controls in quantitative RT-PCR experiments [(Valente *et al.*, 2009) and (Shih *et al.*, 2002)]. The average expression of these two genes was used to normalise LAP2 α expression, as the use of multiple genes as internal controls for Real-time RT-PCR has been shown to

A.



B.

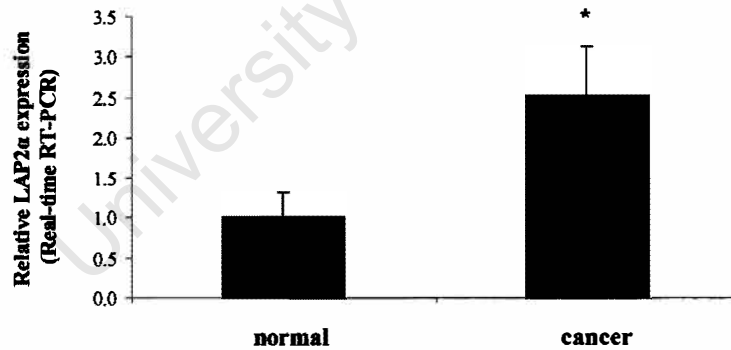


Figure 3.1: Relative LAP2 α mRNA levels in normal and cancer cervical tissue. (A.) LAP2 α mRNA expression determined by Microarray analysis (normal; n = 8, cancer; n = 16) (van der Watt *et al.*, unpublished data). (B.) Relative LAP2 α mRNA expression was determined by Real-time RT-PCR analysis (normal; n = 9, cancer: n = 16). GusB and cyclophilin were used as normalisers of the data. Results shown represent the mean \pm SEM (*, p-value <0.05).

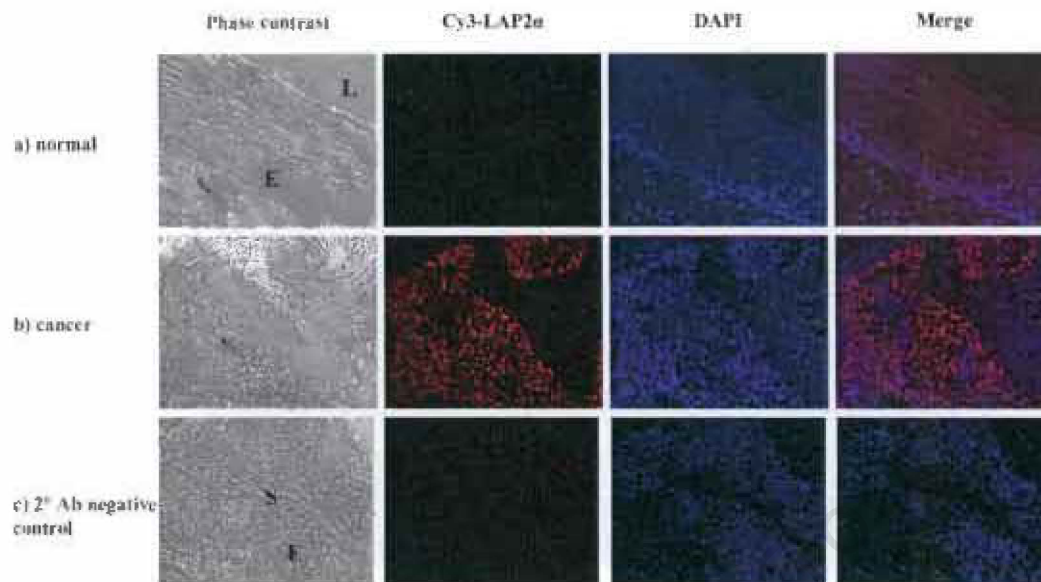
increase accuracy (Vandesompele *et al.*, 2002). Results obtained confirmed the initial microarray studies indicating that there was approximately a 2.5-fold increase in LAP2 α mRNA expression levels in cervical cancer patient material compared to normal cervical epithelial tissue. This corresponded to a statistically significant difference in expression (Fig. 3.1.B).

3.2.1.2 LAP2 α protein expression in normal versus cervical cancer patient specimens

Having established that cervical cancer tissue displayed elevated levels of LAP2 α mRNA expression, the next step was to determine whether this up-regulation was evident at the protein level, as it is proteins that form the functional units within cells. Paraffin-embedded cervical tissue sections (normal and cancer) were obtained from Groote Schuur Hospital and the Department of Anatomical Pathology, UCT, for immunohistochemical analysis. Sections were incubated with a LAP2 α specific primary antibody and a Cy3-fluorescently-labelled secondary antibody, as well as the DAPI nuclear stain. Images were captured using phase contrast microscopy to reveal tissue architecture, and the Cy3-fluorescence channel was used to determine the intensity of LAP2 α staining.

Phase contrast images from sections derived from normal cervical tissue revealed typical stratified squamous epithelial tissue present in the cervix (Fig. 3.2.A). Basal epithelial

A.



B.

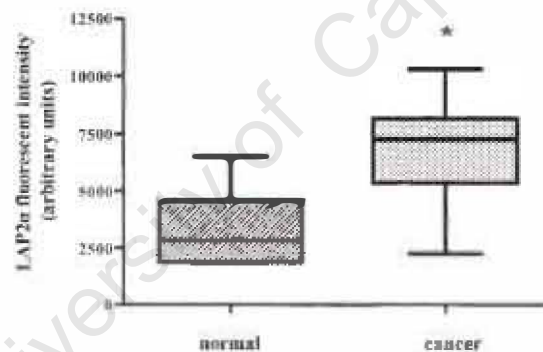


Figure 3.2: LAP2 α protein expression in normal and cancer cervical tissue.

(A.) Representative LAP2 α protein expression in archival tissue sections shown by immunofluorescence where LAP2 α expression is detected in the Cy3 channel and DAPI represents cell nuclei. 2 $^{\circ}$ Ab control (secondary antibody control) has no LAP2 α primary antibody and is shown as an indication of the specificity of the fluorescently tagged secondary antibody. a) Section from normal cervical tissue. b) Section from cervical cancer tissue. c) Section from cervical cancer tissue without primary antibody staining. All images were captured at 400x magnification using the same exposure time. Phase contrast images were captured to show tissue architecture. (E: epithelium, S: stroma, L: lumen). (B.) Quantitation of LAP2 α expression in normal and cancer tissue sections. (normal; n = 9, cancer; n = 16) Quantitation was determined using Axiovision 4.6 software and an average of six fields of view per slide were analysed. Results shown represent the mean \pm minimum and maximum values (*, p-value < 0.05).

cells, which represent proliferating cells, reside on the basement membrane which separates the epithelial layer from the stroma. As cell division occurs, these cells begin to differentiate causing them to flatten before being sloughed off into the lumen of the cervix (Fig. 3.2.A panel a). Corresponding images from sections derived from cancer patients showed abnormal cells throughout the epithelial layer as well as pockets of infiltrating cancer cells within the stroma (Fig. 3.2.A panel b). LAP2 α displayed high expression in the epithelial-derived cancer cells, and its expression was visible compared to that in the surrounding stromal tissue, which showed little staining. In contrast, very low levels of LAP2 α staining were detected within normal cervical tissue sections. A section incubated with secondary antibody alone, as a negative control, confirmed that the fluorescent signal obtained was specific to LAP2 α staining (Fig. 3.2.A panel c).

Quantification of the LAP2 α fluorescent intensity in sixteen cancer and nine normal sections revealed an increase in LAP2 α protein expression levels in the cancer specimens (Fig. 3.2.B). This corresponded to an approximate two-fold increase in LAP2 α expression in cancer compared to normal. An area comprising roughly ten cells per view was used to measure the fluorescent intensity (densitometric intensity/cm²) of LAP2 α staining in the different sections. The fluorescent intensity per slide was calculated on the basis of six fields of view using Axiovision software.

These results further supported the microarray and Real-time RT-PCR data, and showed that LAP2 α displayed elevated levels of expression at the mRNA and protein levels in cervical cancer tissue specimens compared to normal. We next investigated LAP2 α

expression levels in cells in culture, to determine LAP2 α expression in cancer and transformed cell lines of different types.

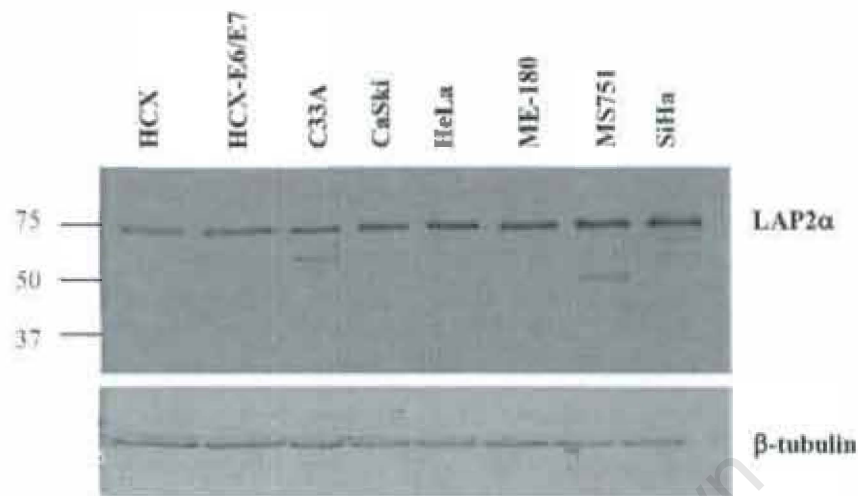
3.2.2 LAP2 expression in cultured cells

3.2.2.1 Western blot analysis of LAP2 expression

A panel of cervical cancer cell lines (CaSki, HeLa, SiHa, ME-180, MS751 and C33A) was used to compare expression of LAP2 α to that in a normal primary cervical epithelial cell culture, HCX, and its transformed counterpart HCX-E6/E7, which has been transformed by the HPV encoded E6 and E7 oncoproteins.

LAP2 α expression was determined using proteins extracted from these cell lines and an antibody that targets the unique C-terminal tail of the alpha isoform. Western blot analysis revealed higher LAP2 α expression in the majority of cancer cell lines compared to the normal primary cervical epithelial cell culture (Fig. 3.3.A). The C33A, HPV negative, cell line displayed the smallest increase in LAP2 α expression compared to HCX. β -tubulin was shown as a loading control to exclude that differences observed may have been as a result of differences in the amount of protein analysed. Densitometric analysis of the western blot band intensity quantitatively showed the increase in LAP2 α expression in cervical cancer cells (Fig. 3.3.B). Interestingly, an approximate two-fold increase in LAP2 α expression was observed in the HCX-E6/E7 transformed human

A.



B.

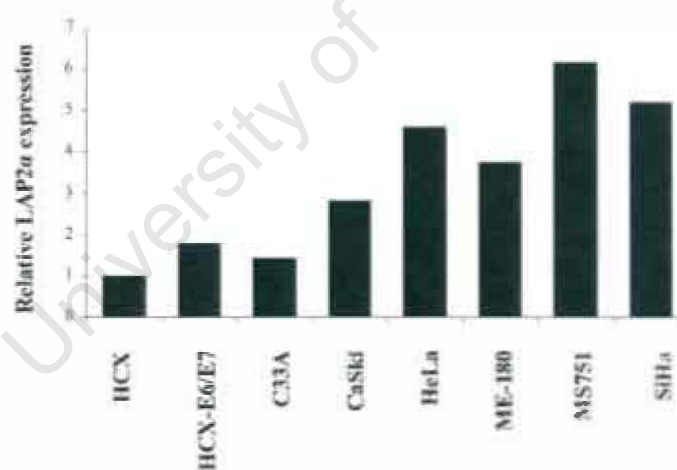


Figure 3.3: LAP2α protein expression in cervical cancer cell lines.

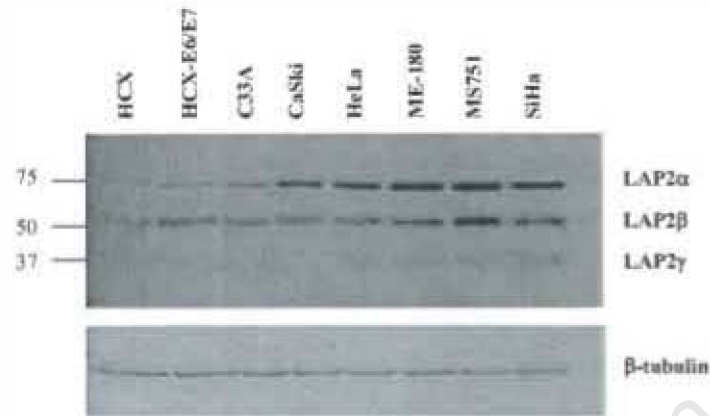
Protein lysates from normal and cancer cervical cell lines were analysed by western blot analysis. (A.) Western blot showing increased LAP2α expression in cancer cell lines using a LAP2 alpha-specific antibody. β-tubulin was included as a control for protein loading. (B.) Quantitation of LAP2α expression relative to β-tubulin determined by densitometry of western blot band intensity using GeneTools software. Results are representative of experiments performed at least two independent times.

cervical epithelial cells compared to the normal control cells (HCX), suggesting that LAP2 α may associate with cellular transformation.

A pan-LAP2 antibody that recognises the common N-terminal region of all three human isoforms of LAP2 was also used to determine whether the same increase could be observed (Fig. 3.4.A). The top band represents the 75 kD alpha isoform of LAP2, the middle band the 55 kD beta isoform, and the lower band the 39 kD gamma isoform. The alpha and beta isoforms seemed to be the predominant LAP2 isoforms, while the gamma isoform was barely detectable in most cell lines, and was virtually absent in the CaSki cell line. There was increased LAP2 α expression in the majority of cancer cell lines used as previously shown with the alpha-specific antibody (Fig. 3.4.A and B). Interestingly, an increase in the expression of the LAP2 β isoform was also observed. Both LAP2 α and LAP2 β (hereafter referred to as LAP2) expression was also increased in the HPV E7-transformed cell line compared to the normal primary cervical epithelial, HCX cells.

To further investigate the possibility that LAP2 associates with cellular transformation, its expression in normal and transformed fibroblasts was determined. LAP2 expression in normal breast skin fibroblasts, CCD1068SK, normal skin fibroblasts, FG $_0$, and normal lung fibroblasts, WI38, was compared to that in the SVW138 and CT-1 transformed lung fibroblasts. LAP2 was barely detectable in CCD1068SK, FG $_0$ and WI38. The transformed fibroblasts showed substantially higher LAP2 levels compared to normal fibroblasts as illustrated by western blot analysis (Fig. 3.5.A) and densitometric quantification of western blots (Fig. 3.5.B).

A.



B.

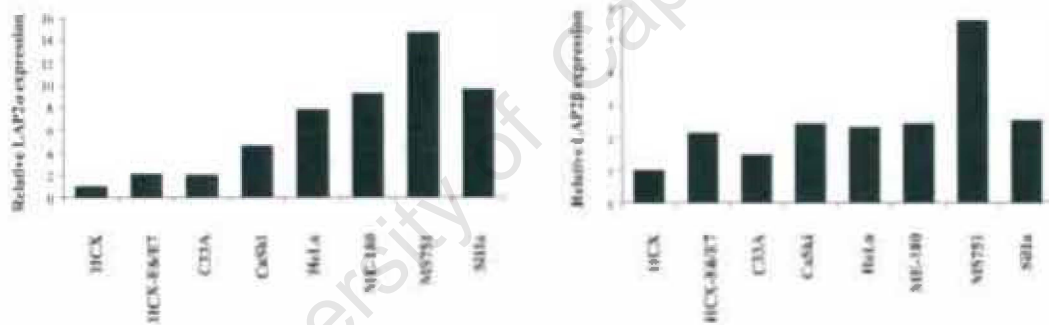
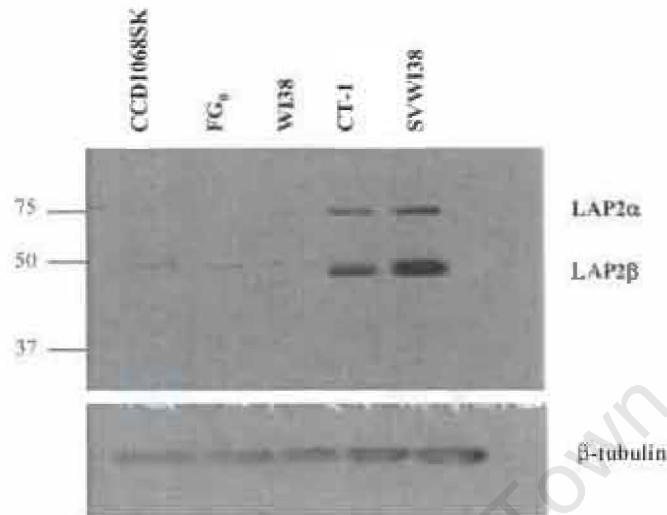


Figure 3.4: LAP2 protein expression in cervical cancer cell lines.

Protein lysates from normal and cancer cervical cell lines were analysed by western blot analysis. (A.) The upper panel shows the expression of all three LAP2 isoforms; 75 kD alpha isoform, 55 kD beta isoform and the 39 kD gamma isoform using an antibody that recognises the common N-terminal tail of all three isoforms. LAP2α and LAP2β show higher protein expression in cervical cancer cell lines (CaSki, HeLa, ME-180, MS751, SiHa) compared to the normal cervical epithelial cell line HCX and the HCX-E6/E7 transformed epithelial cell line. β-tubulin was included as a control for protein loading. (B.) Quantitation of LAP2α and LAP2β expression relative to β-tubulin determined by densitometry. Results are representative of experiments performed at least two independent times.

A.



B.

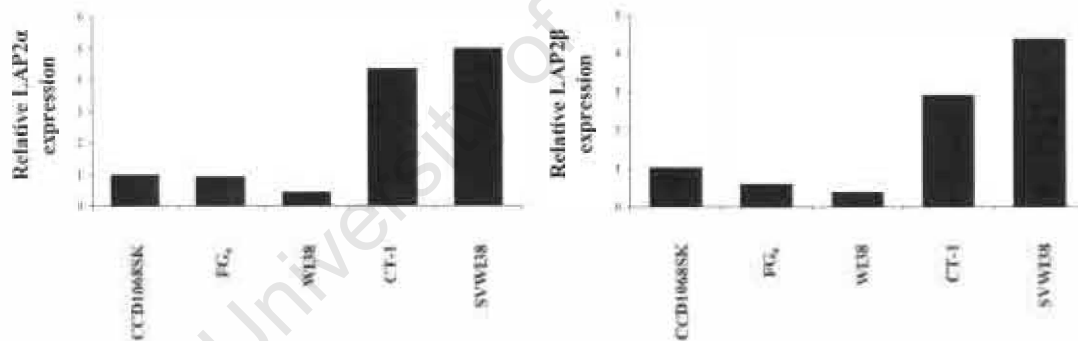


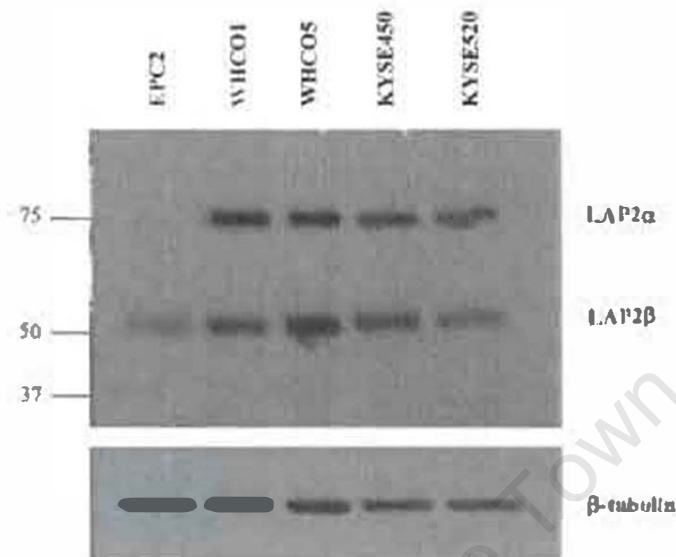
Figure 3.5: LAP2 protein expression in normal and transformed fibroblast cell lines. (A.) Protein lysates from normal and transformed fibroblasts were subjected to western blot analysis for LAP2 expression. LAP2 shows higher expression in transformed lung fibroblast cell lines (CT-1 and SVW138) compared to normal fibroblasts (CCD1068SK (breast), FG₀ (skin) and WI38 (lung)). (B.) Relative LAP2 α and LAP2 β expression in fibroblast cell lines determined by densitometric analysis of LAP2 and β -tubulin western blot band intensity. Results are representative of experiments performed at least two independent times.

Increased LAP2 expression thus appears to associate with both cervical cancer cell lines and transformed fibroblasts. We were therefore interested in determining whether this expression pattern is unique to cervical cancer, or whether it occurs in other cancer types. An investigation of LAP2 expression was thus also performed in a panel of oesophageal cancer cell lines (WHCO1, WHCO5, KYSE450 and KYSE520) and, compared to the corresponding normal oesophageal epithelial cell line EPC2. As observed with normal cervical epithelial and normal fibroblasts, LAP2 expression was shown to be very low in the normal epithelial oesophageal cells, with higher expression levels observed in the cancer cell lines (Fig. 3.6.A and Fig. 3.6.B). Taken together, these results suggest the increase in LAP2 expression associates with cellular transformation, as well as with cancer cells of different origins.

3.2.2.2 Immunocytochemical analysis of LAP2 α expression

The increase in LAP2 α expression in transformed and cancer cell lines was independently confirmed using immunocytochemistry. Paraformaldehyde-fixed cells were incubated with a LAP2 α specific antibody which is recognised by the Cy3-labelled secondary antibody, as well as a DAPI nuclear stain. The highest expression of LAP2 α was observed in the CaSki cervical cancer cell line and the SVW138 transformed fibroblast cell line, while the normal W138 cell line displayed lower levels of LAP2 α (Fig. 3.7.A). The merged image generated from the DAPI and Cy3 (showing LAP2 α) channels indicated nuclear localisation of LAP2 α as expected from previous published reports on LAP2 α localisation (Dechat *et al.*, 1998). The fluorescent intensity of LAP2 α staining was calculated in the different cell lines where ten cells per field of view and ten

A.



B.

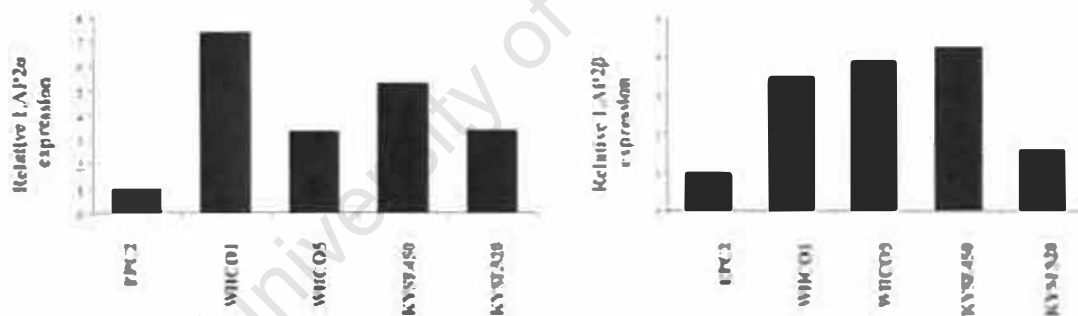
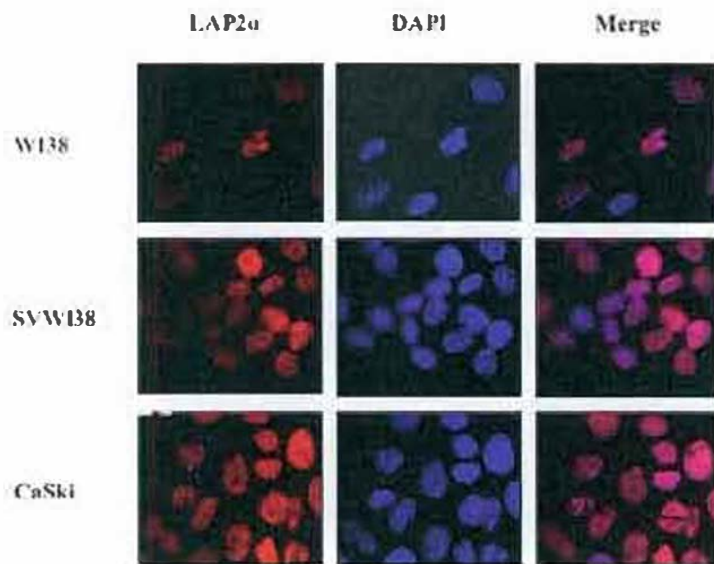


Figure 3.6: LAP2 protein expression in oesophageal cancer cell lines.

(A.) Protein lysates from normal and cancer oesophageal cell lines were subjected to western blot analysis for LAP2 expression. LAP2 shows significantly higher expression in oesophageal cancer cell lines (WHCO1, WHCO5, KYSE450, KYSE520) compared to the normal immortalised oesophageal cell line EPC2. (B.) Quantitation of LAP2 α and LAP2 β expression relative to β -tubulin in oesophageal cell lines. Protein expression was determined by densitometry of western blot band intensity. Results are representative of experiments performed at least two independent times.

A.



B.

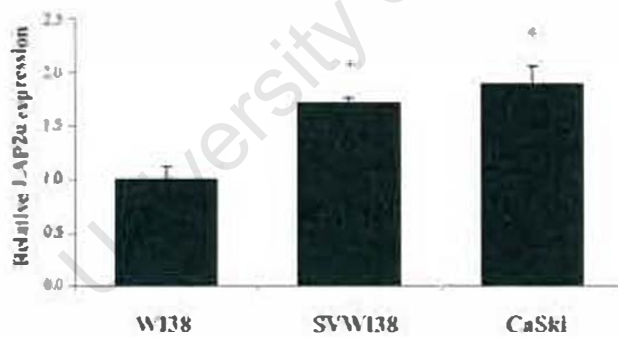


Figure 3.7: LAP2 α expression in a normal, transformed and cancer cell line.

(A.) Immunofluorescent analysis in the normal fibroblast, WI38, transformed fibroblast, SVWI38, and CaSki cervical cancer cell lines. Images were captured using 1000x magnification and the same exposure time was used across all samples. Immunofluorescence shows the nuclear localisation of LAP2 α expression. (B.) Quantitation of LAP2 α expression in WI38, SVWI38 and CaSki cell lines. Fluorescent intensity was calculated using Axiovision software where 10 cells per field of view and 10 fields of view were quantitated per slide. Quantification of LAP2 α expression was thus obtained from at least 100 cells per condition. Results are representative of experiments performed at least two independent times. (*, p-value <0.05).

fields of view per slide were quantitated as described previously. A significant increase in LAP2 α fluorescent intensity was observed in the transformed and cancer cell lines compared to normal fibroblasts (Fig. 3.7.B). These results support the western blot data suggesting that increased LAP2 α expression associates with cellular transformation and cancer cells.

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3.3 Discussion

This study aimed to follow up on expression profiling data that identified genes that are differentially expressed in cervical cancer patient material compared to normal, as potential biomarkers and cancer therapeutic targets. Identification of cervical cancer biomarkers could potentially enable diagnosis, or predict prognosis and patient response to therapy, and hence potentially reduce mortality rates associated with the disease.

The focus of this study are the LAP2 proteins, where the alpha isoform was found to display increased expression in cervical cancer patient material compared to normal tissue, by expression profiling experiments using cDNA microarray analysis. Microarray data should be independently validated using alternative techniques as there are often cases of false positive and false negative results. This present study confirmed an increase in LAP2 α mRNA and protein levels in cancer tissue compared to normal tissue as suggested by microarray analysis. A similar trend of increased LAP2 α expression was also observed in cervical cancer cell lines compared to a primary normal cervical epithelial cell line. Interestingly, western blots also showed an increase in the LAP2 β isoform, while LAP2 γ levels were largely undetected. Higher LAP2 (LAP2 α and LAP2 β) expression was also observed in transformed fibroblast cell lines and suggested that LAP2 expression is associated with cellular transformation. LAP2 was also shown to be overexpressed in oesophageal cancer cell lines compared to normal, suggesting that LAP2 expression associates with multiple cancer types.

A possible explanation for the observed up-regulation of LAP2 in cervical cancer, as an example, could be due to enhanced E2F activity in cervical cancer cells as a result of diminished Rb function. Rb is sequestered and targeted for degradation by the HPV E7 oncoprotein resulting in the activation of the E2F transcription factor (Bosch *et al.*, 2002). This allows for the increased transcription of E2F target genes. As LAP2 has been reported to be regulated by E2F (Parise *et al.*, 2006), this may explain the increased levels in cervical cancer cells. The reason for the C33A, HPV negative cervical cancer cell line, displaying lower levels of LAP2 expression could thus be due to the absence of the E7 oncoprotein; however it has been shown that the Rb protein is mutated in this cell line and thus should perhaps show a similar effect (Scheffner *et al.*, 1991). Rb is similarly often mutated in oesophageal cancer (Huang *et al.*, 1993) and may explain the overexpression observed in the oesophageal cancer cell lines. In the SVWI38 cell line, the simian virus large T antigen has been shown to bind and inactivate Rb (DeCaprio *et al.*, 1988) and thus explains the increased LAP2 expression observed in this cell line.

While our study is a first to identify and confirm increased LAP2 expression in cervical cancer patient material, a similar preliminary genome-wide expression profiling study identified LAP2 as one of the genes in a cervical cancer proliferation gene cluster the expression of which is positively correlated with that of E6 and E7 mRNA (Rosty *et al.*, 2005). LAP2 α mRNA overexpression has also been reported in several primary tumours and its expression has been shown to be correlated with tumour proliferation rate (Parise *et al.*, 2006); however expression has not been determined in cervical or oesophageal cancer cells and overexpression has not been confirmed at a protein level. Our

observation that LAP2 β expression is elevated in cancer cell lines is in agreement with studies showing increased levels in neuroblastomas (Weber *et al.*, 1999) and various haematological malignancies (Somech *et al.*, 2007).

While this study presents evidence of increased LAP2 expression in cancer cells, the use of LAP2 as a biomarker is preliminary. In order for the potential of LAP2 to be determined as a cancer biomarker, a larger patient cohort would be required to assess the clinical relevance of the up-regulation of LAP2. There is often considerable heterogeneity between patient samples and thus a more comprehensive study would need to be conducted to evaluate its suitability. Further laboratory-based studies are required to determine its suitability as a biomarker before the phases of assay development, screening and clinical trials can be considered. This would include the determination of whether its levels could be easily quantified. LAP2, previously known as thymopoietin, has been assayed from blood plasma at concentrations of <0.25 ng/ml and thus should be able to be assayed fairly easily (Twomey *et al.*, 1977).

The observation that LAP2 is overexpressed in cancer patient material compared to normal tissue, as well as in transformed and cancer cells in culture, suggests that this protein may play an important role in cancer cells. Further investigation into the functional significance of this up-regulation was thus performed.

Chapter 4: Effect of LAP2 inhibition on cancer cell biology

4.1 Introduction

In order to determine the biological relevance of increased LAP2 expression in cancer cells, two principle approaches can be followed. Either, the protein of interest can be overexpressed in a cell line which has low endogenous expression of the gene, via the introduction of cDNA plasmids encoding the gene of interest; alternatively, the expression of the gene can be inhibited in cell lines which highly express the gene. This can be achieved by transiently inhibiting expression using molecules such as short interfering RNA (siRNA), or short, stably transfected hairpin (shRNA) molecules.

Because LAP2 was shown to be overexpressed in cancer cells, this study used transiently transfected siRNA targeted to the LAP2 gene to inhibit its expression. This technique takes advantage of the fact that short (21-22 nucleotide) duplexes, when transfected into mammalian cells in culture, can mediate gene silencing (Elbashir *et al.*, 2001). Briefly, transfection with siRNA induces the RNA-induced silencing complex (RISC), which via its endonucleolytic activity, results in the targeting, cleavage and degradation of target mRNA, which ultimately leads to gene silencing evident at the protein level (Aigner, 2006). This process is illustrated in Diagram 4.1.

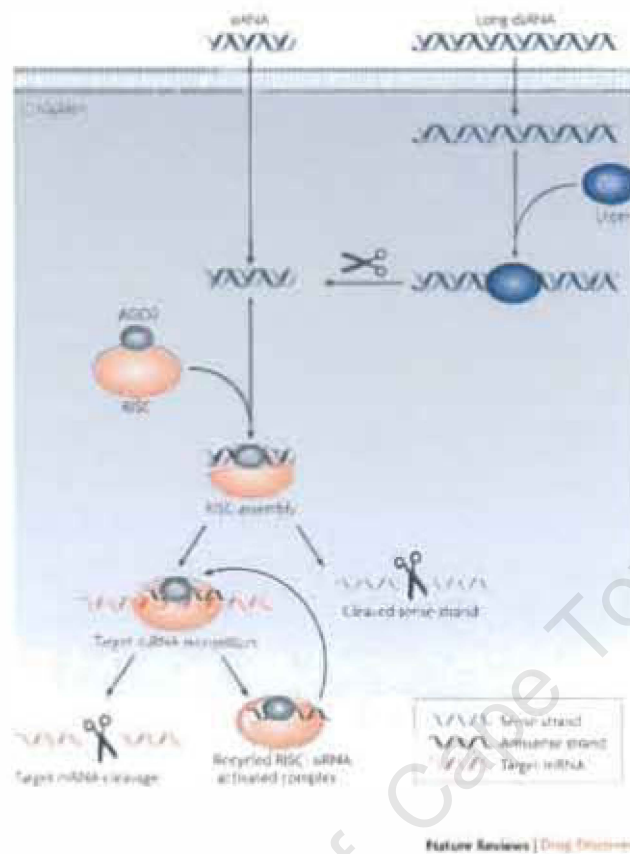


Diagram 4.1: Mechanism of RNA interference in mammalian systems. Long double-stranded DNA molecules enter the cell and are cleaved by the enzyme Dicer to form shorter siRNA molecules. Alternatively, siRNA molecules can be introduced directly into cells where they become incorporated into the RISC complex. This association results in the cleavage of the RNA sense strand by argonaute 2 (AGO2). The activated RISC-siRNA complex targets complementary mRNA, resulting in silencing of the expression of the gene of interest. The RISC-siRNA complex can be recycled and the process repeated on other identical mRNA molecules (Whitehead *et al.*, 2009).

Normal and cancer cells can be in one of four phases of the cell cycle at any point in time (reviewed in Collins *et al.*, 1997). These are the G₁ (gap 1) phase, in which the cell prepares to replicate its DNA, the S (DNA synthesis) phase, in which DNA replication takes place, the G₂ (gap 2) phase, where proteins are synthesised, or the M (mitosis) phase, when cell division occurs. Quiescent, or non-dividing cells, are said to reside in

the G₀ phase and can re-enter the cell cycle when conditions become favourable. Progression through the various phases of the cell cycle is tightly regulated by cell cycle regulatory proteins (reviewed in Murray, 2004). These include cyclins which bind to, and activate cyclin dependent kinases (CDKs), resulting in cell cycle progression via a cascade of phosphorylation modifications. Conversely if DNA damage is detected, or the cell is under stress conditions (eg. serum deprivation), cyclin dependent kinase inhibitors (CDKIs), also known as tumour suppressors are induced, and function to halt cell cycle progression. If the damage is not repaired, or the stress alleviated, the cell will undergo programmed cell death or apoptosis.

Important cell cycle regulatory proteins include the cyclins such as cyclin D1, CDK4, p16, p21 and p53 [reviewed in (Sherr *et al.*, 1999) and (Murray, 2004)]. Cyclin D1 is expressed in response to mitogenic stimuli and its expression is necessary for the induction of subsequent cyclins within the cell cycle. CDK4 is the kinase responsible for phosphorylating and inactivating Rb when complexed to cyclin D1. p16 inhibits the CDK4-cyclinD1 complex required for Rb phosphorylation and release of cells into S-phase. p27 and p21 act to repress cyclinE-CDK2 activity. These CDKIs are induced in response to contact inhibition, replicative senescence and TGF β signalling. In addition, p21 is positively regulated by p53 which is activated in response to DNA damage.

In this chapter, the effects of inhibiting LAP2 expression, via the introduction of LAP2 specific siRNA molecules, will be determined on cancer cell biology, cell cycle regulatory proteins and cell cycle progression.

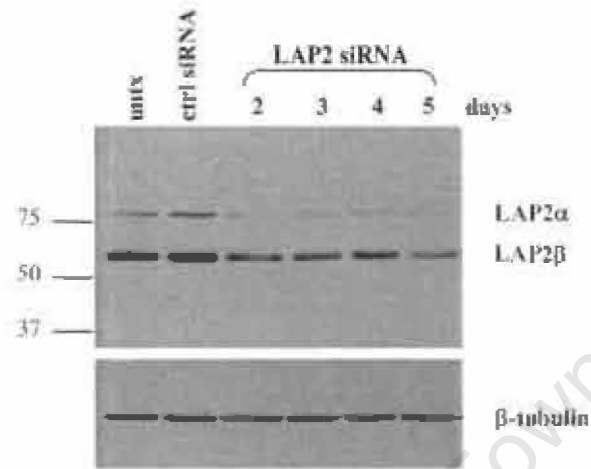
4.2 Results

4.2.1 LAP2 inhibition in cancer and transformed cell lines using siRNA molecules

LAP2 protein expression was silenced by transiently transfecting LAP2 siRNA molecules into cancer cells grown in culture, in order to determine the effects on the biology of cancer cells. The CaSki and HeLa cervical cancer cell lines were chosen as model cell lines for further experiments as they both display high levels of expression of LAP2, and are routinely used in our laboratory as cell lines representative of cervical cancer. LAP2 protein expression was inhibited using a pool of three, target-specific, 20-25 nucleotide, commercially available siRNA molecules (Santa Cruz Biotechnology). The LAP2 siRNA was expected to inhibit the expression of the alpha and beta isoforms as an alpha-specific siRNA is not available at present. A random, non-targeting 20-25 nucleotide, control siRNA sequence was used as a control for non-specific effects on gene expression, as well as to control for any changes induced by the transfection reagent.

Western blot analysis confirmed that there was a significant reduction in LAP2 expression (LAP2 α and LAP2 β), following LAP2 siRNA transfection in CaSki cells, which was sustained over the number of days assayed (Fig. 4.1.A). Transfection with control siRNA had no effect on LAP2 protein levels and LAP2 expression was comparable to levels observed in untransfected cell lysates. A similar decrease in LAP2 expression was observed in HeLa cell lysates following LAP2 siRNA transfection

A.



B.

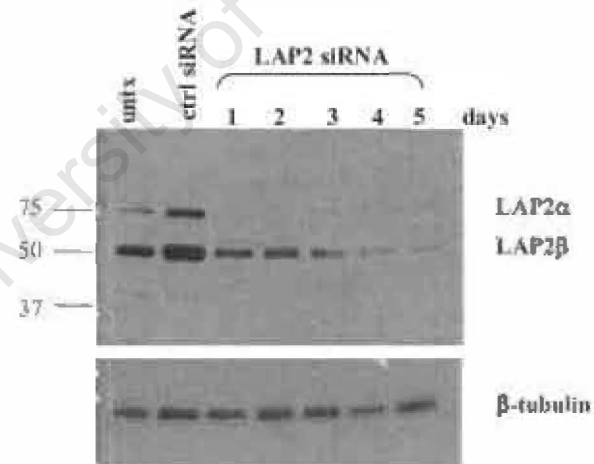


Figure 4.1: Inhibition of LAP2 expression in cervical cancer cell lines.

(A.) Western blot analysis showing LAP2 expression in untreated (untx) CaSki cells and cells transfected with 20 nM control (ctrl) or LAP2 siRNA up until five days post transfection. (B.) Western blot analysis showing LAP2 expression following LAP2 siRNA transfection in HeLa cells. β -tubulin was included as a control for protein loading. Results are representative of experiments performed at least two independent times.

(Fig. 4.1.B). The apparent increase in LAP2 expression in the control, compared to untransfected HeLa lysates, was due to differences in protein loading, as β -tubulin levels were also increased in the control cells.

Western blot analysis thus revealed that LAP2 siRNA transient transfections resulted in a substantial decrease in LAP2 protein levels, making the siRNA suitable for determining the role of LAP2 in the biology of cancer cells.

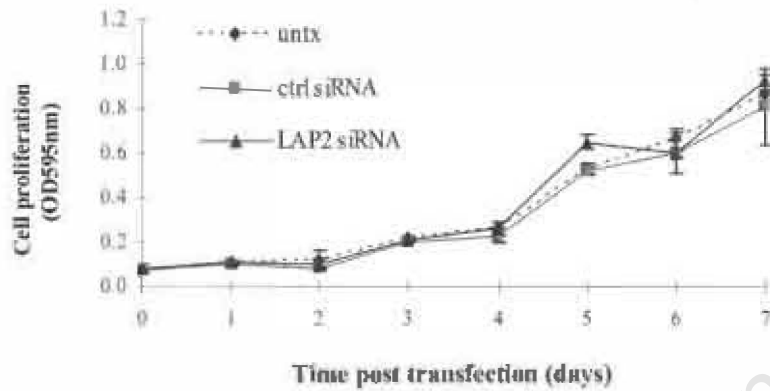
4.2.2 Effects of LAP2 inhibition on cancer cell biology

4.2.2.1 Effects of LAP2 inhibition on cancer and transformed cells grown under adherent cell proliferation conditions

Having established that transfection with LAP2 siRNA significantly reduces the expression of LAP2, functional effects of this inhibition were determined. The fundamental phenotype associated with cancer cells is the ability to proliferate rapidly and uncontrollably, and thus we wanted to determine whether LAP2 plays a role in proliferation of cancer cells when grown under adherent growth conditions in media containing 10% serum.

CuSki cells were transfected with 20 nM LAP2 or control siRNA and cell proliferation monitored by the MTT assay (Fig. 4.2.A). This colourimetric assay measures cell metabolic activity as an indication of cell number. Our results showed no significant

A.



B.

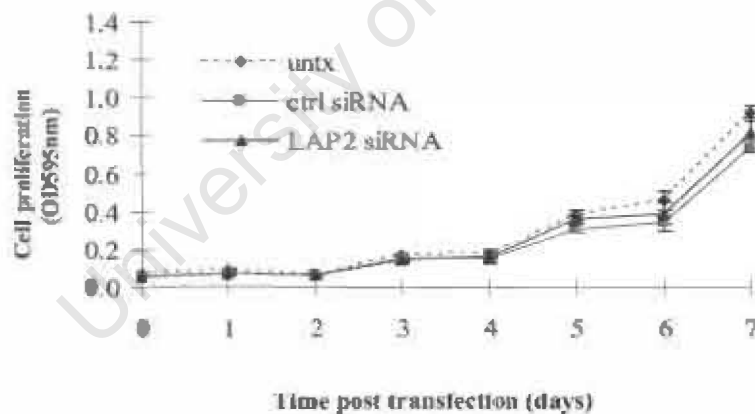


Figure 4.2: Effect of LAP2 inhibition on cervical cancer cell proliferation.

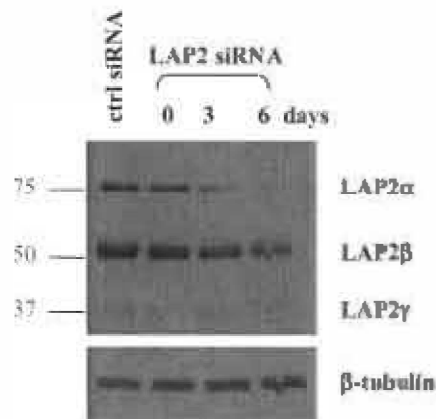
(A.) CaSki cervical cancer cells were either untreated (untx), or transfected with 20 nM of LAP2 or control (ctrl) siRNA and cell proliferation monitored up until seven days post transfection using the MTT cell proliferation assay. (B.) The effect of LAP2 inhibition on HeLa cell proliferation was performed as described in (A.). Experiments were performed in triplicate and repeated at least three independent times. Results shown represent the mean \pm SD.

difference in adherent cell proliferation in LAP2 siRNA transfected CaSki cells compared to untransfected and control siRNA transfected cells (Fig. 4.2.A). Similar results were obtained in HeLa cells showing LAP2 inhibition in this cell line also did not result in an alteration in cell proliferation (Fig. 4.2.B). This assay was repeated a number of times at different cell densities (data not shown), with the results consistently suggesting that LAP2 does not play a role in cervical cancer cell proliferation under adherent growth conditions.

Because LAP2 also showed elevated expression in a transformed fibroblast cell line, SVW138, the effect of LAP2 inhibition on SVW138 cells, was also investigated. SVW138 cells were treated with control and LAP2 siRNA as described for the cervical cancer cell lines. Two time points (three and six days post transfection) were selected for the assay. Western blot analysis showed a reduction in LAP2 protein levels at both three and six days post transfection with LAP2 siRNA (Fig. 4.3.A). MTT assays for SVW138 cell proliferation, when LAP2 expression was inhibited, revealed that there was no difference in proliferation between untransfected, control and LAP2 siRNA transfected cells (Fig. 4.3.B).

Together these data suggest that LAP2 does not appear to play a role in the cell proliferation of cervical cancer cell lines and transformed fibroblasts when grown under adherent growth conditions.

A.



B.

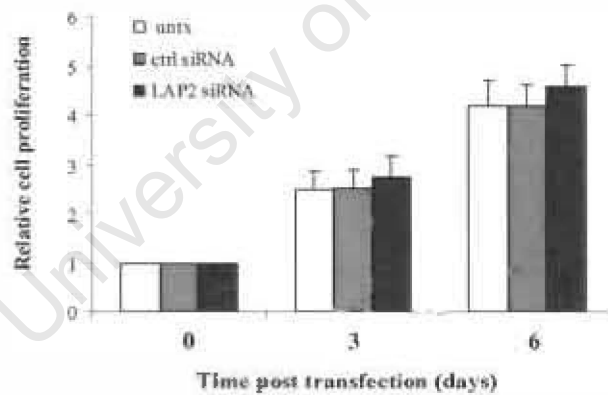


Figure 4.3: Effect of LAP2 inhibition on SVWI38 transformed fibroblast cell proliferation.

(A.) Western blot analysis showing LAP2 expression in SVWI38 fibroblast cells which were transfected with 20 nM control (ctrl) or LAP2 siRNA and protein harvested at zero, three and six days post transfection. (B.) SVWI38 cell proliferation was determined three and six days post transfection using the MTT cell proliferation assay and values expressed relative to Day 0. Experiments were performed in triplicate at least two independent times and results shown represent the mean \pm SD.

4.2.2.2 Effects of LAP2 inhibition on cell proliferation under various conditions of stress

The benefits of treating cancer with combination therapies to synergistically enhance the activities of individual treatments are becoming increasingly evident. As an example, the use of radiation therapy together with chemotherapeutic agents has been shown to improve survival associated with cervical cancer (Moore, 2003). While inhibiting LAP2 alone did not appear to have a significant effect on the proliferation of cervical cancer cells, we next investigated whether inhibition of LAP2 may enhance the growth inhibitory effect observed when cancer cells are placed under stressful conditions; including (1) serum stress, (2) stress induced by chemotherapeutic agents and (3) stress induced by anchorage-independent conditions.

(i) LAP2 inhibition and serum stress

One of the recognised phenotypes of cancer cells is that they possess a self-sufficiency in growth signals (Hanahan and Weinberg, 2000) and can thus proliferate under low serum conditions. This is however, a stress on the cells and they proliferate substantially slower than cells grown in growth factor supplemented medium. We wanted to determine whether inhibiting the expression of LAP2 would have an effect on cell proliferation under serum stress.

CaSki cells were grown in 2, 4 or 10% foetal calf serum containing medium, and cell proliferation monitored over five days. Cells grown in 10% serum proliferated the fastest,

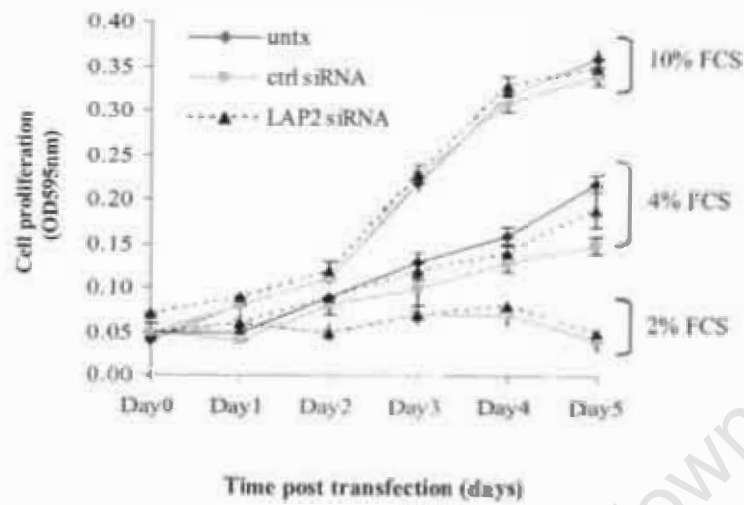
as expected, followed by cells grown in 4% serum. No proliferation was observed for cells grown in 2% serum. Under all three conditions there was no difference in the rate of proliferation between the untransfected, control and LAP2 siRNA transfected cells (Fig. 4.4.A). CaSki cells were also serum starved for 24 hours prior to plating in 10% serum to determine whether this alternative form of serum stress, and synchronization of cells within the G₀ phase, would have an effect on cell proliferation following LAP2 inhibition. The rates of cell proliferation between untransfected and control and LAP2 siRNA transfected cells did not differ under these conditions, suggesting that LAP2 inhibition does not enhance the growth inhibitory effect of serum depletion. We next determined whether inhibiting LAP2 expression could enhance the growth inhibitory effect of other stresses including; response to chemotherapeutic drugs and anchorage-independent growth conditions.

(ii) Cell proliferation in response to LAP2 inhibition and chemotherapeutic drug treatment

The effect of two apoptosis-inducing agents, 5-fluorouracil (5-FU) and *cis*-diaminedichloroplatinum (CP), in combination with LAP2 inhibition, was used to determine whether LAP2 inhibition could enhance the cell death effect induced by these drugs.

5-FU is a S-phase-dependent, pyrimidine analogue, which inhibits the thymidylate synthetase enzyme which is important in DNA synthesis [(Langenbach *et al.*, 1972) and

A.



B.

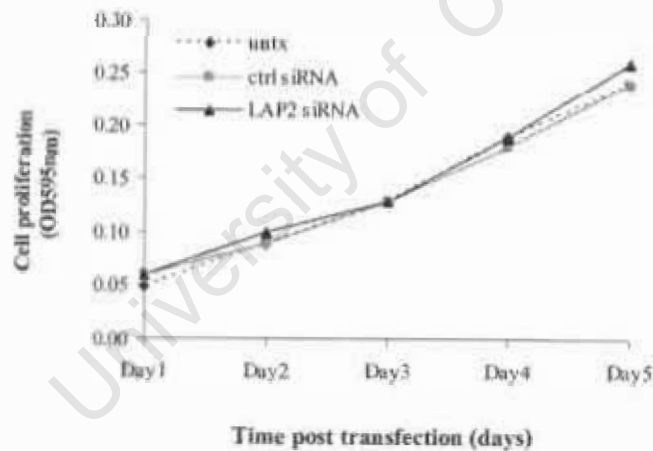


Figure 4.4; Effect of LAP2 inhibition on growth factor independent cell proliferation.

(A.) CaSki cells were either untreated (untx), or were transfected with 20 nM of control (ctrl) or LAP2 siRNA and plated in 2, 4 or 10% serum containing DMEM for the time indicated. Cell proliferation was determined by the addition of MTT reagent at each time point. (B.) CaSki cells were transfected as described above and serum starved for 24 hours prior to plating in 10% serum for the duration of the MTT cell proliferation assay. Experiments were performed in triplicate at least two independent times. Results shown represent the mean \pm SD.

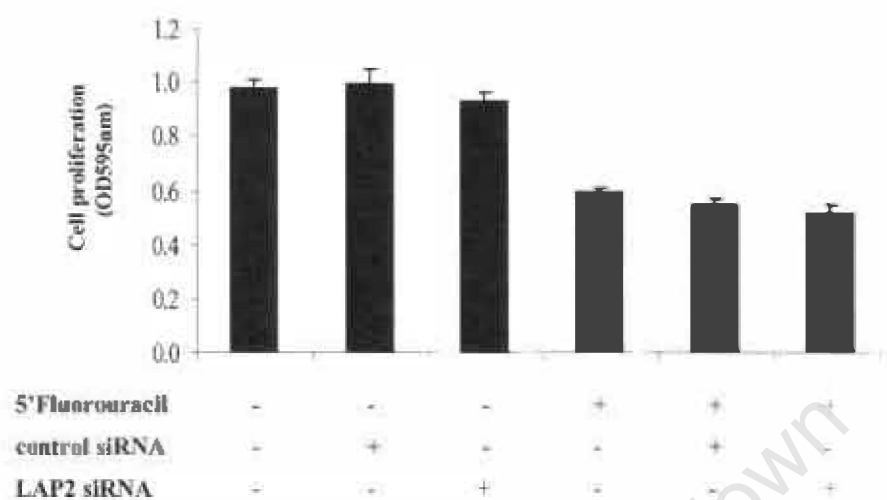
(Houghton *et al.*, 1979)] and is a chemotherapeutic agent commonly used in the treatment of cervical cancer (Moore, 2003). CP, also widely used in the treatment of cervical cancer (Tattersall, 2001), is known to bind DNA and form adducts, which affects the conformation of DNA-associated proteins, thereby preventing the repair of damaged DNA (Zamble *et al.*, 1996).

CaSki cells were transfected with control or LAP2 siRNA and incubated for 48 hours prior to treatment with 5'FU or CP for 96 hours. A concentration of 1 μ M 5'FU and 86 nM CP was found to inhibit CaSki cell growth by approximately 40-50% (Fig. 4.5.A and B). Our results show that inhibiting LAP2 expression does not alter the cell death effect of both 5'FU and CP alone (Fig. 4.5.A and B).

(iii) Cell proliferation in response to LAP2 inhibition under conditions of anchorage-independent cell growth

The ability of cancer cells to proliferate in the absence of a substrate (i.e. in an anchorage-independent manner) is a phenotype observed in cancer cells and is an indication of tumorigenicity (Shin *et al.*, 1975). It is nonetheless a stress condition in tissue culture systems, and the cells proliferate far slower than they would under adherent growth conditions. To grow cells under anchorage-independent conditions, cells are plated on poly-HEMA-coated dishes, which prevents cell adhesion to the culture vessels.

A.



B.

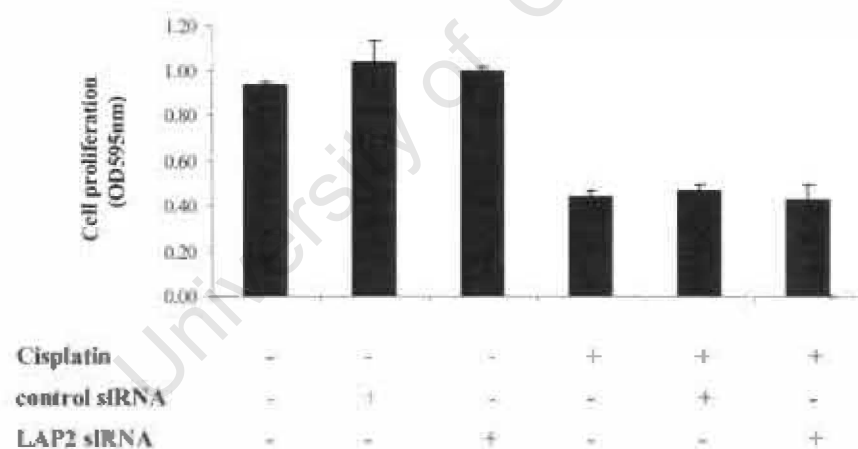


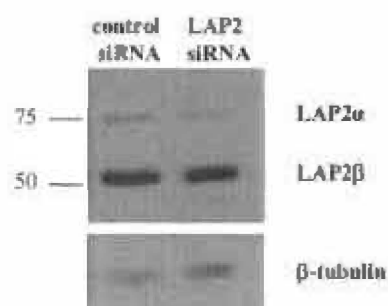
Figure 4.5: Effect of LAP2 inhibition on chemotherapeutic drug sensitivity.

(A.) CaSki cells were transfected with 20 nM of control or LAP2 siRNA. Cells were treated with 1 μ M 5'fluorouracil 48 hours later and cell proliferation measured by the addition of MTT reagent 96 hours post drug treatment. (B.) CaSki cells were transfected with 20 nM of control or LAP2 siRNA. Cells were treated with 86 nM Cisplatin 48 hours later and cell proliferation measured by the addition of MTT reagent 96 hours post drug treatment. Results shown represent the mean \pm SD for experiments performed in triplicate at least two independent times.

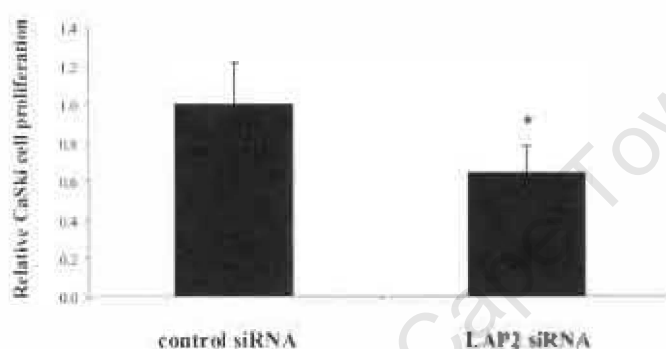
To determine whether LAP2 is required for cancer cell proliferation under anchorage-independent growth conditions, cells were re-suspended in 1% methylcellulose and plated on poly-HEMA-coated 96-well plates and both cell proliferation and colony formation determined. As anchorage-independent cells proliferate at a slower rate than adherent cells, proliferation and colony formation was monitored at eight days. To ensure that LAP2 protein expression was still inhibited eight days post LAP2 siRNA transfection, western blot analysis from control and LAP2 siRNA transfected cells was performed (Fig. 4.6.A). Anchorage-independent cell proliferation, as measured by MTT assay, revealed that inhibition of LAP2 in both CaSki and HeLa cells resulted in a significant decrease in cell proliferation compared to control siRNA transfected cells (Fig. 4.6.B and C). Although significant, LAP2 inhibition resulted in an approximate 30-50% inhibition in anchorage-independent cell proliferation, possibly due to the fact that 100% inhibition of LAP2 with siRNA was not achieved in transient transfection.

In addition to measuring anchorage-independent proliferation by MTT assay, the colonies formed by control and LAP2 siRNA treated cells were viewed by microscopy. Colonies from control and LAP2 siRNA transfected cells were stained with *p*-iodo-nitrotetrazolium and photographed under brightfield microscopy (Fig. 4.7). The results show that colonies derived from control siRNA transfected cells appear larger than colonies derived from LAP2 siRNA transfected cells (Fig. 4.7.1). The colonies from control cells also had a more defined, regular shape compared to the LAP2 knockdown colonies which were amorphous and less defined (Fig. 4.7.2), suggesting that some of these cells were stressed, resulting in the cell death observed.

A.



B.



C.

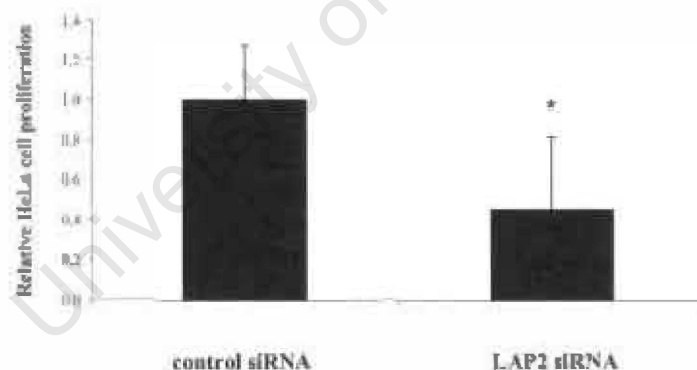


Figure 4.6: Effect of LAP2 inhibition on anchorage-independent cell proliferation. (A.) CaSki cells were transfected with 20 nM control or LAP2 siRNA and protein harvested eight days post transfection. LAP2 protein levels were determined by western blot analysis. (B.) CaSki cells were transfected with either 20 nM control or LAP2 siRNA and re-suspended in 1% methylcellulose prior to plating on poly-HEMA-coated 96-well plates. Cell proliferation was measured eight days post transfection using MTT reagent. (C.) HeLa cells were similarly treated and resulting cell proliferation measured. Results shown represent the mean \pm SD for experiments performed in triplicate, and repeated at least three independent times (*, p-value < 0.05).

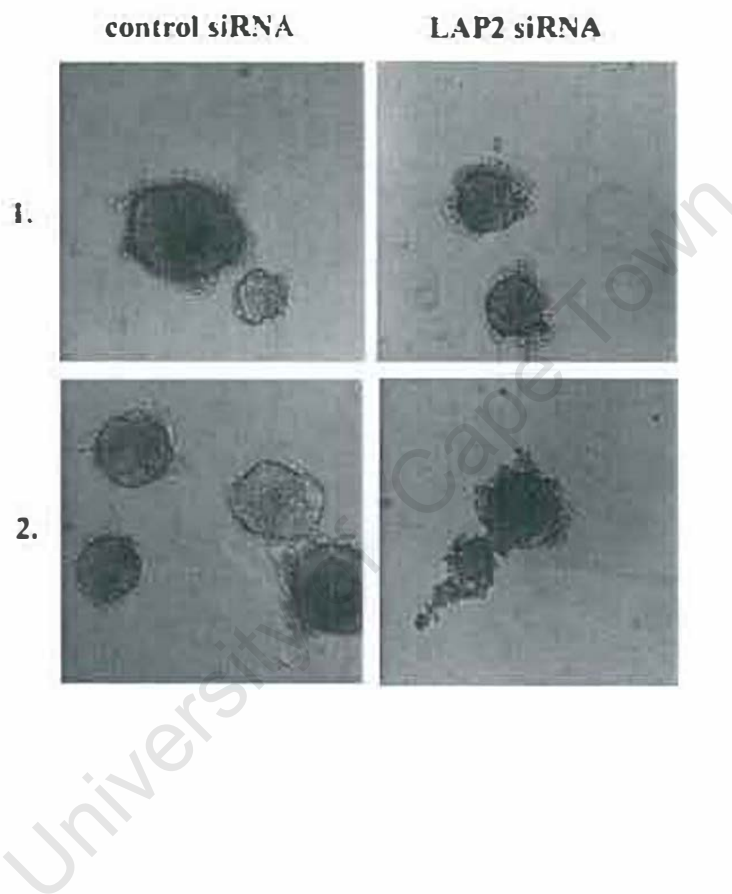


Figure 4.7: Effect of LAP2 inhibition on colony formation.

CaSki cells were transfected with either 20 nM control or LAP2 siRNA, suspended in 1% methylcellulose and plated on poly-HEMA-coated 96-well plates. Eight days post transfection colonies were stained with 1 mg/ml *p*-iodo-nitrotetrazolium for 16 hours at 37°C after which the colonies were photographed under brightfield microscopy at 400x magnification on a Moticam camera. Two fields of view were taken and are denoted 1. and 2. Experiments were performed in triplicate and repeated at least two independent times.

4.2.2.3 Effects of LAP2 inhibition on cell cycle regulatory protein expression

Since inhibiting the expression of LAP2 under anchorage-independent growth conditions resulted in a growth inhibitory effect, we next determined the expression of several cell cycle regulatory proteins that may associate with the observed phenotype. Cell cycle progression is regulated by the complex of cyclins with, either cyclin dependent kinases (CDKs), or cyclin dependent kinase inhibitors (CKIs), which activate cell cycle progression, and initiate cell cycle arrest respectively.

Protein was harvested from control and LAP2 siRNA transfected cells grown under anchorage-independent conditions, and cell cycle protein expression determined by western blot analysis. LAP2 protein expression was confirmed to be decreased in LAP2 siRNA transfected cells (Fig. 4.8). Western blot analysis for the cell cycle proteins; CDK4, cyclin D1, p16, p27, p21 and Rb was performed. While CDK4 and p27 levels remained unchanged in response to LAP2 inhibition, p21, and Rb levels appeared partially inhibited. Cyclin D1 levels decreased and p16 levels increased substantially (Fig. 4.8). The observed decrease in Rb levels is further investigated in Chapter 5. As cyclin D1 levels were decreased, and p16 levels increased, this suggested that the cells may show changes in the cell cycle.

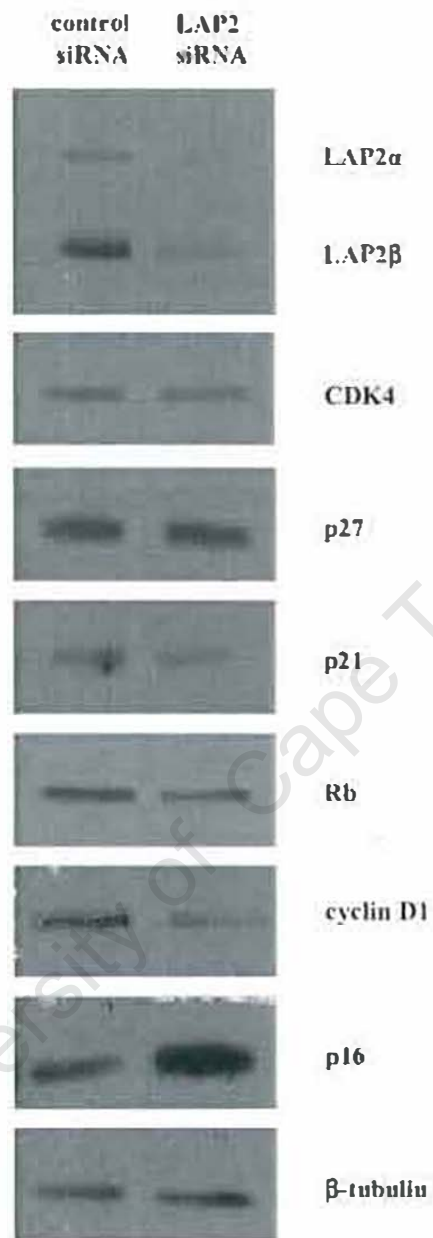


Figure 4.8: Effect of LAP2 inhibition on growth regulatory protein expression. CaSki cells were transfected with either 20 nM control or LAP2 siRNA and re-plated on poly-HEMA-coated dishes to prevent cell adhesion. Protein was harvested and subjected to western blot analysis. LAP2, CDK4, p27, p21, Rb, cyclin D1 and p16 expression was determined using specific antibodies. β -tubulin was included to control for protein loading. Results shown are representative of experiments performed at least two independent times.

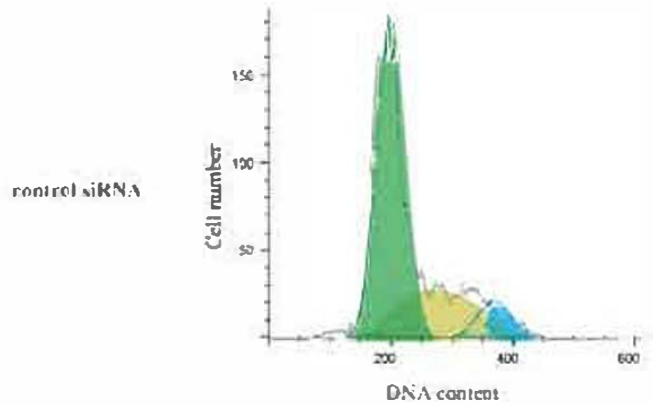
4.2.2.4 Effects of LAP2 inhibition on cell cycle progression under anchorage-independent growth conditions

Inhibition of LAP2 expression under anchorage-independent growth conditions showed that there was an approximate thirty to fifty percent decrease in cell proliferation, and an alteration in expression of cell cycle regulatory genes (cyclin D1 and p16), suggesting a possible alteration in the cell cycle profile of LAP2 knockdown cells. Cell cycle analysis was thus performed using fluorescent activated cell sorting (FACS). Propidium iodide staining was used to measure the DNA content of cells in order for DNA histograms, representative of the cell cycle, to be generated (Krishan, 1975). The cell cycle profile was analysed using FlowJo and ModFit software according to the Dean-Jett-Fox model [(Dean *et al.*, 1974) and (Fox, 1980)], which fits a smooth curve to the S-phase. Background, cell aggregates and cell debris were gated out for analysis.

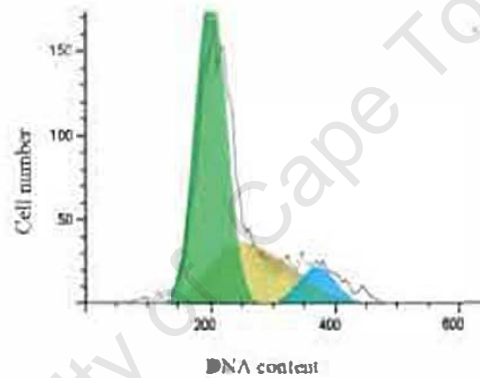
Control or LAP2 siRNA transfected CaSki cells grown in an anchorage-independent manner, were analysed eight days post transfection, the time point at which the growth inhibitory effect was observed. Surprisingly, analysis of the cell cycle profile, when LAP2 expression was inhibited, revealed a similar cell distribution within each phase of the cell cycle compared to controls (Fig. 4.9.A). Quantitative analysis of the cell cycle data showed no significant difference in the percentage of cells within each phase of the cell cycle in control and LAP2 siRNA transfected cells (Fig. 4.9.B).

This data suggests that LAP2 knockdown cells cycle normally, indicating that the decrease in cell proliferation observed, was not as a result of cell cycle arrest. However,

A.



LAP2 siRNA



B.

phase	control siRNA	LAP2 siRNA
% G ₁	59.98 (±1.27)	60.00 (±1.09)
% S	38.13 (±2.69)	37.86 (±1.51)
% G ₂ /M	1.38 (±1.46)	2.13 (±1.63)

Figure 4.9: Effect of LAP2 inhibition on the cell cycle profile.

(A.) The cell cycle distribution of CaSki cells grown under anchorage-independent conditions was determined by flow cytometry eight days following transfection with 20 nM control or LAP2 siRNA. Cells were stained with propidium iodide and the cell cycle profile captured using Cellquest software on a BD Biosciences FACS Calibur and data analysed with FlowJo and ModFit software. (B.) Quantitation of the percentage of cells within each phase of the cell cycle. (G₁; gap phase 1, S; DNA synthesis, G₂; gap phase 2, M; mitosis). Experiments were performed in triplicate and repeated at least two independent times. Results represent the mean ± SD.

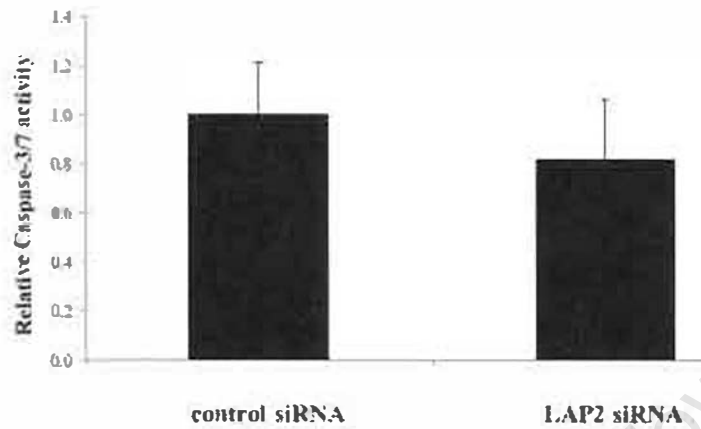
only cycling cells were included in analyses, and it is possible that the decrease in proliferation observed, may have been as a result of cell death.

4.2.2.5 Effects of LAP2 inhibition on cell death through apoptosis in cells grown under anchorage-independent growth conditions

In order to further investigate whether the inhibition of anchorage-independent proliferation observed in LAP2 knockdown cells could be due to cell death induced by apoptosis, a quantitative apoptosis assay, measuring this particular mode of programmed cell death, was performed. Cysteiny l aspartate-specific proteinases, or caspases, are a family of proteases that are essential for mediating the apoptotic phenotype through the targeted cleavage of specific substrates (reviewed in Nicholson and Thornberry, 1997). A commercially available caspase activity assay was thus used to assess the activity of the caspase-3 and -7 enzymes using a luminogenic tetrapeptide substrate characteristic of *in vivo* cleavage sites. Luminescence released is proportional to substrate cleavage by the caspase enzymes and hence apoptotic activity. Caspase activity is known to be an early and transient event in apoptosis (reviewed in Elmore, 2007) and thus caspase activity was measured seven days post LAP2 siRNA transfection, slightly earlier than when the growth effect was observed.

There was no significant difference in caspase activity in control and LAP2 siRNA transfected CaSki cells grown under anchorage-independent conditions for seven days post transfection (Fig. 4.10.A). Caspase activity was also found to be equivalent in

A.



B.

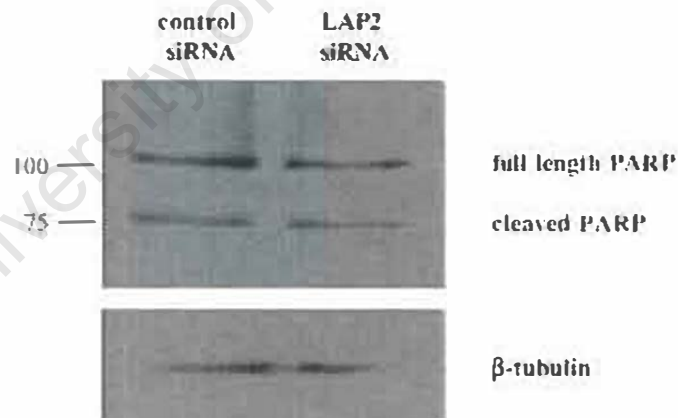


Figure 4.10: Effect of LAP2 inhibition on apoptosis.

(A.) Caspase-3/7 activity relative to protein concentration in control and LAP2 siRNA (20 nM) transfected CaSki cells grown under anchorage-independent conditions for seven days post transfection. (B.) Western blot showing PARP cleavage in CaSki cells transfected with control or LAP2 siRNA and plated under anchorage-independent growth conditions seven days post transfection. Results shown are representative of at least two independent experiments. Results shown represent the mean \pm SD.

control and LAP2 knockdown cells eight days post transfection (data not shown). The levels of poly (ADP-ribose) polymerase (PARP), a known caspase substrate (Nicholson *et al.*, 1995), were also independently determined. Cleavage of the 116 kD PARP protein into a 85 kD fragment has been shown to be an early event in apoptosis (Kauffman *et al.*, 1993). The cleaved 85 kD fragment was evident in both control and LAP2 siRNA transfected cells and no significant difference was observed (Fig. 4.10.B). Our results therefore cannot conclusively link the effect of LAP2 inhibition on anchorage-independent proliferation to an increase in cell death via apoptosis.

It is possible that the decrease in cell number in the LAP2 siRNA transfected cells grown under anchorage-independent conditions was due to an alternative mode of cell death such as necrosis. Necrosis typically results in disruption of the cell membrane leading to inflammation, whereas in the process of apoptosis the cell membrane remains intact and an inflammatory response is not induced (reviewed in Elmore, 2007). A more recently discovered mode of programmed cell death is autophagy, where the cell degrades cell components within its own lysosomes, and alternatively may have been the process responsible for the cell death observed. Due to limited time during the M.Sc. project, these possibilities could not be investigated prior to submission of this M.Sc. thesis.

4.3 Discussion

Having established that LAP2 is overexpressed in cancer samples, and has the potential for use as a cancer biomarker, the functional significance of this up-regulation in cancer cells was investigated. There are conflicting reports in the literature regarding the involvement of the LAP2 alpha isoform in cell proliferation. Pekovic *et al.* (2007) showed that inhibition of LAP2 α expression in human diploid fibroblasts (HDFs) resulted in a decrease in Ki67 staining, a cell proliferation marker, and induced delayed G₂M progression and eventual cell cycle arrest in the G₁ phase. This is in contrast to the work performed by Dornier *et al.* (2006), which showed that inhibiting the expression of LAP2 α in HeLa cells, resulted in an increase in cell proliferation, and in fact favoured cell cycle progression. These authors substantiated this finding by showing that LAP2 α binds to the C-terminus of Rb, and that LAP2 α expression affects transcription of E2F target genes, where overexpressed LAP2 α is shown to repress E2F target gene expression. Vlcek *et al.* (2002) also report that overexpression of LAP2 α results in the inhibition of cell proliferation and G₁/S cell cycle arrest. Our study on both LAP2 isoforms contradicts that of Dornier *et al.* (2006) and Vlcek *et al.* (2002) and appears to be in line with that of Pekovic *et al.* (2007) supporting a hypothesis that LAP2 levels associate with cell proliferation (albeit anchorage-independent).

In our study, it was only under anchorage-independent growth conditions that a growth phenotype change was observed when LAP2 expression was inhibited. This could be due to the fact that different signalling pathways are activated under anchorage-independent

growth conditions (Uttamsingh *et al.*, 2003). Alterations in gene expression have also been reported between adherent and non-adherently growing cells (Kinoshita *et al.*, 2003).

While we have not conclusively identified the mechanism through which LAP2 inhibition results in cell death, our data showing up-regulation of the tumour suppressor, p16 and decreased cyclin D1 expression, suggest that this may have been a cell cycle inhibitory response that we may have missed. As an asynchronously growing population of cells was analysed, it may have been more appropriate to synchronise the cells prior to cell cycle analysis, as the growth difference observed may have been too marginal to register in an asynchronous population of cells.

Because only cycling cells were included for analysis, we postulated that perhaps there was an increase in cell death in the LAP2 knockdown cells, which would not be evident on the cell cycle profile as the sub G₁ peak, representative of cell doublets and debris, was excluded. Two independent apoptosis assays, however revealed no significant difference in apoptosis between control and LAP2 siRNA transfected cells. The decrease in cell number in LAP2 inhibited cells could possibly be as a result of necrosis or autophagy; this forming the basis of future work.

While there are corresponding reports on the functions of LAP2 β , there is contradictory data in the literature around LAP2 α 's exact role in the cell as it seems to perform conflicting functions. Parise *et al.* (2006) identified an E2F inducible antisense transcript

expressed from the first intron of the LAP2 gene. They suggest that given that LAP2 α plays a role in diverse, often contradictory, cellular processes, it is likely that its expression is tightly regulated, and this could be due, in part, to the antisense transcript which can form double-stranded RNA hybrids, resulting in the formation of regulatory siRNA molecules. The LAP2 α isoform is unique to mammals, and is not evolutionarily conserved (Prufert *et al.*, 2004), hinting at the fact that if its expression is inhibited, other proteins may compensate for loss of its expression. While the beta isoform is conserved across several species, a recent study has shown redundancy between inner nuclear membrane proteins such as LAP2 β and the lamin B receptor (LBR) (Anderson *et al.*, 2009). This may explain why inhibition of LAP2 expression did not seem to have a dramatic effect on the cells.

In summary, the current study indicates that while LAP2 has the potential to be a cancer biomarker, it may have limited use as a therapeutic target as inhibition of its expression only resulted in a marginal decrease in anchorage-independent cell proliferation.

Future work that is of interest would be to overexpress LAP2 in a normal cell line and determine the effects on the biology of the cell, as it is elevated levels that are found in cancer cells, and it is possibly only when its expression is increased, can effects be seen. The structural role of LAP2 in nuclear dynamics and post-mitotic nuclear assembly in cancer cells is also of interest. It could be in these processes that LAP2's overexpression in cancer becomes relevant.

Chapter 5: Effect of LAP2 inhibition on Rb expression levels

5.1 Introduction

The retinoblastoma protein (Rb) is an important tumour suppressor, which plays a critical role in regulating cell cycle progression (reviewed in Nevins, 2001). It functions at the G₁/S transition point where, in its hypophosphorylated state, it binds to, and inactivates the transcription factor, E2F (Mittnacht and Weinberg, 1991). When cells in the G₁ phase receive growth promoting signals, cyclin D1 binds to and activates CDK4, which in turn phosphorylates Rb. This causes the induction of cyclinE-CDK2 complexes and results in hyperphosphorylation of Rb, targeting it for degradation; resulting in the release of E2F, thereby stimulating the transcription of S-phase genes and progression into S-phase (Diagram 5.1). Inactivation or targeted degradation of Rb in cancer is thus often integral to the uncontrolled proliferation characteristic of cancer cells. In cervical cancer, the HPV encoded E7 oncoprotein targets the Rb protein for degradation (Boyer *et al.*, 1996), while in oesophageal cancer the Rb encoding gene is often mutated (Huang *et al.*, 1993). In addition to the role of Rb in regulating cell cycle progression, it has also been shown to be important in the processes of senescence (Shay *et al.*, 1991) and differentiation (reviewed in Korenjak *et al.*, 2005).

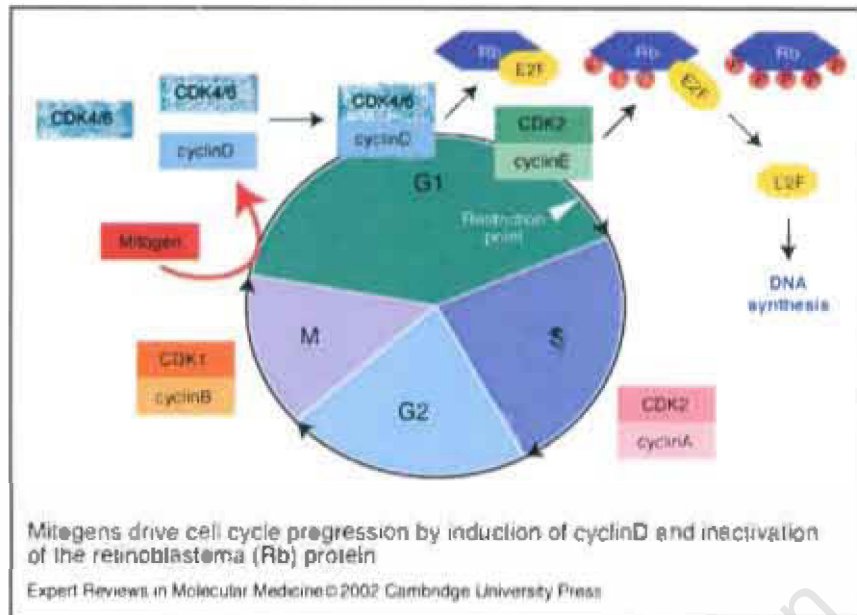


Diagram 5.1: The eukaryotic cell cycle and the role of the Rb tumour suppressor protein. The G₁ (gap 1), S (DNA synthesis), G₂ (gap 2) and M (mitosis) phases of the cell cycle are represented as a pie chart with sections indicating relative time spent within each phase. G₁ cells, upon stimulation by mitogens, cause cyclin D1 to bind to, and activate CDK4, which phosphorylates Rb. This causes cyclin E to bind CDK2 and hyperphosphorylate Rb, resulting in the release of E2F and allowing progression into S-phase. The cyclinA-CDK2 complex functions within the S-phase and the cyclin B-CDK1 complex within mitosis (Kolch *et al.*, 2002).

Rb has also been shown to interact with A-type lamins as well as the LAP2 alpha isoform, and it has been suggested that it is through these interactions that Rb is tethered in the nucleus enabling it to perform its regulatory functions [(Johnson *et al.*, 2004) and (Markiewicz *et al.*, 2002)]. However, the exact nature of the interaction between LAP2 alpha and Rb, and the functional relevance thereof is not clear. The interaction between Rb and LAP2 α occurs within the unique C-terminal tail of the alpha isoform (Markiewicz *et al.*, 2002), and there is thus no evidence in the literature to suggest an association between Rb and LAP2 β .

Having observed a decrease in Rb expression when LAP2 is inhibited, the aim of this chapter was to investigate this observation further. Our results indicate that while

inhibition of LAP2 has no effect on Rb mRNA levels, it does result in a more rapid degradation of the Rb protein.

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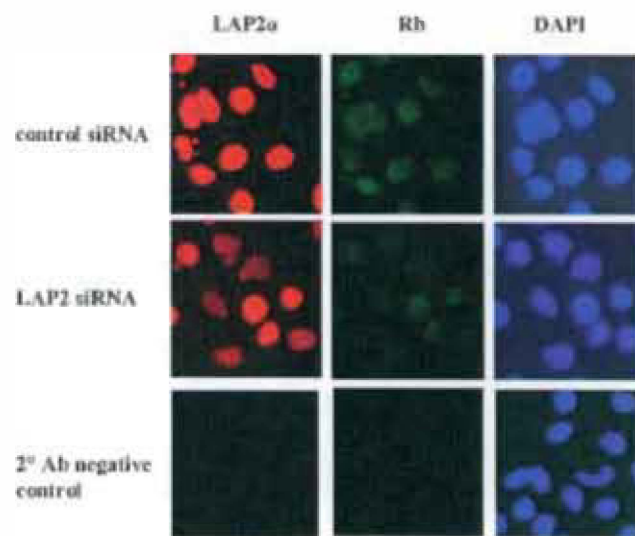
5.2 Results

5.2.1 Effects of LAP2 inhibition on Rb protein expression

As Rb is a protein associated with growth regulatory mechanisms (Nevins, 2001), we had previously (Chapter 4) monitored its expression in response to LAP2 inhibition. Interestingly, our results indicated that inhibition of LAP2 expression resulted in an associated decrease in Rb levels. This observation was therefore further explored by transiently transfecting CaSki cells with control or LAP2 siRNA and Rb expression determined when LAP2 was inhibited. Immunocytochemistry was performed on paraformaldehyde-fixed LAP2 siRNA transfected cells, where LAP2 α expression was visible in the Cy3 fluorescent channel and Rb in the Alexa488 channel (Fig. 5.1.A). Our results showed that in cells in which LAP2 had effectively been inhibited, a corresponding decrease in the levels of Rb was observed. As this was a transient LAP2 siRNA experiment, not all cells in the panel showed LAP2 inhibition. Thus shown in this panel are cells where LAP2 was not inhibited, and it is notable that these cells have correspondingly higher levels of Rb. Quantification of the fluorescent images revealed a significant decrease in Rb levels when LAP2 was inhibited (Fig. 5.1.B).

This finding was independently confirmed by western blot analysis, where a timecourse following transfection with LAP2 siRNA showed a corresponding reduction in Rb expression (Fig. 5.2.A).

A.



B.

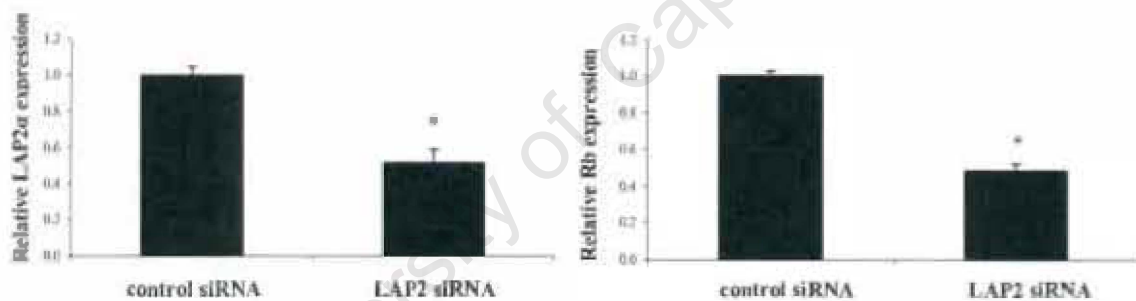


Figure 5.1: Effect of LAP2 inhibition on Rb protein expression.

(A.) Immunofluorescence showing LAP2 α and Rb expression in control and LAP2 siRNA transfected cells paraformaldehyde-fixed four days post transfection. A Cy3-conjugated goat anti-rabbit secondary antibody was used to detect the LAP2 α specific primary antibody and an Alexa488-conjugated goat anti-mouse secondary antibody was used to detect Rb. DAPI staining indicates cell nuclei. A 2° Ab negative control (secondary antibody only control) was included to show specificity of the fluorescently tagged secondary antibodies. Images were captured on a Zeiss fluorescence microscope at 400x magnification using Axiovision 4.6 software. (B.) Quantitation of LAP2 α and Rb expression in at least 100 LAP2 siRNA transfected cells was performed using Axiovision 4.6 software. Results shown represent the mean \pm SD for experiments repeated at least two independent times. (*, p-value < 0.05).

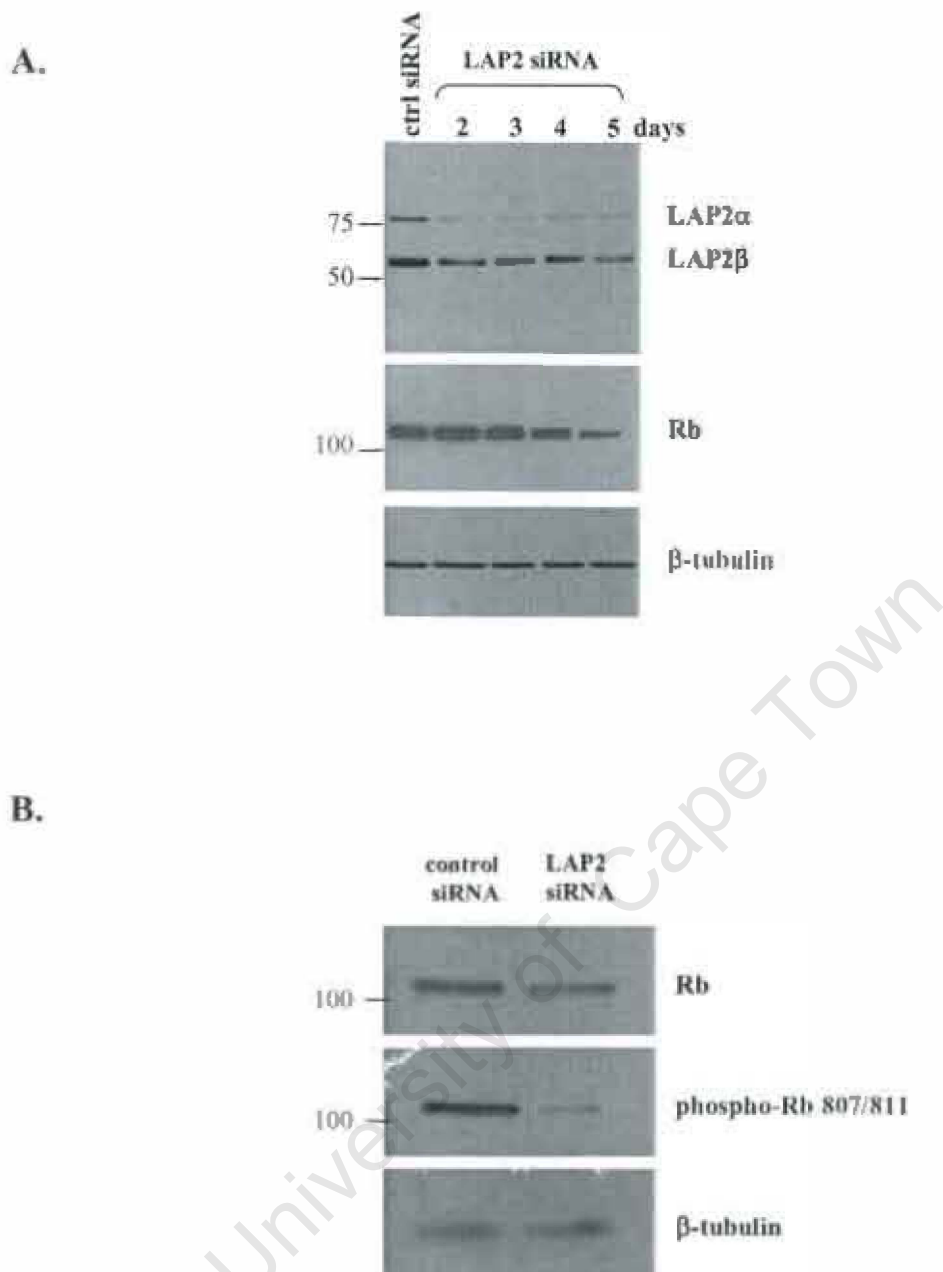


Figure 5.2: Effect of LAP2 inhibition on Rb protein expression.

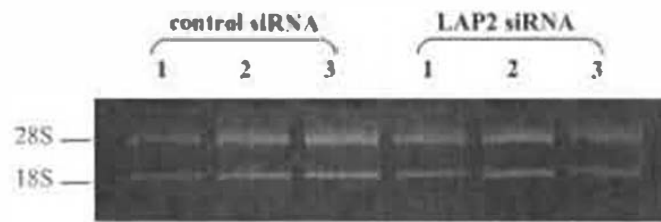
(A.) Rb expression following LAP2 siRNA transfection. Protein was harvested at two, three, four and five days post transfection and analysed by western blot analysis for Rb expression. β -tubulin was included as a control for protein loading. (B.) Rb and phospho-Rb levels in LAP2 knockdown cells. Protein was harvested from cells five days post control or LAP2 siRNA transfection and Rb levels determined by western blot analysis. A phospho-Rb antibody recognising the 807 and 811 phosphorylated serine residues was used as an indication of phospho-Rb levels. β -tubulin was included as a protein loading control. Results shown are representative of experiments performed at least two independent times.

Rb can either be in a phosphorylated or unphosphorylated state. In order to determine whether LAP2 inhibition alters the phosphorylation status of Rb, an antibody targeting the 807 and 811 serine residues (RbS807/811), which are known Rb phosphorylation sites, was used in western blot analysis. CaSki cells were transfected with control or LAP2 siRNA, protein extracted, and Rb and phospho-Rb levels determined. Concomitant with the decrease in total Rb levels, was a decrease in phospho (S807/811) Rb (Fig. 5.2.B).

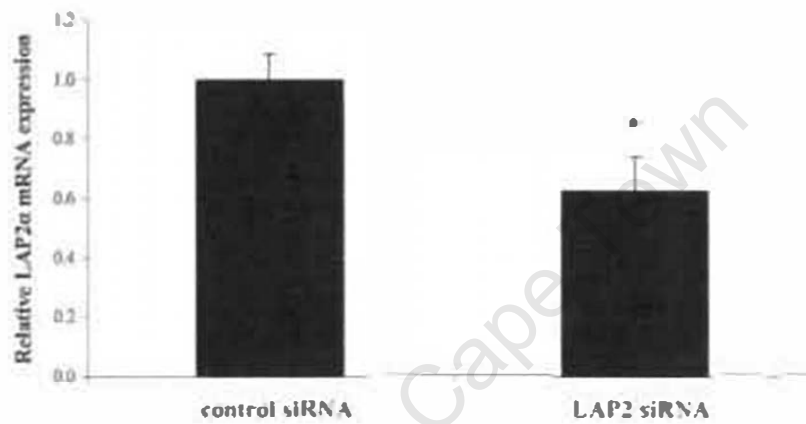
5.2.2 Effects of LAP2 inhibition on Rb mRNA expression

Having observed that Rb protein levels were reduced when LAP2 expression was inhibited, we investigated whether LAP2 inhibition resulted in a transcriptional change in Rb expression. In order to ascertain this, Rb mRNA levels were determined in LAP2 knockdown cells. RNA was extracted from control and LAP2 siRNA transfected cells and RNA integrity confirmed on a formaldehyde-agarose gel, where a 2:1 ratio of 28S to 18S rRNA and an absence of smearing indicated good quality RNA (Fig. 5.3.A). RNA was subsequently reverse transcribed into cDNA and expression of LAP2 α and Rb determined relative to the cyclophilin housekeeping gene by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2000) by Real-time RT-PCR analysis. Transfection with LAP2 siRNA resulted in a significant decrease in LAP2 α mRNA levels (Fig. 5.3.B), while no difference in Rb mRNA expression was observed under these conditions (Fig. 5.3.C). These results indicate that LAP2 does not regulate Rb at a transcriptional level, as there was no change in mRNA expression, and suggest a post-transcriptional regulation of Rb expression in LAP2 inhibited cells.

A.



B.



C.

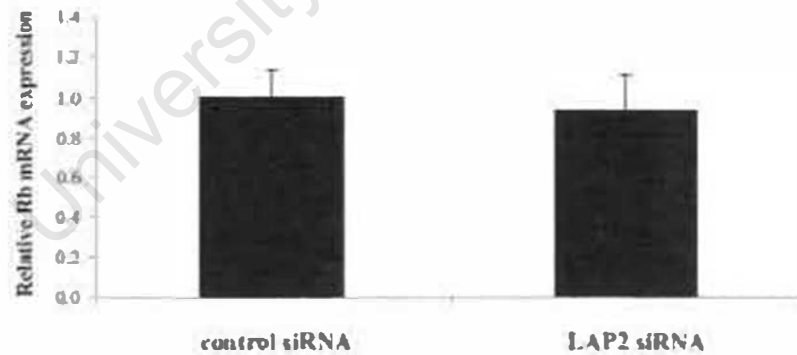


Figure 5.3: Effect of LAP2 inhibition on Rb mRNA expression.

(A.) RNA was extracted from control and LAP2 siRNA transfected CaSki cells in triplicate four days post transfection and RNA integrity determined on a 1.5% formaldehyde-agarose gel. 28S and 18S are representative of ribosomal subunits. (B.) cDNA was synthesized from RNA obtained from control and LAP2 siRNA transfected cells and LAP2a mRNA expression was quantitatively determined relative to the cyclophilin house-keeping gene by Real-time RT-PCR analysis using the $2^{-\Delta\Delta CT}$ method. (C.) Quantitation of Rb mRNA expression relative to cyclophilin as described in (B.). Experiments were performed in triplicate and repeated at least two independent times. Results shown are the mean \pm SD (*, p-value < 0.05).

5.2.3 Effects of LAP2 inhibition on Rb protein stability

There is evidence in the literature to suggest that Lamin A affects the protein stability of Rb (Johnson *et al.*, 2004), and since LAP2 α is known to interact principally with Lamin A (Dechat *et al.*, 2000a) and Rb (Markiewicz *et al.*, 2002), we next determined whether LAP2 plays a role in regulating Rb protein stability which could explain why protein, and not mRNA levels, were reduced when LAP2 was inhibited. In order to test this theory, cells were transfected with either control or LAP2 siRNA for 48 hours and treated with 50 μ g/ml cycloheximide (CHX) to inhibit new protein synthesis. Protein was harvested at various time points following LAP2 inhibition and CHX treatment and Rb and β -tubulin protein levels determined by western blot analysis (Fig. 5.4.A). After treatment with CHX for the indicated times, a decrease in Rb expression in both control and LAP2 inhibited cells was observed (Fig. 5.4.A). This decrease appeared to be to a greater extent in the LAP2 knockdown cells compared to the control cells. Rb expression was quantitated relative to β -tubulin by densitometric analysis of the western blot band intensity and the values expressed relative to the values at Day 0, to ensure that protein stability, and not expression, was being measured. Log values were plotted on a scatter plot and a linear trendline fitted to the data points (Fig. 5.4.B). The results show that Rb was less stable in LAP2 siRNA transfected cells, as the gradient of the LAP2 siRNA graph is steeper than that of the control siRNA graph, and extrapolation to a point where there is no Rb expression, would occur at an earlier time point. As log values were plotted, the following equation was used to quantitatively determine the half-life ($t_{1/2}$) of the Rb protein under these conditions: $t_{1/2} = \log 2/m$ where $m = (y-c)/x$ on a linear graph. The half-life of Rb in control transfected cells was calculated to be ~ 13 hours while the half-life in LAP2 siRNA transfected cells was ~ 7 hours, indicating that Rb was less stable, and

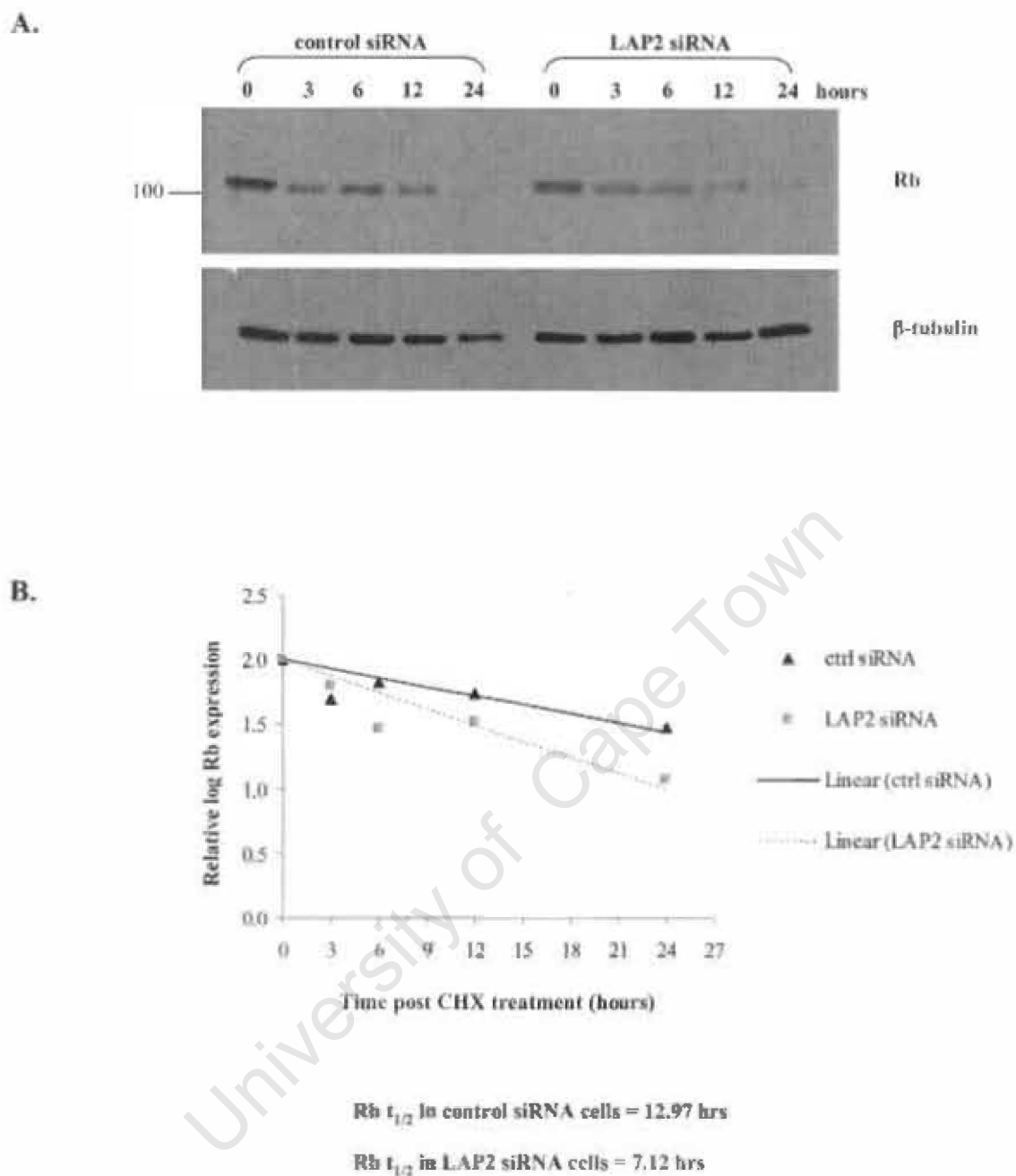


Figure 5.4: Effect of LAP2 inhibition on Rb protein stability. (A.) CaSki cells were transfected with 20 nM control or LAP2 siRNA and incubated for 48 hours prior to treatment with 50 μ g/ml cycloheximide (CHX). Protein was harvested at 0, 3, 6, 12 and 24 hour intervals following CHX treatment and Rb levels determined by western blot analysis. (B.) Quantitation of Rb expression relative to β -tubulin. Densitometric analysis of western blot band intensity was performed using GeneTools software and the half-life of Rb calculated based on the gradients of the two graphs. Results shown are representative of at least two independent experiments.

more rapidly degraded, when LAP2 was inhibited. This data suggests that LAP2 plays a potential role in Rb protein stability and degradation.

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5.3 Discussion

Our observation that LAP2 inhibition results in decreased Rb levels is in agreement with that made by Pekovic *et al.* (2007). These authors also analysed phospho-Rb (RbS780) expression when LAP2 α was inhibited, and found an even greater decrease in expression of this phospho-form. This paper also shows that LAP2 α expression affects the localisation of any remaining Rb, where inhibition of LAP2 α expression causes the phospho-form, RbS795, levels to remain unchanged, but rather its distribution is re-localised from the nuclear compartment to nuclear speckles. We similarly showed a decrease in Rb and phospho-Rb (RbS807/811) levels in LAP2 inhibited cervical cancer cells. To date, no further studies on the mechanism behind this observed decrease in Rb expression when LAP2 is inhibited have been reported.

To determine whether LAP2 was involved in regulating Rb at a transcriptional level, Real-time RT-PCR analysis was performed to determine Rb mRNA levels in LAP2 knockdown cells. Our results revealed that Rb mRNA transcript levels were not altered, suggesting LAP2 regulates Rb expression at a post-transcriptional level. It has been shown that LAP2 α binds Lamin A (Dechat *et al.*, 2000a) and that together they can form a complex with Rb (Markiewicz *et al.*, 2002). Work by Johnson *et al.* (2004) showed that inhibiting Lamin A expression resulted in a decrease in Rb levels. They found that this decrease in expression was as a result of proteasomal degradation of Rb, and that Lamin A plays a role in mediating Rb stability. This association was found to be functionally relevant as the stabilisation of Rb by A-type lamins was shown to be necessary for p16-induced cell cycle arrest (Nitta *et al.*, 2006).

The work on Lamin A's involvement in Rb stability indicated that this proteasome-dependent degradation of Rb occurs independently of Gankyrin and MDM2, which are known mediators of Rb degradation (Nitta *et al.*, 2007). However, interestingly LAP2 α has been shown to associate with the B and C pockets of Rb (Markiewicz *et al.*, 2002), and as these authors noted, this is the same site to which MDM2 binds (Xiao *et al.*, 1995). Together, these results suggest that LAP2 α is important for tethering Rb in the nucleus, and perhaps when LAP2 α is inhibited, the MDM2 site is available for MDM2 to bind, and Rb is targeted for degradation resulting in a decrease in Rb protein levels. The involvement of MDM2 in Rb stability, when LAP2 α is inhibited, could in future experiments, be determined by overexpressing p14, a CDK inhibitor known to inhibit MDM2 function.

In summary, there seems to be an association between LAP2 and Rb expression. It is proposed that LAP2 plays a role in stabilising the Rb protein, thereby preventing its degradation; however further studies are required to support this theory and to determine the significance of this interaction.

Chapter 6: Conclusion

6.1 Expression of LAP2 in cancer

Expression profiling to determine differences in gene expression between normal and cancer patient samples with the aim of finding novel tumour markers, is an important area of cancer research. These tumour markers can be used as a diagnostic tool if the change in expression is shown to be an early event in cancer development, or if only found to be expressed later in cancer development, can be used to assist prognosis and patient management or predict responses to cancer therapy (Kulasingam and Diamandis, 2008).

Earlier work in our laboratory has shown that there are a range of genes differentially expressed in cervical cancer tissue compared to normal tissue using such expression profiling technology (van der Watt *et al.*, 2009 and unpublished data). One of the cDNAs shown to be highly expressed in the cancer samples was one encoding the LAP2 α protein. This finding is in accordance with a similar expression profiling study performed by Rosty *et al.* (2005). The LAP2 gene encodes alpha, beta and gamma protein isoforms which interact with nuclear lamins; thereby playing a role in nuclear architecture, as well as nuclear dynamics (Gruenbaum, 2000).

The work in the present study validated the increase in LAP2 α expression observed following analysis of the microarray data. Real-time RT-PCR analysis performed from

cervical biopsy tissue, confirmed an increase in LAP2 α mRNA expression in the cancer tissue compared to normal tissue. Similarly, an increase in LAP2 α protein expression was observed following immunohistochemical staining of archival patient tissue sections. These findings were extrapolated to cell lines, where increased expression of both the LAP2 alpha and beta isoforms was observed in both cervical and oesophageal cancer cell lines, as well as transformed fibroblasts, indicating an association between LAP2 and cellular transformation.

This study is unique in validating the up-regulation of LAP2 expression in cervical cancer tissue. There have only been a few reports correlating LAP2's expression with cancer and this data is derived from large-scale genome-wide arrays [(Agrawal *et al.*, 2002), (LaTulippe *et al.*, 2002), (Welsh *et al.*, 2002), (Pomeroy *et al.*, 2002) and (Yokota *et al.*, 2004)]. Our study revealed an association between LAP2 and transformed and cancer cells and suggests that LAP2 may be a useful tumour marker. These findings are, however preliminary and a larger sample size will have to be used in order to predict clinical relevance.

6.2 LAP2 inhibition and cancer cell biology

While there have been a few genome-wide analyses identifying the LAP2 gene to be associated with transformed cells, to our knowledge no functional studies have clearly described LAP2's role in cancer. There is, in fact, controversy surrounding the role that LAP2 plays in normal cells. There are some indications that overexpression of the LAP2

apoptosis in LAP2 siRNA transfected cells, as shown by caspase and PARP assays, and it is thus likely that the cell death observed was as a result of necrosis or autophagy.

6.3 LAP2 inhibition and decreased Rb expression

Interestingly, we found that LAP2 inhibition resulted in a decrease in Rb expression. This is in agreement with a study by Pekovic *et al.* (2007) that suggested that there may be an association between LAP2 α and Rb. Work presented in this project showed a decrease in Rb protein expression in cells in which LAP2 was inhibited. It was also shown that LAP2 played a role in maintaining the stability of the Rb protein, as mRNA levels were unaffected in LAP2 knockdown cells; whereas treatment with a protein synthesis inhibitor, reduced the half-life of Rb in LAP2 knockdown cells. It is likely that the LAP2 alpha isoform was responsible for exerting these effects, as it has been shown to interact with Rb (Markiewicz *et al.*, 2002).

Inhibition of LAP2 α 's interaction partner, Lamin A, has also been shown to negatively affect Rb stability (Johnson *et al.*, 2004), and together these data suggest that this LAP2 α -LaminA complex is at least partly responsible for protecting Rb from degradation. This decrease in Rb expression did not seem to have an effect on cell proliferation which would have been expected, as if Rb is targeted for degradation, E2F would be free to activate transcription of S-phase genes. The role of LAP2 α in maintaining Rb stability, and the effects of this interaction on the cell cycle and in the cancer state, clearly requires further investigation.

The precise role of LAP2 in cancer cells is still debatable. The apparently contradictory data in the literature and found in our study suggests that LAP2's role in both normal and cancer cells, is a complex , and probably multifaceted one.

6.4 Concluding statement

In summary, this study has validated the up-regulation of LAP2 α in cervical cancer tissue compared to normal tissue as previously suggested by expression profile analysis. This up-regulation was shown at both the mRNA and protein levels in biopsy samples, as well as in various cancer and transformed cell lines. The data indicates an association between the expression of the LAP2 alpha and beta isoforms and cellular transformation, and suggests that LAP2 has potential for use as a cancer biomarker. The functional significance of this overexpression in cancer cells was not fully elucidated; however the study did suggest that there would be limited potential for the use of LAP2 as a therapeutic target, as inhibition of its expression only partially altered the proliferation rate of cancer cells used in this study.

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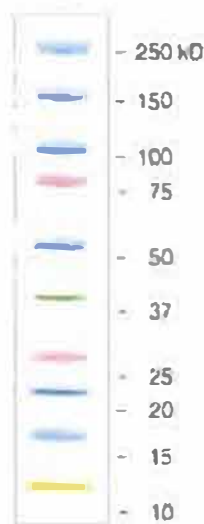
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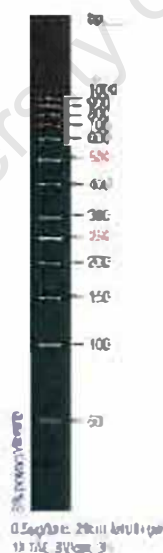
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Appendix A: Protein and DNA markers



A.1: Protein molecular weight marker: Precision Plus Protein Kaleidoscope Standards (Bio-Rad) used to determine the molecular weight of proteins subjected to polyacrylamide gel electrophoresis and western blot analysis.



A.2: DNA molecular weight marker: Generuler 50 bp ladder (Fermentas) used to determine the size of Real-time RT-PCR products.

Appendix B: Solutions

B.1 Tissue cell culture

10X PBS (500ml)

40 g NaCl
1 g KCl
5.75 g Na₂HPO₄·7H₂O
1g KH₂PO₄
Up to 500 ml with dH₂O

Heat Inactivated Foetal Calf Serum (FCS)

Heat FCS at 56°C for 30 minutes
Store at -20°C until use

Complete DMEM medium

450 ml Dulbecco's modified Eagle's medium (DMEM)
50 ml FCS
5 ml Penicillin and streptomycin (P/S)

Complete KSFM medium

500 ml Keratinocyte Serum-Free Medium (KSFM)
5 ml P/S
Bovine Pituitary Extract: 50 µg/ml for EPC2, HCX, HCX-E6/E7
Epidermal growth factor: 1 ng/ml for EPC2, 26 ng/ml for HCX and HCX-E6/E7

Trypsinisation solution

0.5 g Trypsin
8 g NaCl
1.45 g Na₂HPO₄·2H₂O
0.2 g KCl
0.2 g KH₂HPO₄
10 mM EDTA
Make up to 1 litre with PBS

Cell freezing medium (DMEM)

5 ml FCS

5 ml dimethyl sulphoxide (DMSO)
40 ml complete DMEM medium

Primary cell culture cell freezing medium

9 ml FCS
1 ml DMSO

Hank's balanced salt solution

4.5 mM KCl
0.3 mM Na₂HPO₄
0.4 mM KH₂PO₄
1.3 mM CaCl₂
0.5 mM MgCl₂
0.6 mM MgSO₄
137 mM NaCl
5.6 mM D-glucose

Hoechst stain (0.5 µg/ml)

5 mg Hoechst No. 33258
100 ml Hank's Balanced Salt Solution
Store at 4°C in foil

Fixing solution

1:3 glacial acetic acid-methanol

Mounting fluid

22.2 ml 0.1 M citric acid
27.8 ml 0.2 M Na₂HPO₄·2H₂O
50 ml glycerol
pH 5.5
Store at 2-8°C

B.2 Real-time RT-PCR

DEPC dH₂O

100 µl DEPC in 1 litre dH₂O

10X MOPS buffer (1 litre)

41.86 g MOPS (0.2 M)
16.6 ml 3 M NaAcetate (0.005 M)
20 ml 0.5 M EDTA (pH 8.0) (0.01 M)

RNA loading buffer

0.72 ml formamide
0.16 ml 10X MOPS buffer
0.26 ml formaldehyde (37%)
0.18 ml dH₂O
0.1 ml 80% glycerol
0.08 ml bromophenol blue (0.025%)
Store at -80°C

1.5% formaldehyde-agarose gel (100 ml)

1.5 g agarose
10 ml 10X MOPS buffer
89 ml dH₂O
5.4 ml 37% glycerol
5 µl (10 mg/ml) ethidium bromide

2% agarose gel (100 ml)

2 g agarose
10 ml 10X Tris borate EDTA (TBE)
90 ml dH₂O
5 µl (10 mg/ml) ethidium bromide

B.3 Western blot

RIPA buffer

3 ml NaCl (150 mM)
1 ml Triton X-100 (1%)
1 g Na deoxycholate
1 ml 10% SDS (1%)
1 ml 1 M Tris pH 7.4 (10 mM Tris)
1X protease inhibitor (200 mM Phenylmethylsulfonyl fluoride (PMSF), 100 mM EDTA,
1 mg/ml leupeptin, aprotinin and pepstatin) (added just before use)
1X phosphatase inhibitor (1 mM Na₃VO₄) (added just before use)
Store at 4°C

30% acrylamide solution

30 g acrylamide

0.8 g bisacrylamide

0.1 g SDS

Make up to 100 ml with dH₂O

Store at 4°C covered in foil

8% denaturing polyacrylamide separating gel (10ml)

3.39 ml dH₂O

3.75 ml 1 M Tris pH 8.8

100 µl 10% SDS

2.7 ml 30% acrylamide

150 µl 10% Ammonium persulphate (APS)

15 µl N,N,N-tetramethyl-ethylene diamine (TEMED)

4% denaturing polyacrylamide stacking gel (5ml)

3.65 ml dH₂O

0.625 ml 1 M Tris pH 6.8

0.650 ml 30% acrylamide

50 µl 10% SDS

60 µl 10% APS

6 µl TEMED

2X Laemmli buffer

40 ml 10% SDS

2.5 ml β-mercaptoethanol

5 ml glycerol

1.25 ml 1 M Tris pH 7.0

Few grains of bromophenol blue

1.25 ml dH₂O

10X running buffer (500ml)

20 g glycine

31.6 g Tris

50 ml 10% SDS

Make up to 500 ml with dH₂O

10X transfer buffer (500ml)

72 g glycine

19 g Tris

Make up to 500 ml with dH₂O

1X transfer buffer (1 litre)

100 ml 10X transfer buffer
200 ml methanol
700 ml dH₂O

TBST (1 litre)

50 ml 1 M Tris pH 7.5 (50 mM)
30 ml NaCl (150 mM)
500 µl Tween20 (0.05%)
920 ml dH₂O

Rapid Coomassie stain

10 ml acetic acid
0.006 g Coomassie brilliant blue
90 ml dH₂O

Destain solution (200ml)

10 ml methanol
14 ml acetic acid
176 ml dH₂O

B.4 Immunofluorescence

4% paraformaldehyde

Prepare 16% paraformaldehyde (PFA)
16 g PFA to 80 ml dH₂O
Cover with foil and stir for 1 hour not letting temperature exceed 60°C
Add few drops 10 M NaOH until solution becomes clear
Filter through 0.45 µM filter
Adjust to pH 7.0 with concentrated HCl
Store in 2.5 ml aliquots at -20°C
Add 7.5 ml 1X PBS to make 4% PFA

50 mM NH₄Cl

0.265 g NH₄Cl
100 ml 1X PBS

0.2% gelatin

100 mg gelatin in 10 ml 1X PBS

Sudan Black

0.03 g in 10 ml 70% ethanol

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

10 mg DAPI in 20 ml 1X PBS

Mowiol 4-88

2 g Mowiol 4-88

2 ml glycerol

4 ml dH₂O

Leave at room temperature overnight

8 ml 0.2 M Tris pH 8.5

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 1200 g for 5 minutes to remove insoluble particles before mounting

B.5 Cell proliferation assays

MTT Reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

5 mg/ml in PBS

Weigh out 100 mg MTT in sterile 50 ml conical tube

Add 20 ml sterile 1X PBS

Vortex the mixture and incubate at 37°C for 15 minutes

Filter sterilize the solution through a 0.2 µM filter

Store in the dark at 4°C

Use within 1 month

Solubilisation Reagent

10% Sodium Lauryl Sulphate (SLS) in 0.01 M HCl filter sterilized

Dissolve 25 g of SLS in 250 ml dH₂O

Add 76.6 µl concentrated HCl

B.6 Colony forming assay

1% methylcellulose

Weigh 1 g of methylcellulose in 100 ml glass bottle
Autoclave powder
Warm 100 ml complete DMEM medium and add to powder
Shake until clumps dissolve
Shake overnight at 4°C

12mg/ml poly-(HEMA)

Weigh 2.4 g poly (2-hydroxyethyl-methacrylate)
Dissolve in 20 ml 96% ethanol by rotating at 65°C until melted (~1-2 hours)
Dilute 1:10 in 96% ethanol i.e. 20 ml in 200 ml

B.7 Cell cycle

RNase (50 µg/ml)

300 µl 1 mg/ml RNase
5.7 ml 1X PBS

0.1 M PIPES pH 6.8

3.02 g PIPES in 100 ml dH₂O
pH to 6.8 to become clear

Propidium Iodide Solution

0.1 M Triton X-100
0.002 M MgCl₂
0.1 M NaCl
0.01 M PIPES pH 6.8
0.01 M Propidium Iodide