AN INVESTIGATION INTO THE REGULATION AND EXPRESSION OF THE TUMOUR SUPPRESSOR GENE CLUSTERIN IN ORAL, CERVICAL AND NASOPHARYNGEAL CANCER

by

STEPHANIE LOUISE MALONEY

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School of Cancer Sciences
College of Medical & Dental Sciences
The University of Birmingham

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ABSTRACT

Clusterin is a multifunctional glycoprotein widely expressed as two isoforms. One isoform, sCLU, is secreted, cytoplasmic and anti-apoptotic, the other, nCLU, is nuclear and pro-apoptotic. Seven genes, DKK3, TIMP1, CADM1, AKAP12, KLF4, RNASET2 and CLU were identified to be candidate tumour suppressor genes in cervical neoplasia. Following validation of expression and methylation patterns of these genes CLU was taken forward.

The regulation and expression of CLU has been investigated at three sites of squamous cancer: the oral cavity, cervix and nasopharynx. Down-regulation of CLU at both transcriptional and protein levels was demonstrated in nasopharyngeal cancer (NPC) and oral cancer. Loss of one CLU allele and methylation of the other is demonstrated in the NPC cell line C666-1. This defect has been repaired in this NPC cell line and showed that CLU overexpression of the nuclear isoform of CLU resulted in reduced proliferation and decreased cell viability.

Overexpression of both isoforms of CLU was achieved in C666-1 cells and their knockdown in HeLa cells regulates NF-kB activity. Further evidence that CLU may regulate NF-kB activity was demonstrated by stabilisation of IkBα following CLU overexpression in C666-1. Although sCLU is now considered a promising therapeutic target because of its anti-apoptotic function, with an antisense oligonucleotide currently undergoing clinical evaluation, results suggest that further consideration needs to be given to the possible tumour suppressor function of nCLU.

For Grandad

‘To live in the hearts of those we love is not to die’

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INTRODUCTION

MATERIALS AND METHODS

RESULTS 1

Abbreviation	Full description
^{o}C	degrees celcius
µl	Microlitre
x *g*	gravitational force
5-Aza	5'-Azacytidine
18S	18S ribosomal RNA
Ab	antibody
ALT	alternative lengthening of telomerases
ASO	antisense oligonucleotides
B2M	Beta-2 microglobulin
BGS	bisulfite genomic sequencing
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CLU	Clusterin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferases
dNTP	deoxynucleotide-tri-phosphate
EBV	Epstein Barr virus
ECL	enhanced chemiluminescence system
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERBB2	erythroblastic leukemia viral oncogene homolog 2
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gy	Gray
HAC	histone acetylase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HDAC	histone deacetylase

HPV	human papilloma virus
hr	hour
HSIL	high grade squamous intraepithelial lesions
HSP	heat shock protein
IkB	inhibitors of kB
IR	ionising radiation
kb	kilobase
kDa	kilodalton
L	Litre
LSIL	low grade squamous intraepithelial lesions
mA	milliamp
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
mg	microgram
MHC	major histocompatibility complex
min	minute
ml	millilitre
MMP	matrix metalloproteinases
ms	millisecond
MSP	methylation-sensitive PCR
nCLU	nuclear clusterin
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPC	nasopharyngeal carcinoma
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGK1	Phosphoglycerate kinase 1
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
qRT-PCR	quantitative real-time RT-PCR
RCC	renal cell carcinoma
RNA	ribonucleic acid
RNAi	RNA interference

RT	room temperature
RT-PCR	reverse-transcription PCR
s	second
SAM	S-adenosylmethionine
SCC	squamous cell carcinoma
sCLU	secreted clusterin
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
TBS	tris buffered saline
TCC	transitional cell carcinoma
TGFβ	transforming growth factor beta
TNF	tumour necrosis factor
TP53	tumour p53 gene
TSG	tumour suppressor gene
TSA	trichostatin A
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
WHO	World Health Organisation

Chapter 1

Introduction

1. Cancer

Cancer accounts for around 13% of all deaths and it is estimated that 1 in 5 of us will die from cancer, with its incidence predicted to increase globally from 10 million in 2000 to 15 million by 2010 (IARC-WHO). Cancer cells are neoplastic and proliferate in defiance of normal cell controls, preventing normal multicellular function and tissue and organ formation, with the potential to become malignant (invasive) and colonise surrounding tissues (Abbott, Forrest et al. 2006).

Cancers can be characterised by the tissue and cell type they occur in. Around 90% of cancers are classed as carcinomas, which arise from epithelial cells, and common sites of occurrence are the breast, prostate, lung and colon (Petersen, Gudjonsson et al. 2003). Carcinomas are thought to account for the majority of cancers as the epithelial cells are a site of active cell proliferation and are frequently exposed to both physical and chemical carcinogens. Tumours that arise from mesenchymal cells or connective tissue are termed sarcomas. A third type of cancers are lymphomas and leukaemias, which arise from hemopoietic cells. Other tumour types include germ cell tumours derived from totipotent cells, and blastomas that commonly occur in children and resemble embryonic tissue. All of these types of cancer can be further subdivided according to specific cell type, location in body and the structure of the tumour.

The majority of cancers result from initial somatic hypermutation of a single cell that then must acquire a number of mutations in order to become cancerous, a process which has been described

in Hanahan and Weinberg's 'hypothesis of 6 cumulative alterations' (Hanahan and Weinberg 2000). Benign tumours can be removed surgically and consist of neoplastic cells which remain clustered together in a single mass. As more aggressive cancer cells divide, they may penetrate the basement membrane. This local invasion leads to neoplastic cell adherence to adjacent tissue, and through production of proteolytic enzymes and factors such as matrix metalloproteinases (MMPs) degradation of the extracellular matrix (ECM) is caused in surrounding tissue. This allows cancer cells to cross tissues into neighbouring stroma, facilitating deeper invasion and metastasis. It is through this process that a tumour becomes 'malignant', as the cells invade the surrounding tissue and cross the basal lamina to enter the blood stream. Metastatic tumours have the ability to survive in the circulation, and are able to travel through blood vessels to other organs and through the lymphatic vessels to lymph nodes. Consequently, development of secondary tumours at a site remote from the primary tumour is a hallmark of more aggressive cancers, making them much harder to eradicate (Laerum 1997; Liotta and Kohn 2001) .

1.1 Pathogenesis of cancer

Hanahan and Weinberg's hypothesis of 6 cumulative alterations that define malignancy include self-sufficiency in growth signals, insensitivity to growth-inhibition signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000).

Normal cells require mitogenic growth factors to stimulate proliferation, but some cancer cells have acquired autonomy from this mode of down-regulation. Cancer cells can gain self-sufficiency for growth signalling by overexpression or constitutive activation of growth factor receptors, synthesising their own growth factors and deregulating downstream signalling targets (Peters, Loud et al. 2001). Cancers cell must evade negative anti-growth signals, which act by forcing cells out of the cell cycle into a resting, quiescent state (G0), or by inducing cells to undergo differentiation into a post-mitotic state (Zeimet, Riha et al. 2000). This can also be achieved by abrogation of growth-inhibitory receptor function.

Mechanism	Example
Self-sufficency of growth signals	Over production of platlet-derived growth factor (PDGF) by glioblastomas
	Up-regulation of epidermal growth factor receptors (EGFR) by breast cancer
	Alteration of Ras signalling proteins in cancers
Insensitivity of anti-growth signals	Disruption of retinoblastoma protein (pRb)
	down-regulation of TGFβ
Evading apoptosis	Inactivation of TP53
	Mutation of Caspase 8 in hepatocellular carcinomas
Limitless replicative potential	Up-regulation of telomerase enzyme
Sustained angiogenesis	Up-regulatuion of vascular endothelial growth factor (VEGF)
	Up-regulation of fibroblast growth factors (FGF)
	Down-regulation of thrombospondin-1 (THBS-1)
Tissue invasion and metastasis	Loss of E-cadherin function
	Isoform switch of N-CAM

Table 1.1. Hanahan and Weinberg's hypothesis of 6 cumulative alterations that define malignancy.

Cancer cells evade programmed apoptotic cell death by abrogation of pro-apoptotic 'sensor' receptor signalling that monitors the extracellular and intracellular environment, such as CD95/CD95L pathway (Strand and Galle 1998). Where abnormalities are detected or survival signals are insufficient, effector molecules, such as caspases, are triggered to execute programmed cell death, with over-expression of anti-apoptotic molecules such as Bcl-2. Telomere maintenance is evident in many malignant cells and provides a mechanism of controlling replicative potential, by up-regulating telomerase enzymes leading to cell immortalisation with stimulation of the alternative lengthening of telomerases (ALT) pathway (Kelland 2007). The survival of cells in virtually every tissue depends upon their close proximity to a blood vessel to supply oxygen and nutrients. Cancer cells have developed mechanisms of encouraging growth of new blood vessels from pre-existing vessels (angiogenesis), by changing the balance of angiogenesis inducers such as VEGF and inhibitors such as thrombospondin-1 (Cheung, Singh et al. 1995; Hanahan and Folkman 1996).

Eventually, tumours may acquire the ability to invade adjacent tissue and travel via the lymphatic and blood vessels to distant sites in the body to form secondary tumours where space and nutrients are not limiting. This is achieved through changes in the balance of cell-cell adhesion molecules, overexpression of extracellular proteases, such as matrix metalloproteases, and by suppression of protease inhibitors (Crawford and Matrisian 1994).

1.2. Causes of cancer

Causes of cancer include a number of genetic and environmental factors such as carcinogens in tobacco smoke, hormones, chemicals, mutagens such as UV radiation, viral infection, all of which can lead to such oncogenic mechanisms as loss of heterozygosity and epigenetic changes affecting gene expression, including silencing of tumour suppressor genes (TSGs) and activation of proto-oncogenes (Braakhuis, Snijders et al. 2004; Fukui, Kondo et al. 2005). Activation of proto-oncogenes to oncogenes stimulates cell proliferation and transformation and prevents cells from undergoing normal programmed cell death by apoptosis. Inactivation of TSGs prevents the normal cellular control and inhibition of the cell cycle, DNA replication and cell adhesion.

Smoking is considered to be the most significant risk factor for cancer; however this is closely followed by viral infection that can be attributed to 15% of all cancers. Many oncogenes and TSGs have been shown to interact with tumour virus proteins and carry corrupted genes into other host cells. An example of this is papillomaviruses and SV40 which sequester products of Retinoblastoma and p53 proteins, which would normally regulate cell division (Werness, Levine et al. 1990; Howley, Munger et al. 1991; Lechner, Mack et al. 1992; Tommasino and Crawford 1995).

1.2.1. Oncogenes

Certain genes have the ability to acquire transforming potential, often as a result of alterations in

the gene coding regions of regulatory sequences or through an increase in copy number and are referred to as oncogenes. When present in normal cells they are referred to proto-oncogenes, and are sporadically modified to become an oncogene which results in changes in gene expression or product activity. A single mutant allele of an oncogene can affect the phenotype of the cell and there are several ways in which oncogenes can become activated, resulting in either an increased rate of production or production of an altered gene product. Overproduction can be achieved through gene amplification, such as amplification of the proto-oncogene ERBB2 in breast cancer (tyrosine kinase-type cell surface receptor HER2) (Singleton and Strickler 1992). Alternatively point mutations can lead to increased gene product activity. Activation can occur by translocation to create novel, chimeric proteins. Translocation into a transcriptionally active region of chromatin can cause oncogene activation, such MYC (Wiseman 2006).

1.2.2. Tumour suppressor genes

TSGs have been identified in a range of cellular processes and are regulators of normal growth and development. However, in carcinogenesis this function is lost. Mutation of TSGs usually exhibits recessive effects as the cell still contains one normal functioning gene copy (Payne and Kemp 2005). Inheritance of a mutation in combination with a single somatic hypermutation can lead to cancer by completely ameliorating TSG function. There is evidence that many TSGs undergo epigenetic inactivation (see 1.3) by DNA hypermethylation, demonstrating that mutation and chromosomal loss or intragenic deletion are not the sole mechanisms of TSG inactivation (Ibanez de Caceres and Cairns 2007).

Gene	Oncogene/TSG	Function	Type of cancer
PDGFB	Oncogene	Platelet-derived growth factor	Dermatofibrosarcoma
RAS	Oncogene	GTPase	Pancreatic, colon, lung, ovarian and thyroid
ERBB2	Oncogene	Growth factor receptor	Breast and ovarian
EGFR	Oncogene	Growth factor receptor	Breast
SRC	Oncogene	Tyrosine kinase	Colorectal
RAF	Oncogene	Serine/threonine kinase	Renal cell carcinoma
MYC	Oncogene	Transcription factor	Burkitt's lymphoma and breast carcinoma
FOS	Oncogene	Transcription factor	Fibrous dysplasia
RB1	TSG	Cell cycle	Retinoblastoma, Wilm's tumoure, oestosarcoma
TP53	TSG	DNA repair, apoptosis, allotoxin associated	Hepatocellular carcinoma, osteogenic sarcoma, colorectal and lung
p16INK4A	TSG	cell cycle/p53 function	Melanoma, pancreatic adenocarcinoma, leukaemia, bladder
BRAC2	TSG	DNA repair	Breast, ovarian and prostate
APC	TSG	Cell-cell recognition	Colorectal

Table 1.2. Exampes of oncogenes and tumour suppressor genes

(adapted from Kings and Robins, 2006).

The gene most frequently altered in human cancers in p53. In normal cells p53 is thought to be a 'guardian of the genome' as it protects DNA from damage by co-ordinately blocking cell proliferation, stimulating DNA repair and promoting apoptotic cell death (Wahl and Carr 2001). p53 inactivation can occur by mutation, which usually disrupts DNA binding, either directly by preventing interaction with DNA bases or indirectly by destabilising the loop structures required for this interaction (Lim, Lim et al. 2007) and may be responsible for the genetic instability of many full-blown metastasizing cancers (Wahl, Linke et al. 1997).

1.3. Epigenetics and cancer

Epigenetics is defined as 'heritable changes in gene expression that are not due to any alteration in the DNA sequence'(Tanaka, Watanabe et al. 2008). Epigenetic changes include methylation of DNA, histone deacetylation, ubiquitination, and phosphorylation. These mechanisms play an important role in regulating gene expression and are said to constitute an 'epigenetic code'; for example depending on the type of modification a particular gene can either be expressed or silenced. DNA methylation plays an important role in normal cells and is responsible for X-chromosome inactivation and silencing alleles of imprinted genes (Esteller 2007; Esteller 2008).

1.3.1. DNA methylation

DNA methylation, catalysed by the enzymic activity of DNA methyltransferases (DNMTs), is the addition of a methyl group from S-adenosylmethionine (SAM, a universal methyl donor) to the fifth carbon of a cytosine ring that precedes a guanine, known as a CpG dinucleotide (Bernstein, Meissner et al. 2007). CpG dinucleotide is under-represented in most of the genome, but stretches of sequence that are rich in cytosines proceeded by guanines are referred to as CpG islands. CpG islands span the 5' region of nearly half of the genes in the mammalian genome and can be defined as less than 0.5kb stretches of DNA comprising of ≤55% CpGs, and an over expected frequency of the dincleotide CG greater than 0.6 (Gardiner-Garden and Frommer, 1987). In normal mammalian somatic cells most promoter associated CpG islands are unmethylated with an open, transcriptionally active chromatin structure, whereas CpG dinucleotides elsewhere in the genome, such as in repetitive elements, are generally methylated (Bestor, 2000).

1.3.2. Epigenetic mechanisms of transcriptional control

A reciprocal relationship between density of methylated cytosine residues in the promoter region of genes and the transcriptional activity of a gene has been widely documented (Razin and Cedar, 19991; Kass et al., 1997). In genes which have unmethylated promoters, nucleosomes are widely spaced and associated with a transcriptionally active state. Histone acetylases (HACs) are found in close proximity to unmethylated promoters and catalyse the addition of acetyl groups to lysine residues close to the N-terminus of histone proteins. Conversely, in genes that have methylated promoters, members of methylated cytosine DNA binding protein (MBP) family, such as MeCP2, MBD1, MBD2, MBD3 and MBD4, bind the DNA and recruit histone deacetylases (HDACs) and the chromatin remodelling complex human SWI/SNF (Meehan et al., 1989; Harikrishnan et al., 2005).

DNA methylation leads to deactylation of histones by HDACs and can directly impede the binding of transcription factors to their target sites with a closed compacted chromatin structure, thereby preventing the transcription of genes (Nakayama and Takami 2001; Noma, Allis et al. 2001). Other specific histone modifications include the methylation of lysine 9 on histone H3. Silencing of TSGs that are involved in molecular pathways including apoptosis, cell cycle control and DNA repair (e.g. RASSF1A) by DNA methylation plays an important role in multistep carcinogenesis, along with loss of heterozygosity. Tumour suppressor gene hypermethylation is accompanied in cancer cells by the deacetylation of histones H3 and H4, gain of H3K9 methylation and H3K27 trimethylation, and the loss of H3K4 trimethylation (Gronbaek, Hother

et al. 2007). CpG methylation and a series of chromatin remodelling enzymes together allow chromatin to adopt an open, transcriptionally active structure (Jenuwein and Allis, 2001; Strachan and Read, 2004).

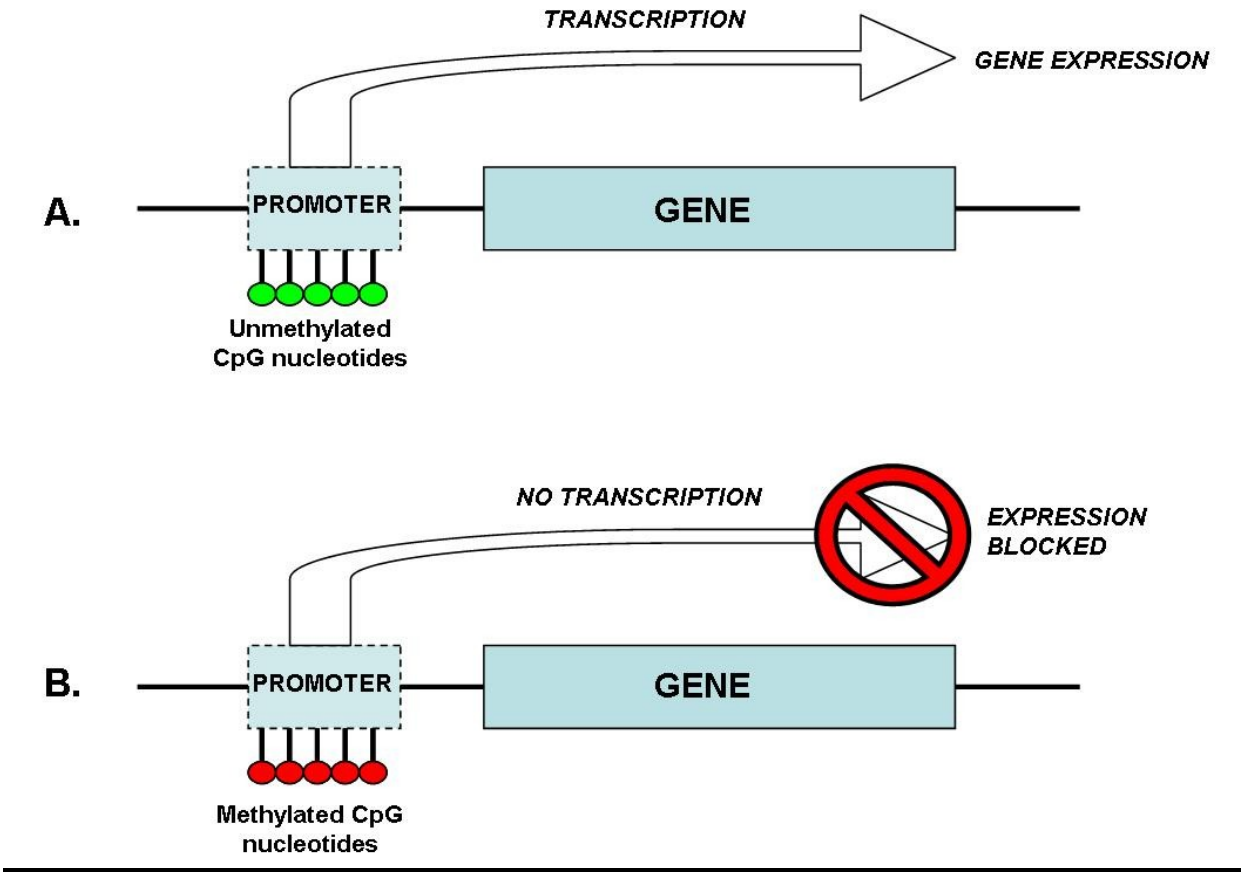


Figure 1.1. Methylation of CpG islands in gene promoter region regulates transcription.
A. Unmethylated CpG nucleotides in the gene promoter region permit transcription and expression of the gene. **B.** Methylation of CpG nucleotides in the promoter region blocks transcription by rendering the area inaccessible to cellular transcription

1.3.3. Epigenetic events during normal development

The pattern of DNA methylation is crucial for the normal function and development of mammalian organisms, and changes in DNA methylation processes have been associated with a wide range of human diseases (Robertson, 2005). The genomes of the primordial germ cells are largely methylated by pre-existing methylation marks become erased by a wave of genome-wide demethylation during development. During differentiation and germ cell development, reprogramming of gene expression patterns occurs by *de novo* methylation, with the sperm genome becoming more heavily methylated than the oocyte's genome. After fertilisation a second wave of genome-wide demethylation occurs in the maternal genome in a replication dependent manner due to exclusion of DNMT1 from the nucleus (Mayer, Niveleau et al. 2000; Oswald, Engemann et al. 2000; Santos, Hendrich et al. 2002). In this way, the mammalian genome undergoes major reprogramming of modification patterns between parental germ cells and the developing embryo.

Differences in gene expression can be seen between tissues of the body and are associated with methylation status, with tissue-specific expression of genes able to undergo hypomethylation as well as hypermethylation during tumour development. Sex-specific differences in the methylation pattern of germ cells occur at imprinted loci, where a specific parental allele is epigenetically modified to allow differential expression of the two alleles in somatic cells of offspring produced. There are at least 60 imprinted genes in mammals and errors in this process can lead to disease

and disorders such as Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Wiedemann syndrome (BWS).

X-inactivation occurs during the pre-implantation stage of development in all female mammals, and results in the selective inactivation of one of the two X-chromosomes by hypomethylation; providing a mechanism to allow males and females to have equal expression of X-chromosome genes (Panning, Dausman et al. 1997; Riggs 2002). Incorrect X-chromosome inactivation can predispose females to recessive X-linked diseases such as incontinentia pigmenti (Bruckner, 2004). DNA methylation also regulates diverse immune processes such as hematopoietic cell development and lineage decisions, immune competence, antigen reactivity and autoimmunity; in particular major histo-compatibility complex (MHC) and class II transactivator (CIITA) expression in antigen-specific immune responses and immune surveillance (Reiner, 2005). It has been suggested that genomic hypomethylation aids the acquisition of mutations in cancer cells by destabilising the genome and promoting loss of heterozygosity in regions containing tumour suppressor genes (Ehrlich, 2002; Feinberg and Tucko, 2004).

It has been proposed that epigenetic modifications such as methylation primarily arose as a mechanism of host defence against inter-genomic parasites, which can cause gene dysregulation and mutation (Walsh et al., 1998). It has been suggested that Alu elements, short stretch of DNA that are the most abundant mobile elements in the human genome in the human genome, may act as modifiers of gene expression through changes in their own methylation status (Batzer and

Deininger, 2002), and inappropriate insertions of these elements accounts for at least 20 known human diseases (Ostertag and Kazazian, 2001).

1.3.4. DNA methylation in cancer

Both hypermethylation and hypomethylation have been observed in a number of cancers, with hypomethylation increasing with increased malignancy at a number of sites such as breast (Jackson, Yu et al. 2004). Hypomethylation causes chromatin decondensation and results in the upregulation of proto-oncogenes, increased recombination and mutation, X-chromosome inactivation, loss of imprinting, reactivation of transposable elements, and demethylation of xenobiotics; ultimately promoting cancer (Franco, Schoneveld et al. 2008). In relation to squamous cancer, TSGs have been shown to be hypermethylated in cervical carcinogenesis, such as CADM1 (Steenbergen, Kramer et al. 2004). It is well established that cervical squamous cell carcinoma is closely correlated with HPV infection, particularly that of HPV 16 and 18. DNMT1 expression increases in cervical intra-epithelial neoplasia (CIN) and progression to cervical carcinoma. Interestingly *in vitro* studies have shown a direct interaction between HPV-16 E7 and DNMT1, stimulating its activity (Burgers, Blanchon et al. 2007).

1.3.4.1. Hypomethylation in cancer

In 1983 Feinberg and co-workers at the John Hopkins University reported widespread loss of CpG DNA methylation in tumour samples, which provided the first evidence for the

involvement of aberrant methylation patterns in cancer (Feinberg and Vogelstein 1983). Loss of methylation at a genome wide level is a frequent and early event in cancer leading to increased mitotic recombination events and genome instability and in many tumour types correlates with disease severity and metastatic potential (Widschwendter, Jiang et al. 2004). In addition to the effects of widespread demethylation, specific genes may become hypomethylated at promoter-associated CpG islands. Tumour-specific transcriptional activation by hypomethylation has been demonstrated for a number of key genes in caner; such as DNAJ domain-containing protein 9 gene (MCJ), which is a marker of response to chemotherapy in ovarian cancer (Strathdee, Vass et al. 2005).

1.3.4.2. Hypermethylation in cancer

Gene silencing by promoter hypermethylation is a frequent and important mechanism of TSG inactivation in cancer, with the RB1 gene being the first TSG shown to be methylated in cancer (Ohtani-Fujita, Fujita et al. 1993). Hypermethylation exemplifies genes involved in tumour developmental processes, including cell-cycle regulation, apoptosis, DNA repair, cell signalling, invasion, chromatin remodelling and transcription (Robertson 2001; Robertson 2005). Nearly half of all genes mutated in inherited tumours are known to undergo silencing by methylation including the BRCA-1 gene in breast cancer (Esteller, Silva et al. 2000) and von Hippel-Lindau syndrome (VHL) in renal cancer (Herman, Latif et al. 1994). Critical TSG in cancer are inactivated by a combination of methylation and mutation, however there is emerging evidence of biallelic hypermethylation distant from regions of chromosomal loss, such as the protocadherin

10 gene (PCDF10) in nasopharyngeal carcinoma (Yin, Xie et al. 2002); demonstrating that epigenetic inactivation by promoter hypermethylation alone is sufficient to inactivate a TSG and promote tumourigenesis (Knudson 1971). It has been suggested that the dynamic process of promoter CpG island hypermethylation may gradually spread from the latent edges of heavily methylated DNA flanking a CpG island and progressively edge towards the transcription start site of the gene (Graff, Gabrielson et al. 2000). Methylation of RASSF1A in normal and cancer tissue is an example of diffusion of methylation from a normally methylated region into previously unmethylated promoter (Yan, Shi et al. 2003).

1.3.5. Reversal of epigenetic modifications

Genetic mutations are transmitted through DNA replication; however epigenetic modifications must be actively maintained and therefore are reversible. By inhibiting epigenetic maintenance molecules it is possible to reactivate genes vital to suppression of a malignant phenotype that have lost function by DNA hypermethylation-mediated gene silencing in cancer. Pharmacological agents can therefore be used to target DNA methyltransferase (DNMT) enzymes maintaining DNA methylation marks and HDAC enzymes responsible for modifying histones resulting in chromatin remodelling and transcriptional genes activity (Stresemann, Bokelmann et al. 2008; Stresemann and Lyko 2008). A number of HDAC inhibitors are currently being tested in phase I and II clinical trials and in 2004 the US Food and Drug Administration (FDA) licensed the archetypal DNMT inhibitor 5-azacytidine (Vidaza) for use as an anti-tumour agent in the pre-leukaemic disorder, myelodysplastic syndrome.

1.3.5.1. 5-Azacytidine

5-Azacytidine is a cytosine nucleoside analogue that is incorporated into the DNA of dividing cells, where it inhibits methylation by forming a covalent bond with DNMT1 and preventing its activity. Over the past 20 years, many clinical trials have included the use of both 5-Azacytidine and 5-Aza-2'-deoxycytidine and a phase III trial showed great clinical benefit to patients with myelodysplastic syndrome treated with low dose 5'-Azacytidine (Kornblith, Herndon et al. 2002; Oki and Issa 2006; Oki, Aoki et al. 2007). A phase I trial demonstrated significant demethylation of the viral promoter in EBV-associated tumours with 5-Azacytidine, however there was no recordable clinical response with little reactivation in EBV expression (Ben-Sasson and Klein 1981; Countryman, Gradoville et al. 2008). Nonetheless, the identification of hypermethylated DNA in a carrier of BRCA-1 mutation in breast cancer is of great potential use as a diagnostic tool since DNA hypermethylation is an early event in cancer.

In vitro studies with 5-Azacytidine and its deoxyribose derivative 5-Aza-2'deoxycitidine in cancer cells have shown a time and dose dependent inhibition of proliferation, and multiple studies have demonstrated the restoration of gene function of methylated TSG during culture with these drugs. 5-Aza-2'deoxycitidine (Decitabine) is possibly a more specific DNMT inhibitor than 5-Azacytidine as it can be directly incorporated into DNA; 5-Azacytidine needs to be converted to a deoxyribonucleoside first, and before this occurs it can be incorporated into RNA, affecting a variety of RNA functions independent of demethylation. However, both drugs have toxic effects and 5-Aza-2'deoxycitidine treatment induces myelosuppression with neutropenia-related fever

(Demakos and Linebaugh 2005). The inherent toxicity of cytosine nucleoside analogues has been the main factor limiting their use as anti-cancer agents.

1.4. Squamous cancer and adenocarcinoma

Squamous cell carcinoma (SCC) is a malignant tumour of the squamous epithelium, affecting the mouth, skin, oesophagus, bladder, prostate, vagina and cervix. Human papillomavirus has been associated with SCC of the oropharynx, lung, fingers, and anogenital region. The incidence of SCC peaks at 66 years with males twice as likely to be affected as females and exposure to sunlight being a further risk factor in skin SCC. The majority of head and neck cancers are SCC and are associated with smoking, alcohol and HPV in 25% of mouth and 35% of throat cancers (Ragin and Taioli 2007). Adenocarcinoma originates from glandular tissue and develops in cells lining glandular types of internal organs, such as the lungs, breasts, colon, prostate, stomach, pancreas, and cervix. 10-15% of all adenocarcinomas are mucinous and aggressive at sites such as colon (Buetow, Buck et al. 1995).

1.4.1. Cervical cancer

Cervical carcinoma is strongly correlated with sexually transmitted HPV infection with disease incidence increased in women who have had multiple sexual partners or whose partner is promiscuous; the disease is absent in virgins. 471,000 new cases of cervical cancer are diagnosed each year worldwide, with approximately 2300 women per year in the UK. Around 80% of cervical cancers occur in the developing world where it is the most common cause of cancer related deaths (Beaudenon and Huibregtse 2008). Although cervical cancer is the second most common cause of cancer in women worldwide, first being breast cancer, the incidence overall is falling in the UK, as in the western world. A 40% decrease in deaths from cervical cancer in the

last 20 years can be attributed to the implementation of cervical screening programmes (Herbert 2007). The incidence of squamous cell cervical carcinoma is falling; however the incidence of adenocarcinoma is now increasing with 95% cases occurring in women over the age of 35.

1.4.1.1. Histology of cervical cancer

The normal cervix is comprised of columnar and stratified non-keratinising squamous epithelium, the ectocervix is lined by stratified squamous epithelium and the endocervix lined by mucinous columnar glandular epithelium with ciliated cells. Cell proliferation is normally restricted to the basal zone. Squamous metaplasia of the cervix is the process whereby columnar glandular epithelium of the transformation zone is gradually replaced by a mature squamous epithelium and is a normal process taking place during the reproductive years. The border between the metaplastic epithelium arising during the reproductive years and the original squamous epithelium is called the original squamous cell junction (Ehrmann 1996). The transformation zone, between the original and current squamocolumnar junctions, is the site of squamous metaplasia of the endocervical glands. Over 90% of cervical cancer arises from the transformation zone (Tranbaloc 2008). The risk of abnormal cell changes is increased by metaplasia during puberty and pregnancy due to the rapid cell turnover.

1.4.1.2. Classification of premalignant invasive lesions

Proliferation of cells in the lower layers of the epithelium leads to a disordered development of the squamous epithelium, referred to as cervical intraepithelial neoplasia (CIN). (Richart 1973; Ambros and Kurman 1990). This is accompanied by an increase in nuclei size and mitoses, hyperchromasia and abnormal mitotic forms. Premalignant lesions can also be classified as low grade squamous intra-epithelial lesions (LSIL), equivalent to CIN I, and high grade squamous intra-epithelial lesions (LSIL), equivalent to CIN III; with the traditional CIN II classification falling between the two (La Vecchia, Tavani et al. 1997; Sherman, Tabbara et al. 1999; Saffer, Wahed et al. 2002; Ha and Califano 2006; Mokhtar, Delatour et al. 2008; Hunter, Duggan et al. 2009; Lukic, Iannaccio et al. 2009) .

1.4.1.2.1. CIN I/LSIL

Proliferating parabasal-like cells are confined to the lower basal third of the epithelium, with the upper two thirds showing good differentiation; however a number of nuclear abnormalities persist and so superficial cells demonstrate koilocyte atypia. The vast majority of low grade lesions are transient and 65% of CIN I naturally regress, with only 15% progressing as far as CIN III (Moore, Cofer et al. 2007).

1.4.1.2.2. CIN II

When proliferating parabasal-like cells have extended in the middle third of the epithelium, with a relative decrease in koilocytotic atypia this is referred to as moderate dysplasia, with differentiation and maturation of the upper half of the epithelium. When the upper one third of the epithelium becomes involved the dysplasia becomes severe. 50% of CIN II regress with 35% progression to high grade disease (Tranbaloc 2008).

1.4.1.2.3. CIN III/HSIL

In CIN III there is an aneuploid cell population, with proliferating cells having extended through the entire thickness of the epithelium. Keratinised dysplasia with nuclear abnormalities and mitotic figures are present throughout the epithelium, referred to as carcinoma *in situ*. 20% CIN III become invasive and a number of studies has shown that progression of CIN to invasive disease can take decades, with CIN incidence peaking at 30-34 years and that of invasive disease 55-59 years.

1.4.1.2.4. Adenocarcinoma *in situ*

Less common than CIN, adenocarcinoma *in situ* is the precursor of most endocervical adenocarcinomas and is localised to the squamocolumnar junction (Christopherson 1979; Boon, Baak et al. 1981; Gloor and Hurlimann 1986; Farnsworth, Laverty et al. 1989). It is characterised by hyperchromasia, chromatin alteration, nuclear pseudo-stratification, decreased mucin

secretion, mitotic activity and apoptotic bodies. It has a 40-70% association with HSIL and strong association with HPV (Wilczynski, Walker et al. 1988; Farnsworth, Laverty et al. 1989; Duggan, McGregor et al. 1995).

1.4.1.3. Squamous cell carcinoma of the cervix

SCC is the most frequently diagnosed histological type of cervical cancer, accounting for 85% carcinomas and shows strong HPV association (Nair and Pillai 1992; Kwasniewska, Gozdzicka-Jozefiak et al. 2004). It can be classified as keratising, large cell non-keratising and small cell types. Keratinising carcinomas are characterised by keratin spheres of infiltrating squamous epithelial cells with hyperchromatic pyknotic nuclei and infrequent mitoses. Large cell non-keratinising carcinomas account for 75% of SCC and constitute infiltrating sheets of undifferentiated polygonal cells forming masses with pushing borders, enlarged nuclei with granular chromatin and occasional keratisation of only single cells, with more frequent mitoses than keratinising types.

1.4.1.4. Adenocarcinoma of the cervix

Adenocarcinoma, arising from the endocervical epithelium, accounts for 15% of all invasive carcinomas and is associated with HPV (Young and Scully 1990; Duggan, McGregor et al. 1995). It is usually well differentiated and may produce mucin, consisting of columnar cells with gland-like spaces. Subtypes of adenocarcinoma account for 30% glandular cancers and include

endometriod, clear cell, papillary villoglandular, papillary serous and mesonephric. This form of cervical cancer is difficult to diagnose with few early stage symptoms meaning that it is frequently diagnosed at much later stage than that of SCC. Unlike SCC this tumour does not have strong correlation with sexual behaviour or smoking but show association with obesity.

1.4.1.5. Causes of cervical cancer

Multiple pregnancies, smoking, exposing to radiation and viral infection are risk factors for cervical cancer, with HPV infection being the most significant. Almost all cervical cancers are HPV positive , an involvement which was first hypothesised in the 1970s (Wheeler 2008). Despite the success of the screening programme, women with minor cytological abnormalities continue to be treated unnecessarily because light microscopy cannot distinguish those women who will progress to invasive cancer and those women in whom epithelial abnormalities will spontaneously regress. Therefore, there are compelling reasons for the identification of other predictors of disease progression.

1.4.1.5.1. HPV and cervical cancer

Human papillomavirus (HPV) is a small double stranded DNA virus, of which there are over 130 different types, 40 of these infecting the genital tract. Those most commonly associated with cervical intraepithelial neoplasia and invasive cancer and classified as high risk types are HPV 16 and HPV 18, followed by other oncogenic types HPV 31, 33, 35, 52, 58, 39, 45, 59, 56, 66 and

51. HPV 16 and HPV 18 together are responsible for the development of approximately 80% of the cervical carcinomas worldwide, with HPV 16 shown to dominate with increasing grade of CIN (Bosch, Manos et al. 1995; Walboomers, Jacobs et al. 1999). HPV 33 and HPV 35 are classified as moderate risk types and low risk HPV 6 and HPV 11 are present in 90% genital warts and do not generally progress toward cancer. Other types of HPV are known to cause cancers at other sites such as head and neck. HPV is present in 99.7% biopsies of invasive carcinoma of cervix with 90% of CIN III positive for high risk HPV types (Walboomers, Jacobs et al. 1999).

The HPV DNA genome is enclosed in a capsid comprised of 2 proteins, L1 and L2; infecting most women under the age of 25 shortly after they become sexually active. However, as a result of cell-mediated immunity, infection of the genital tract with HPV is usually transient, frequently showing viral clearance within a year of detection (Evander, Edlund et al. 1995; Ho, Bierman et al. 1998; Ho, Kadish et al. 1998; Woodman, Collins et al. 2001; Sellors, Karwalajtys et al. 2003). Infection does appear to show a second incidence peak in middle-aged women, when cervical cancer incidence is maximal, which may be due to reactivation of the latent virus. HPV is a necessary but not sufficient cause of cervical neoplasia and only a very small minority of women infected with these types will progress to invasive disease. Progressive disease follows the accumulation of genetic abnormalities, which may be dependent upon HPV integration status or infecting HPV type.

Adenocarcinoma of the cervix is most highly correlated with HPV 18 infection and HPV 16 followed by 18 most frequently detected in SCC; however unlike HPV 16, HPV 18 is rarely detected at the time of diagnosis of high grade CIN (Zehbe and Wilander 1997). HPV 16 can be found in both episomal and integrated forms whereas HPV 18 is always integrated in malignant tissue and women with HPV 18 positive SCC have poorer survival than those who are HPV 16 positive. When in a purely integrated population HPV 16 has worse prognosis than episomal forms or combination of episomal and integrated. This is suggestive of disease progression being determined by the integration status of the infecting HPV type (Pett and Coleman 2007).

Oncogenic genital HPV encodes 2 potent oncogenes, E6 and E7, that disable cell cycle control mediated by inactivation of p53 and retinoblastoma tumour suppressors, allowing high-level amplification of the viral genome (Doorbar 2006). A bivalent (HPV 16, 18) and quadravalent (HPV 6, 11, 16, 18) prophylactic HPV vaccine has now been approved for use and offers great promise in prevention of cervical cancers when used in combination with successful cervical screening programmes. High-risk HPV types also show 25% associated with head and neck carcinomas and so the vaccine may also show promise at other sites.

1.4.1.5.2. Other viruses and cervical cancer

Genital herpes is primarily caused by HSV 2 infection and it has been shown that patients with cervical cancer have elevated HSV 2 levels. An interaction has been postulated between HSV and HPV 16/18 along with alteration of p53, with Herpes virus infection accelerating the viral

replication of HPV and increases its integration (Haverkos, Rohrer et al. 2000; Haverkos 2005). EBV is most commonly associated with Hodgkin's and Burkitt lymphoma and lymphomas derived from immuno-compromised individuals with an established association with nasopharyngeal and gastric cancers. There have been a number of studies suggesting an association with cervical carcinoma, with an absence of EBV in normal cervix and CIN I and 8% of CIN II and III and 43% invasive carcinomas being EBV positive (Landers, O'Leary et al. 1993). Studies have also shown that EBV latent genes EBNA2, LMP1 and EBER1 to be elevated in CIN and invasive carcinoma when compared with normal cervix. It has been suggested that EBV-infected tumour infiltrating lymphocytes might contribute to cervical carcinogenesis (Al-Daraji and Smith 2009).

It has been shown that in the presence or absence of viral infection that immunosuppressed HIV-positive females have higher incidence of cervical cancer. The HIV specific Tat protein up-regulates HPV E6 and E7 oncogenes and enhances their oncogenetic transformation efficacy. The disease is more advanced in HIV-positive women with an increased incidence of relapse post treatment (Al-Daraji and Smith 2009). A negative correlation has been shown between Adeno-associated viruses (AAV) and incidence of cervical cancer, with AAV displaying anti-oncogenic activity, inhibiting the oncogenic properties of HPV (Al-Daraji and Smith 2009). Prior infection with Chlamydia has been correlated with increased progression to CIN III and invasive disease (Koskela, Anttila et al. 2000; Luostarinen, Lehtinen et al. 2004).

1.4.1.5.3. Smoking and cervical cancer

Smoking has been shown to double the risk of cervical cancer, as it results in exposure to many harmful substances that are absorbed by the lungs and carried in the bloodstream throughout the body. Tobacco by-products have been found in the cervical mucus of women who smoke and it has been suggested that this results in DNA damage, contribute to the progression of cervical cancer (DeMarini 1983; DeMarini 2004).

1.4.1.5.4. Epigenetic component of cervical cancer

The presence of epigenetically inactivated TSGs in cervical smears or tissue is potentially a useful predictor of the natural history of the disease, as epigenetic changes are time dependent and heterogeneous. Aberrant methylation of CpG islands in the promoter regions of TSGs has already been reported in cervical intraepithelial neoplasia (CIN) (Virmani, Muller et al. 2001; Steenbergen, Kramer et al. 2004; Singh, Indra et al. 2007; Terra, Murta et al. 2007; Overmeer, Henken et al. 2008). However, it is unknown if these epigenetic changes are necessary or sufficient for the initiation or progression of disease.

1.4.2. Oral carcinoma

Carcinoma of the oral cavity is the eighth most common cancer worldwide, with 274 000 new cases in 2002 (Parkin 2006) accounting for 4% cancers and 2% cancer deaths and possessing a higher death rate than carcinoma of the cervix, brain, liver, testes, kidney and skin due to its diagnosis at late stage (World Health Organization (WHO) cancer database, 2000). Approximately two-thirds of cases occur in men, with an overall incidence rate in the UK of 4.5 in 100 000 (Blot, Devesa et al. 1994). Cancer of the oral cavity is rare in people under 40 years old, with peak incidence at 60 years of age. 10-30% patients go on to develop second tumours of the aero digestive tract (Day and Blot 1992). Prior to development of a metastatic phenotype, cells undergo a number of genetic and phenotypic changes, with patients presenting mouth ulcers, warty nodules, pigmented lesions, enlarged neck nodes and difficulty swallowing, which provides a window of opportunity for intervention before the development of malignancy. Current treatment is surgery in combination with chemotherapy and radiotherapy (Arora, Matta et al. 2005).

1.4.2.1. Histology of oral carcinoma

Oral cancer can be classified as spindle-cell carcinoma, papillary squamous cell carcinomas, adenosquamous carcinoma, acantholytic squamous cell carcinomas and carcinoma cuniculatum, with 90% of malignant lesions being SCC (Johnson, Ranasinghe et al. 1993; Johnson and Warnakulasuriya 1993). Classification and staging includes the size of the tumour primary site,

spread of the tumour to regional lymph nodes and whether the tumour has spread or metastasized to distant sites such as the lung, brain or liver.

1.4.2.1.1. Oral squamous cell carcinoma

Clinically, SCC can present as a minor nodule and can extend in severity to a chronic ulcer. The degree of keratinisation, cellular and nuclear pleomorphism and mitotic activity provides a basis for the grading of SCC as grade 1 which is well differentiated, grade 2 being moderately differentiated and poorly differentiated carcinomas termed grade 3 (Pindborg, Reibel et al. 1985). Smoking and alcohol consumption are well established risk factors and abnormalities in the p53 gene have been suggested to be early events in the development of oral SCC. The involvement of HPV is well defined in head and neck cancers and is suggestive of HPV 16 involvement in SCC development. Basaloid squamous cell carcinoma (BSCC) is a rare and aggressive form of SCC that presents as an aggressive, ulcerated, exophylic firm mass (Wain, Kier et al. 1986). It has a worse prognosis than conventional SCC with early disease recurrence rates and decreased survival rates.

1.4.2.1.2. Oral verrucous carcinoma

Oral verrucous carcinoma is a much less aggressive and well differentiated form of SCC, also known as Ackerman's tumour, with excellent prognosis due to its slow cauliflower-like exophylic growth and low rate of metastasis (Indudharan, Das et al. 1996). Tumours occurring in

the lower lip and hard palate are warty with a greyish surface and erythematous areas and treatable by excision. Pathogenesis has been associated with tobacco chewing and an association has been suggested with HPV (Lubbe, Kormann et al. 1996).

1.4.2.2. Causes of oral carcinoma

Compared with other sites, the oral cavity is easy to access for examination and sampling yet despite the progress in therapeutic procedures, the five-year survival rate for oral cancer patients remains unchanged. Identification and characterisation of risk factors for the disease is crucial to both prevention and development of improved treatment regimes. A number of genetic and environmental/lifestyle factors have been shown to have prognostic significance. Poor oral heath and hygiene have been shown to contribute to disease with dental plaques interacting with saliva and potential being mutagenic. A synergistic and dose dependent effect of smoking and alcohol consumption upon oral cancer rates has been shown in Europe and South Africa, and a further risk of chewing of betel quid (which can activate NF-kB) has been demonstrated in Asia and Melanesia (Graham, Dayal et al. 1977; Elwood, Pearson et al. 1984; Somers 1985; Blot, McLaughlin et al. 1988; La Vecchia, Tavani et al. 1997). 75% of patients are smokers with heavy smokers, as compared with non-smokers, having a 6-fold increase in disease (Cawson 1969). Other physical factors such as accumulative expose to x-rays and UV radiation have been demonstrated in lip cancer and several head and neck cancers. The fact that some patients who have not been exposed to such risk factors still develop carcinomas would suggest a genetic and viral component to pathogenesis.

1.4.2.2.1. HPV and oral carcinoma

Human Papillomavirus (HPV) infects the skin and mucosas and is associated with the formation of benign and malignant tumours. 25 of the 130 established types of HPV have shown association with oral carcinoma; HPV 1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35, 40, 45, 52, 55, 57, 58, 59, 69, 72 and 73, with oral warts being most commonly positive for HPV 2 and HPV 57. Latent infection has also been demonstrated in normal oral mucosa for HPV 6 and HPV 11. Oral squamous cell carcinoma shows high prevalence of HPV 13 and 32, and HPV 16, with detection of at least one of these in around 30% tumours (Saffer, Wahed et al. 2002; Syrjanen 2003; Castro and Bussoloti Filho 2006; Abdul-Rahman, Lim et al. 2007; Anaya-Saavedra, Ramirez-Amador et al. 2008).

1.4.2.2.2. Genetic components of oral carcinoma causation

Oral carcinogenesis is a multi-step process and can be the result of the progressive alteration in genes that regulate cell proliferation, apoptosis, genome stability, angiogenesis, invasion and metastasis (Hanahan and Weinberg 2000). Oral cancer has been linked with a down regulation of the tumour suppressors p53, p16, retinoblastoma (RB1), cyclin dependent kinase inhibitors and adenomatous polyposis coli (APC) (Somoza-Martin, Garcia-Garcia et al. 2005) and an over expression of oncogenes such as cyclin family members, epidermal growth factor receptor and Ras. Of these, perhaps one of the most significant is p53 that is a G1/S block in the cell cycle and is involved in DNA damage repair and apoptosis (Williams 2000). p53 mutation in head and neck cancer has been linked with smoking and its restoration *in vitro* and *in vivo* has been shown to

reverse the malignant phenotype (Langdon and Partridge 1992; Brennan, Boyle et al. 1995; Schantz 1995).

Allelic loss has been demonstrated in oral cancer at 3p, 9p and 17p which are though to be early markers of carcinogenesis due to their common occurrence in dysplastic regions and at 13q and 8p in later stage carcinogenesis (Braakhuis, Leemans et al. 2004; Braakhuis, Snijders et al. 2004). LOH has also been reported at the APC locus by a number of groups (Largey, Meltzer et al. 1994; Huang, Chiang et al. 1997). Chromosomal gain has been demonstrated at 3q26 and 11q13 (Forastiere, Koch et al. 2001; Gollin 2001). Stat3 has been demonstrated to up-regulate vascular endothelial growth factor (VEGF) and promote angiogenesis which gives rise to a poor prognosis phenotype. Pre-existing oral inflammatory disease and lichen planus have also been shown to predispose patients to development or oral cancer.

1.4.2.2.3. Epigenetics component of oral carcinoma

Hypermethylation of circulating DNA has shown promise as a tumour marker in oral cancer and with recent development in techniques used to detect methylation, such as pyrosequencing, the sensitivity and specificity are high and can be used in a non-invasive manner, e.g., on blood specimens (Shaw 2006). Methylation of a number of tumour suppressor genes has been reported in oral cancer pathogenesis including E-cadherin, CDH1, MGMT and DAPK1 (Wu, Roz et al. 1999; Ha and Califano 2006; Shaw 2006)

1.4.3. Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma arising from the epithelial cells that line the nasopharynx, usually developing around the ostium of the Eustachian tube in the lateral wall. NPC differs from other head and neck cancers in terms of its geographic distribution, pathology and etiology including genetic susceptibility, carcinogen exposure and prior viral infection with Epstein Barr Virus (EBV) (Chang and Adami 2006; Agulnik and Epstein 2008). Close association with EBV makes NPC unique from other head and neck cancers. NPC is rare yet it is endemic in a few well defined populations, presenting with swelling of the neck, nasal obstruction and epistaxis; with tradition assessment by transnasal biopsy and fine needle aspiration biopsy. Tumours have a characteristic lymphoid infiltrate, with expression of inflammatory cytokines in malignant and infiltrating cells. Infiltrating lymphocytes have been suggested to play a role in disease pathogenesis, however there is no efficient immune response against the tumour. The size of primary tumour, lymph node involvement, skull base erosion/invasion and cranial nerve involvement can be used to predict the probable final outcome of NPC patients (Liu, Hsieh et al. 2003).

During tumour growth, breach of the basement membrane and interaction with stroma causes a reaction involving recruitment of a variety of cell types, including fibroblasts, dendritic cells and a range of leukocytes, which in turn secrete a variety of chemokines, enzymes and growth factors (Le Bitoux and Stamenkovic 2008). This leads to a process which is similar to tissue injury, causing inflammation and surrounding tissue damage through processes such as mast cell

activation of the complement cascade, release of reactive oxygen species (ROS) by neutrophils, and release of matrix metalloproteinases (MMPs) by macrophages that contribute to extracellular matrix (ECM) breakdown. Virus induced transformation is also known to particularly stimulate immune responses – for example, EBV creates a strong cytotoxic T cell response which may contribute to breakdown of surrounding tissues (Comoli, De Palma et al. 2004; Yip, Abdullah et al. 2009). Tumours progression can often be restricted by an associated immune response characterized by the presence of tumour infiltrating lymphocytes (TILs). Control of tumour growth by TILs is often cytotoxic; this involves the production of cytokines which potentiate an immune response, characterized by high numbers of CD4 and CD8 positive T cells. High levels of TILs have been associated with a good prognosis in patients with cancer of the colon (Murray, Hreno et al. 1975).

NPC shows a first peak in occurrence at 15–25 years and peaks again at 40-60 years of age, and has a 2 to 3 fold higher incidence in men compared with women. NPC, usually type III, accounts for around 50% of nasopharyngeal malignancies in children. In the United Kingdom the incidence of NPC is rare, around 1 in 100,000, and in North America it accounts for around 0.2% of all malignancies. The incidence of NPC is highest in southern China and southeast Asia, with a peak incidence of 20-30 cases per 100,000 (Yu 1991; Tse, Yu et al. 2006). There is a 50-fold regional variation in incidence across China, generally increasing from northern China, including Beijing and Tianjin, to southern China, such as in Hong Kong. In 2002, NPC was found to be the 23rd most common new cancer in the world and the fourth in Hong Kong, with a 24-fold higher incidence in Hong Kong Chinese males than US males. Emigrants of a Chinese decent still have

an elevated incidence of NPC, but one that is lower than China if they are born in North America (Lung, Chang et al. 1994).

1.4.3.1. Histology of NPC

Two major systems of classification for NPC have been put forward by the American Joint Committee on Cancer (AJCC) and the World Health Organization (WHO). AJCC classification is clinically orientated, based on the status of the primary tumour, the status of the regional lymph nodes and the presence or absence of distant metastasis. This classification method is concerned with the stage of the tumour, not the histological tumour type, and is given a category code known as TNM staging; where T is the size or extent of the primary tumor, N is degree of spread to regional lymph nodes and M is the presence of distant metastasis (Kalogera-Fountzila, Karanikolas et al. 2006). Each of these categories is given a number ranging from 0 to 4 relating to the stage of each, with 4 being the most advanced. For example, a 1cm breast cancer that has not spread to the lymph nodes can be categorised as T1, N0, M0.

Alternatively, the WHO classification is based on morphological characterization which incorporates the viral load of EBV in NPC. Keratinising squamous cell carcinoma, type I, is characterised well differentiated cells that produce keratin. Non-keratinising squamous carcinoma, type II, varies in cell differentiation but does not produce keratin. Type III is also non-keratinising but less differentiated with highly variable cell types such as clear cell, spindle cell and anaplatic carcinoma. Type II and type III NPC are invariably EBV positive (Liu and Yeh

1998), while type I and normal nasopharyngeal mucosal cells rarely harbour EBV (Baumforth, Young et al. 1999). Types II & III NPC have a better prognosis than type I. In high incidence areas 95% NPC are type III, with the majority of the rest being type II and in low-incidence regions type I is predominant.

1.4.3.2. Causes of NPC

Epidemiological evidence, such as that of Chinese emigrants, suggests that the pathogenesis of NPC can be attributed to genetic susceptibility and environmental factors (Zheng, Yan et al. 1994; Young and Rickinson 2004). Exposure to nitrosamines in salted fish and pickled foods (a staple food in endemic areas), smoking, frequent dust exposure, herbal medicines, formaldehyde and wood fires used for cooking have all been associated with a 2-6 fold increased risk of NPC. Eating lots of fresh fruit and vegetables on a regular basis, particularly in childhood, has been suggested to reduce the risk of NPC. Genetic susceptibility from the inheritance of mutated alleles or genes also increases the risk of NPC, such as polymorphisms in a subset of human leukocyte antigen types. A number of tumour suppressor genes have been reported to be down-regulated in NPC compared with normal nasopharyngeal epithelium; RARRES1, FHIT, IGSF4, ZMYND10, CDKN2A and BRD7 (Lo, Kwong et al. 2001; Lo and Huang 2002). Equally, a large number of oncogenes and proto-oncogenes have been shown to be up-regulated in the pathogenesis of NPC; CSE 1L, MYB, KIT, LAMC2, MYC, HRAS, MST1R, CCND2, BCL2, CTTN, CXCL5, EGFR, PTGS2, FOSL, ID1, MDM2, PIK3CA and TNFRNSF5 (Chou, Lin et al.

2008). A number of pathways, such as the Wnt pathway, have been also been shown to be de-regulated in NPC.

1.4.3.2.1. EBV and NPC

EBV infection is thought to be one of the main causative factors in the pathogenesis of NPC and it is the association with EBV that makes NPC distinct from other head and neck cancers. EBV transmission occurs mainly through saliva and targets the B lymphocytes. 90% of the population worldwide are thought to be EBV positive with infection being asymptomatic and life long as the virus is harmless until the balance between host and virus is altered. The fact that the majority of adults are EBV positive and only a small number develop NPC highlights that EBV alone is not a sufficient cause of disease and requires the contribution of environmental and genetic cofactors. Other diseases associated with EBV include those of lymphocytic origin such as infectious mononucleosis, Hodgkin's disease, Burkitt's lymphoma and those of epithelial origin, such as oral hairy leukoplakia and undifferentiated gastric carcinoma. 95% NPC tumours are correlated with EBV infection with the highest EBV titers being in undifferentiated carcinomas (Young and Murray 2003; Young and Rickinson 2004).

EBV is a gamma-herpes virus carrying a 172kbp double-stranded DNA genome that enters the nucleus of infected cells to form a circular episome. EBV displays three patterns of latency based on the expression of its latent genes. In latency I, occurring in Burkitt's Lymphoma, only the EBERS and EBNA1 are expressed; Latency II such as in Hodgkin's Lymphoma, expresses the

EBERS, EBNA1, LMP1 and LMP2; whereas in infectious mononucleosis and immunosuppressed-lymphoproliferative disease a latency III pattern of expression is displayed and all the latent genes are expressed (Niller, Wolf et al. 2008). NPC has a latency II pattern of gene expression of the EBNA1 nuclear antigen, the latent membrane proteins, LMP2A and LMP2B, and the BamH1A transcripts. 80% of NPC tumours also express the oncogenic LMP1 protein which is essential for cell immortalisation (Dawson, Rickinson et al. 1990). The monoclonality of the EBV genome would imply that its infection of the host occurs prior to clonal expansion of a malignant cell clone (Raab-Traub and Flynn 1986).

1.4.3.2.2. Epigenetic component of NPC

Recent findings suggest that epigenetic inactivation of a number of tumour suppressor genes plays an important role in the tumourigenesis of NPC, with these epigenetic changes influencing cellular pathways including apoptosis, cell cycle control and DNA repair in NPC cells (Lo and Huang 2002). TSGs found to be methylated in NPC include CDKN2A, CDKN2B, RASF1A, MGMT and ARF.

1.4.3.3. Detection and treatment of NPC

In situ hybridisation techniques for EBERs are employed to detect EBV (Yamamoto, Nakamura et al. 1999; Kimura, Miyake et al. 2009) and since increased EBV-specific antibody titers have been shown in high-incidence areas antibodies against EBV capsid antigen are used in

combination with anti-EBV DNase antibodies (Li, Tan et al. 1985; Zeng, Zhang et al. 1985). NPC patients initially undergo flexible endoscopy, CT and MRI and patients with advanced disease are screened for distant metastasis by thoracic and abdominal CT and bone scan. NPC is a treatable disease, however it is usually diagnosed at late and advanced stage when there is a 5-year survival rate of only 38% (Chen, Liu et al. 2008). NPC is usually treated by radiotherapy and recent studies have shown increased survival rates with chemotherapy, such as cisplatin and 5-fluorouracil, administered prior to irradiation of the tumour. Unlike other malignancies of the head and neck, NPC is highly sensitive to radiation and chemotherapy when diagnosed at stage I or II disease. Less toxic compounds such as taxanes and cetuximab may prove useful when use in combination with current treatment regimes for advanced disease. Clinical trials are now investigating the use of demethylating agents such as 5-Aza-2'-deoxycitidine which de- repress lytic latent genes. Other potential targets are currently being explored such as Wnt antagonists in HNSCC and COX2 inhibitors which have been shown to reduce the growth of NPC.

1.5. Candidate tumour suppressor genes

Several other candidate TSGs were identified in cervical cancer which had relevant function and expression patterns identified. However, the results of further investigation into each of these persuaded me not to take them forward and to exclude them from this investigation.

1.5.1. DKK3

DKK3, located on chromosome 11p15.2, is a secretary glycoprotein and member of the Dickkopf gene family, which are antagonists of Wnt, a member of the Wnt pathway. It inhibits Wnt signalling, leading to the suppression of cell growth and differentiation, development and cancer (Niehrs 2006). DKK3 specifically negatively modulates Wnt7A signalling, and tumour-specific DNA hypermethylation of DKK3 has been identified in prostate cancer, breast cancer and acute lymphoblastic leukaemia (Roman-Gomez, Jimenez-Velasco et al. 2004; Lodygin, Epanchintsev et al. 2005; Veeck, Bektas et al. 2008). It has been suggested that DKK3-induced apoptosis in prostate cancer cells is a consequence of JNK phosphorylation (Kashiwakura, Ochiai et al. 2008; Kawasaki, Watanabe et al. 2009). In addition DKK3 has been shown to have a TSG role in certain cancers, with its introduction into cell lines where it is silenced resulting in reduced colony formation and a slowing of growth attributed to an increased rate of apoptosis, notably in cervical cancer and hepatocellular carcinoma cell lines (Hsieh, Hsieh et al. 2004). DKK3 was identified as a candidate tumour suppressor gene in cervical cancer as it was up-regulated in our array following demethylation of HeLa and SiHa cervical cancer cell lines. Since the time of this study it has been demonstrated by Lee et al that DKK3 is down-regulated in cervical cancer and is a negative regulator of β-catenin, with the down-regulation of DKK3 in cancer contributing to the activation of β-catenin signaling pathway (Lee, Jo et al. 2009). In contrast, in head and neck

cancer LOH of DKK3 has recently been shown to increase cancer patient survival rates associated with lower lymph node metastasis (Katase, Gunduz et al. 2008).

1.5.2. KLF4

KLF4 was identified as a candidate tumour suppressor gene in cervical cancer as it was up-regulated in our array following demethylation of CaSki and SiHa cervical cancer cell lines. KLF4, located on 9q31, is a member of a family of zinc-finger containing transcription factors that is highly expressed in epithelial tissues (Zhang, Geiman et al. 2000; Bieker 2001). KLF4 is an inhibitor of the cell cycle, blocking G1/S progression and suppressing cell proliferation through p21 (Shields, Christy et al. 1996; Chen, Johns et al. 2001). Expression of KLF4 is increased following DNA damage and is thought to mediate p53 G1/S checkpoint function (Yoon, Chen et al. 2003). LOH has been demonstrated in colorectal cancer and in gastric cancer KLF4 is down-regulated as a consequence of hypermethylation of its promoter and LOH (Wei, Gong et al. 2005). These evidence suggests that KLF4 may be a TSG, however there are a small number of studies that identify it as on oncogene, being frequently over-expressed in squamous cell carcinoma, with over expression in 70% of primary breast cancers (Foster et al., 1999) (Foster et al, 2000). It has been suggested that these conflicting roles may be dependent on p21 status, with loss of p21 function seemingly causing a switch from KLF4 inhibiting cellular proliferation to promoting proliferation (Rowland and Peeper 2006).

1.5.3. RNASET2

RNASET2, located on 6q27, is a 27 kDa T2-Rnase gylcoprotein that is able to bind actin *in vitro* (Smirnoff, Roiz et al. 2006). Deletion at this locus has been demonstrated in a number of

malignancies, including ovarian (Cooke, Shelling et al. 1996; Lin and Morin 2001), breast (Devilee, van den Broek et al. 1991; Devilee, van Vliet et al. 1991), stomach (Queimado, Seruca et al. 1995), uterine (Chappell, Lydon et al. 1997), kidney (Morita, Ishikawa et al. 1991), colon (Honchel, McDonnell et al. 1996) and liver (De Souza et al., 1995). Expression of RNASET2 has been demonstrated to be significantly reduced in both ovarian tumours and lines (Lin and Morin 2001). RNASET2 was identified as a candidate tumour suppressor gene in cervical cancer as it was upregulated in our array following demethylation of the HeLa cervical cancer cell line.

1.5.4. TIMP1

TIMP1, xp11.3-p11.23, is an inhibitor of matrix metalloproteinases (MMPs) that has variable X-chromosome inactivation in females, which has been suggested to be regulated by acetylation of histone H3 (Anderson et al., 2005). An important balance is maintained between MMPs, which degrade extracellular matrix, and the TIMP family of proteins in order to control matrix turnover rate (Gomez, Alonso et al. 1997). A number of diseases have been associated with a disruption in this balance with upregulation of MMPs and downregulation of TIMP1 in Crohn's disease and a number of sites of cancer (Lukashev and Werb 1998; Sykes, Bhogal et al. 1999). TIMP1 was identified as a candidate tumour suppressor gene in cervical cancer as it was upregulated in our array following demethylation of the HeLa cell line.

1.5.5. AKAP12

AKAP12, located on chromosome 6q24-25.2, is an anchoring protein controlling cell signalling, adhesion and differentiation (Gelman, Lee et al. 1998; Lin, Nelson et al. 2000). This locus is the site of LOH at a number of sites of cancer (Tibiletti, Sessa et al. 2000), with downregulation of

AKAP12 in cancers of the breast, prostate, ovary and colon (Yildirim, Paydas et al. 2007). AKAP12 has not previously been reported to be methylated in cervical tissue but methylated forms have been reported in colorectal and gastric cancers (Xia, Unger et al. 2001; Choi, Jong et al. 2004; Mori, Cai et al. 2006). AKAP12 was identified as a candidate tumour suppressor gene in cervical cancer as it was upregulated in our array following demethylation of HeLa, C33a and SiHa cervical cancer cell lines.

1.5.6. CADM1

CADM1, 11q23, encodes an immunoglobulin superfamily cell adhesion molecule involved in cell to cell interactions (Gomyo, Arai et al. 1999). It has been shown to be inactivated by promoter methylation and LOH in non-small cell lung cancer and other sites (Murakami 2005; Murakami 2005). It has been shown by other groups that CADM1 gene silencing via promoter hypermethylation is a frequent event in the progression from high-risk HPV-containing, high-grade CIN lesions to invasive cervical cancer (Steenbergen, Kramer et al. 2004; Overmeer, Henken et al. 2008). Deletion of 11q is a frequent event in the pathogenesis of NPC and silencing of CADM1 by promoter hypermethylation has been observed in 36% primary NPC tumours and 60% NPC cell lines (Hui, Lo et al. 2002). CADM1 was added to this list despite not being present on the array because it had recently been reported to be epigenetically silenced in cervical cancer (Steenbergen, Kramer et al. 2004).

1.6. Clusterin

Located on 8p21-12 Clusterin (CLU) is coded by a single gene spanning 17kb with a single promoter, N-terminal hydrophobic leader sequence, 9 exons and 8 introns coding. The gene encodes 3 CLU isoforms, and differences in subcellular localisation dictate their function (Pajak and Orzechowski 2006). CLU is a multifunctional and widely expressed glycoprotein found in a diverse range of tissues, such as breast, prostate and kidney (Shannan, Seifert et al. 2006). Such an expression pattern is suggestive of a central role in cellular homeostasis, and the protein is known to have both pro-apoptotic and anti-apoptotic functions. This functional dichotomy may be related to the different isoforms, two of which are translated into a secreted, cytoplasmic and anti-apoptotic clusterin protein (sCLU), and one of which is alternately spliced and translated into a nuclear and pro-apoptotic clusterin protein (nCLU).

1.6.1. Clusterin isoforms

Databases such as the National Center for Biotechnology Information (NCBI) reference sequence database shows two N terminal differing cytoprotective isoforms of CLU to be expressed in humans and chimpanzees designated Isoform 1 (NM_001831) being translated from the first exon and Isoform 2 (NM_203339) being translated from exon II. Of these only isoform 1 is able to produce the third CLU protein, nCLU, through alternative splicing and exclusion of exon II (Cochrane, Wang et al. 2007). This classification has lead to much confusion as in the literature because CLU isoforms are referred to as sCLU and nCLU and in public databases they are referred to as isoform 1 and isoform 2. To clarify at this stage, both the publicly available

sequences designated isoform 1 and 2 are anti-apoptotic and code for sCLU. The sequence for nCLU is not available in the public database, however from the literature we know that is produced alternative splicing of isoform 1 and is pro-apoptotic.

1.6.1.1. Cytoplasmic and secreted CLU

Translation from the first ATG codon generates a full length 60kDa CLU transcript (449 amino acids) that is targeted to the endoplasmic reticulum by the 22mer leader peptide. Once in the ER this precursor protein is glycosylated and cleaved to generate α- and β-chains, held together by five disulfide bonds. This mature protein can also be secreted from the cell as a 70-80 kDa heterodimeric glycoprotein which has chaperone activity. The mature protein contains a nuclear localisation signal which is not utilised under normal homeostatic conditions (O'Sullivan, Whyte et al. 2003).

There is a variety of evidence that both the mature 80kDa glycosylated CLU protein secreted from the cell and the unglcosylated 60kDa cytoplasmic precursor are anti-apoptotic (Hara, Miyake et al. 2001; You, Ji et al. 2003). These anti-apoptotic effects prevail even when induced by BAX (Zhang, Kim et al. 2005), H_2O_2 (Miyake, Hara et al. 2004), TNF alpha (Sensibar, Sutkowski et al. 1995; Sintich, Steinberg et al. 1999; Zhang, Zhou et al. 2006), $1,25(OH)_2D_3$ (Shannan, Seifert et al. 2007), lethal heat shock dose (Wu, Park et al. 2002) and ionising radiation (Zellweger, Chi et al. 2002). It has also been reported to contribute to resistance to Fas-mediated apoptosis (Miyake, Hara et al. 2001).

There is also evidence that the secreted clusterin protein has anti-proliferative effects. Transient transfection of full length rat CLU in PNT2 a non-tumourigenic immortalised prostate cell line expressing very low levels of CLU led to an inhibition of proliferation (Bettuzzi, Scorcioni et al. 2002). In the PC-3 androgen independent human prostate cancer cell line expressing low or undetectable levels of CLU over-expression of intracellular CLU increased cell death and decreased proliferation (Scaltriti, Santamaria et al. 2004) Reduced proliferation was also observed with over-expression of full length CLU (Scaltriti, Bettuzzi et al. 2004) and nCLU in which there was also reduced motility and disruption of actin cytoskeleton (Moretti, Marelli et al. 2007). Conversely, the same authors showed that over-expression of sCLU had no effect on cell proliferation, mobility, or the actin cytoskeleton.

1.6.1.2. Nuclear CLU

Alternative splicing generates an N-terminal truncated pro-apoptotic 45kDa CLU, synthesised from the second in-frame AUG codon eliminating exon II. This isoform of CLU does not have the protein export sequence in the leader peptide and so the protein does not enter the ER, and therefore does not undergo glycosylation and α/β cleavage (Leskov, Klokov et al. 2003).

The precursor protein resides in cytoplasm and translocates to the nucleus in response to pro-apoptotic signals to induce apoptosis. Evidence for this comes from a variety of model cell lines – in the PC-3 androgen independent human prostate cancer cell line, which expresses low or undetectable levels of CLU, stable transfection of full length or intracellular CLU results in

nuclear localisation of the protein and gives rise to reduced proliferation and increase in cell death (Scaltriti, Bettuzzi et al. 2004). The same authors showed that a non-tumourigenic immortalised prostate cell line, PNT1A, stably transfected with full length CLU also had the same localization and effect. In PC-3 prostate cancer cells nCLU activity is associated with decreased motility and disassembly of the actin cytoskeleton. nCLU binds to alpha-actinin in the cytoplasm causing disassembly of the cytoskeleton and an explanation for nCLU-induced cell death (Moretti, Marelli et al. 2007). When LNCaP androgen independent human prostate cancer cell line expressing low or undetectable levels of CLU was stably transfected with full-length CLU without leader sequence, the nuclear isoform could be identified in cytoplasm and nucleus, which increased sensitivity to TNF alpha-induced apoptosis *in vitro* and *in vivo* (Zhang, Zhou et al. 2006).

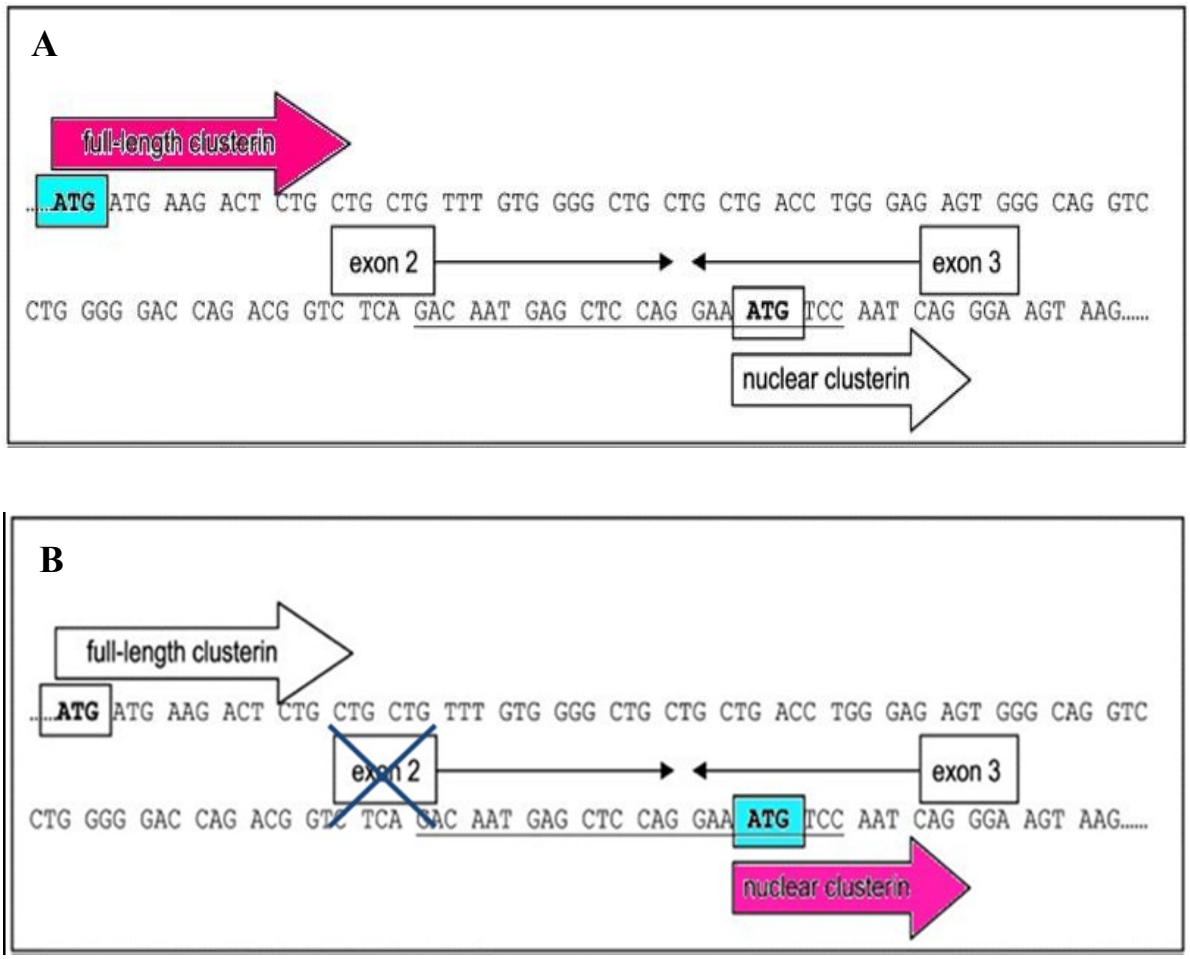


Figure 1.2. Alternative splicing of the clusterin gene produces two different forms of the protein.
A. Secreted clusterin is translated from the first ATG codon to generate a 60kDa CLU transcript. **B.** In the case of nuclear clusterin, alternative splicing generates an N-terminal truncated pro-apoptotic 45kDa CLU, synthesised from the second in-frame AUG codon eliminating exon 2. [Modified from Savkovic et al 2007, *Biochem Biophys Res Comm* **356**, 431-437]

1.6.2. A role for clusterin in carcinogenesis?

Over the past several years, significant data has been generated on the expression and activity of clusterin and its role in malignant disease – CLU expression has been associated with tumourigenesis and the progression of various malignancies. However, much of the available data remains conflicting at present, with evidence of overexpression of CLU in some cancers indicating an oncogenic role, while its repression in other cancers conversely indicates that some aspects of its activity may have a tumour suppressor role.

1.6.2.1. Evidence for clusterin as an oncogene

There is a variety of evidence using animal models of disease progression, transfection studies in cell lines and expression studies in disease and normal tissues which has highlighted a significant over-expression of CLU in tumourigenesis and provides strong evidence that it may be an oncogene. Furthermore, not only is its overexpression associated with cancer rather than normal tissue, but increased expression correlates with disease severity in a number of cases.

In colon, hepatocellular, breast, ovarian, endometrial, melanoma and follicular dendritic cell tumours cytoplasmic CLU over-expression was associated with an increased histological grade (Chen, Halberg et al. 2003; Xie, Sham et al. 2005) (Xie, Lau et al. 2005); (Abdul-Rahman, Lim et al. 2007); (Hoeller, Pratscher et al. 2005); (Grogg, Macon et al. 2005); however this was not necessarily predictive of prognosis or disease reoccurrence (So, Sinnemann et al. 2005; Zhang,

Zhang et al. 2006; Kruger, Ola et al. 2007); (Kang, Hong et al. 2004); (Hsieh, Chen et al. 2005). In bladder cancer overexpression of CLU was found to be significantly associated with recurrence-free survival (Miyake, Gleave et al. 2002); (Kruger, Mahnken et al. 2006), with one contradicting study showing that recurrence-free survival of patients with overexpression of CLU was significantly shorter (Luo, Xie et al. 2005). In renal cancer multivariate analyses revealed that strong expression of clusterin was an independent predictor of tumour recurrence and overall survival (Miyake, Hara et al. 2002). In hepatocellular carcinoma univariate analysis showed recurrence-free survival was significantly lower in patients with strong clusterin expression (Kurahashi, Muramaki et al. 2005).

In various human cancers the tumour suppressor gene *PTEN* is one of the most commonly mutated/deleted genes and its alteration is strongly implicated in prostate cancer development. Disease progression in humans can be demonstrated in the murine Pten prostate cancer model which progresses from hyperplasia to prostatic intraepithelial neoplasia (PIN), to invasive adenocarcinoma, followed by metastasis. CLU is up-regulated in Pten null prostate cancers and is thought to be regulated by a Pten-controlled signalling pathway contributing to initiation and progression of disease (Wang, Gao et al. 2003). This up-regulation of CLU is accompanied by down-regulation of Nkx3.1, which is interestingly also located on 8p21. In prostate cancer patients who had not received androgen ablation CLU staining intensity correlated with Gleason score, corresponding to disease severity but not predictive of disease reoccurrence (Steinberg, Oyasu et al. 1997); (Zhou, Yang et al. 2003); (Ronquist, Carlsson et al. 2006) (Zhang, Kim et al. 2005) (Miyake, Muramaki et al. 2006). Over-expression of clusterin reduces proliferation and

motility and cause disruption of the actin cytoskeleton in androgen independent prostate cancer cell lines (Bettuzzi, Scorcioni et al. 2002; Zhou, Janulis et al. 2002; Scaltriti, Bettuzzi et al. 2004; Scaltriti, Santamaria et al. 2004; Moretti, Marelli et al. 2007).

Clusterin expression and regulation has not been reported in nasopharyngeal or oral cancers, however two studies have looked at the expression of clusterin in cervical neoplasia and demonstrated its overexpression. CLU was expressed distinctly higher in cancers, with strong positive staining throughout each of the cervical cancer tissue samples (Park, Yeo et al. 2006). In a recent study of invasive cervical cancer specimens from patients who underwent radical hysterectomy and systematic lymphadenectomy, CLU positive patients showed significantly worse prognosis than those who did not express CLU (Watari, Ohta et al. 2008).

1.6.2.2. Evidence for clusterin as a TSG

Although there is a significant amount of evidence for overexpression of CLU in a wide range of human cancers, cell lines and animal models, indicating that it may have an oncogenic role, there is also a notable body of evidence for its underexpression (i.e. repression) in a variety of animal models of disease progression and expression studies in disease and normal tissues. These studies have highlighted a significant down-regulation of CLU in tumourigenesis, and provide evidence that it may potentially act as a tumour suppressor gene.

In the colon, oesophagous, skin, AML and breast a down-regulation of CLU has been associated with malignancy (Chen, Turner et al. 2004); (Zhang, Ying et al. 2003); (Goufman, Moshkovskii et al. 2006); (Chung, Kwak et al. 2004). Interestingly in a study of colon cancer there was loss of one CLU allele in 67% of cases (Andersen, Schepeler et al. 2007). Clusterin expression in skin is restricted to differentiating but not proliferating cell layers, suggesting a possible negative role in cell division, influencing the choice between proliferation and differentiation (Thomas-Tikhonenko, Viard-Leveugle et al. 2004). Clusterin-positive patients with pancreatic cancer survived significantly longer and hence down-regulation of CLU may be involved in the progression of pancreatic cancer (Xie, Motoo et al. 2002). However, many studies have found that expression may not necessarily be related to disease progression or tumour size (Grutzmann, Boriss et al. 2005; Jhala, Jhala et al. 2006; Mourra, Couvelard et al. 2007).

Although it has been suggested that CLU is an oncogene in prostate cancer progression, CLU has been shown to be down-regulated in malignant tissue from patients undergoing radical prostectomy after androgen ablation (Bettuzzi, Davalli et al. 2000); (Scaltriti, Brausi et al. 2004). Studies in transgenic adenocarcinoma mouse prostate (TRAMP) mice that spontaneously develop prostate cancer provide a contradiction to the rest of the literature, indicating that CLU is down-regulated during prostate cancer onset and progression, and therefore may have a possible role as a novel tumour-suppressor gene in the prostate (Caporali, Davalli et al. 2004). The authors have suggested that lack of CLU increases the susceptibility to tumourigenesis after carcinogenic challenge and hence is considered to be a tumour attenuator acting predominantly at early stages of neoplastic growth. The apparent contradiction in the literature concerning CLU regulation in

prostate cancer may be explained by the cellular localization of CLU and the recent study showing that in these models of prostate cancer CLU is hypermethylated (Rauhala, Porkka et al. 2008).

CLU expression has been reported in cervical cancer, with one study reporting down-regulation (Choi, Kim et al. 2007). This down-regulation is contradictory to two other studies of cervical cancer, which demonstrate its up-regulation (Park, Yeo et al. 2006). A further study also shows that there is no change in CLU expression in squamous and adenocarcinomas relative to disease free controls (Abdul-Rahman, Lim et al. 2007).

It is probable that this down-regulation observed is of the nuclear isoform of the protein, as some studies discussed have indeed suggested. Therefore, in current treatment regimes for a number cancer types discussed it would be of great advantage to over-express the nuclear isoform as a means of reducing chemo- and radio-resistance. What is not known in these studies is whether the intracellular localization of the protein is cytoplasmic CLU or a pre-cursor for the nuclear isoform, not yet translocated to the nucleus. It would therefore be of great use if an antibody was developed to distinguish between the cytoplasmic and nuclear isoform of CLU to clarify the discrepancies in the literature of CLU being an oncogene and a tumour suppressor. These latter studies suggest caution in the current use of anti-sense oligonucleotides target to CLU until the isoforms responsible for its down-regulation and over-expression have been clarified. A biphasic action of CLU has been proposed; a tumour attenuator acting predominantly at early stages of

neoplastic growth and possibly as an enhancer of the malignant phenotype in full-fledged tumours (Thomas-Tikhonenko, Viard-Leveugle et al. 2004).

Site of Cancer	CLU expression
Breast	↑
HCC	↑
Pancreas	↓
Oesphageous	↓
Colon	↑↓
Cervix	↑↓
Ovary	↑
Endometrium	↑
Prostate	↑↓
Glioma	↑
Haemagioma	↑
Germ cell	↓
FDC	↑
ALCL	↑
VHL defective tumour	↓
Melanoma	↑
Larynx	↑
Lung	↑
Bladder	↑
Kidney	↑

Table 1.3. Summary of the expression of CLU observed in malignant tissue

1.6.2.3. The role of CLU isoforms in patterns of expression in malignant tissues

As described previously, clusterin has two main isoforms – a nuclear isoform which is pro-apoptotic and a cytoplasmic, secreted isoform which is anti-apoptotic. The expression of these two variants in malignant tissue may provide us with some insight into the potentially conflicting evidence for CLU both as a tumour suppressor gene and an oncogene, with overexpression of the cytoplasmic, secreted form conferring oncogenic effects while the nuclear form has TSG effects.

1.6.2.3.1. Isoform expression providing evidence of CLU as an oncogene

Tumour cell survival and progression has been shown to be correlated with an overexpression of cytoplasmic and secreted CLU, and a downregulation of nuclear CLU (Pucci, Bonanno et al. 2004). For example, activation of the p53 TSG suppresses basal and stress-induced sCLU by repressing CLU promoter activity and transcription (Criswell, Klokov et al. 2003). In a study of patients with stage III serous ovarian adenocarcinomas cytoplasmic and secreted CLU, but not nuclear CLU, were more highly expressed in tumours from survivors than in those of deceased patients (Partheen, Levan et al. 2008). Over-expression of full length CLU in bladder cancer prolongs cell survival, resulting in enhanced metastatic potential *in vivo* (Miyake, Gleave et al. 2002). The chemopreventive action of green tea catechins in lowering the incidence of malignancy in the disease progression transgenic adenocarcinoma mouse prostate (TRAMP) model was found to be coupled with clusterin over-expression and its nuclear localisation (Caporali, Davalli et al. 2004). This is in keeping with data showing that when CLU translocates from the nucleus to the cytoplasm in colon adenocarcinoma it promotes tumour progression.

1.6.2.3.2. Isoform expression providing evidence of CLU as a TSG

Many reports in the literature correlate the nuclear localisation of CLU with a TSG role in cells. For example, transient transfection of full length CLU in HT29, a colon cancer cell line which does not express CLU and MCF-7:WS8 oestrogen dependent led to nuclear localisation of the protein and directly induced apoptosis or enhanced chemotherapy-induced apoptosis (Chen, Turner et al. 2004). (Yang, Leskov et al. 2000). A recent study showed that loss of CLU in its nuclear localization was linked with initiation and advancement of spontaneous breast cancer in Tientsin albino 2 mice (Sun, Zhang et al. 2007). In colon cancer specimens CLU was found in the nucleus in healthy colonic mucosa but not in high-grade and metastatic carcinoma, where it appears in cytoplasm, indicating that CLU location and intensity of expression varies with disease status and its severity (Pucci, Bonanno et al. 2004). This shift in localisation is an unusual finding given that clusterin is a marker of apoptosis and conflicts with the rest of the literature with regards to its role in tumourigenesis at other sites, where its localisation in normal tissue is predominantly cytoplasmic. However, the expression of nuclear CLU is normal colonic mucosa suggests that it is regulating cell cycle and apoptosis, especially given that a loss of nCLU and concurrent increase in sCLU has been associated with enhanced metastatic potential.

CLU is translocated to nucleus in response to cytotoxic stimuli such as TGFbeta stimulation, IR (Leskov, Klokov et al. 2003) and overexpression of full length CLU with nuclear localisation inhibited cell growth and increased cell death (Yang, Leskov et al. 2000; Scaltriti, Bettuzzi et al. 2004; Scaltriti, Santamaria et al. 2004; Trougakos, Lourda et al. 2005). Nuclear localisation of

CLU may have a possible impact on DSB repair by causing a decrease in Ku70/Ku80 DNA end binding activity with its overexpression reducing cell growth and colony-forming ability associated with increased G(1) cell cycle checkpoint arrest and increased cell death (Yang, Yeh et al. 1999; Yang, Leskov et al. 2000). Nuclear CLU is a pro-apoptotic protein, whose function may in part be due to its C terminal interaction with Ku70/Ku80. CLU initially binds to Ku70 to form the trimeric protein complex, without direct interaction between CLU and Ku80. This binding activity is thought to be responsible for loss of DNA repair in severely damaged cells (Yang, Leskov et al. 2000), and accumulation of damaged DNA may be directly responsible for apoptotic cell death. There has been much debate in previous years by groups such as Betuzzi as to whether nuclear localization of CLU was actually real or just an artefact of transfection. These studies demonstrate that CLU can be expressed in the nucleus with distinct phenotypic effects.

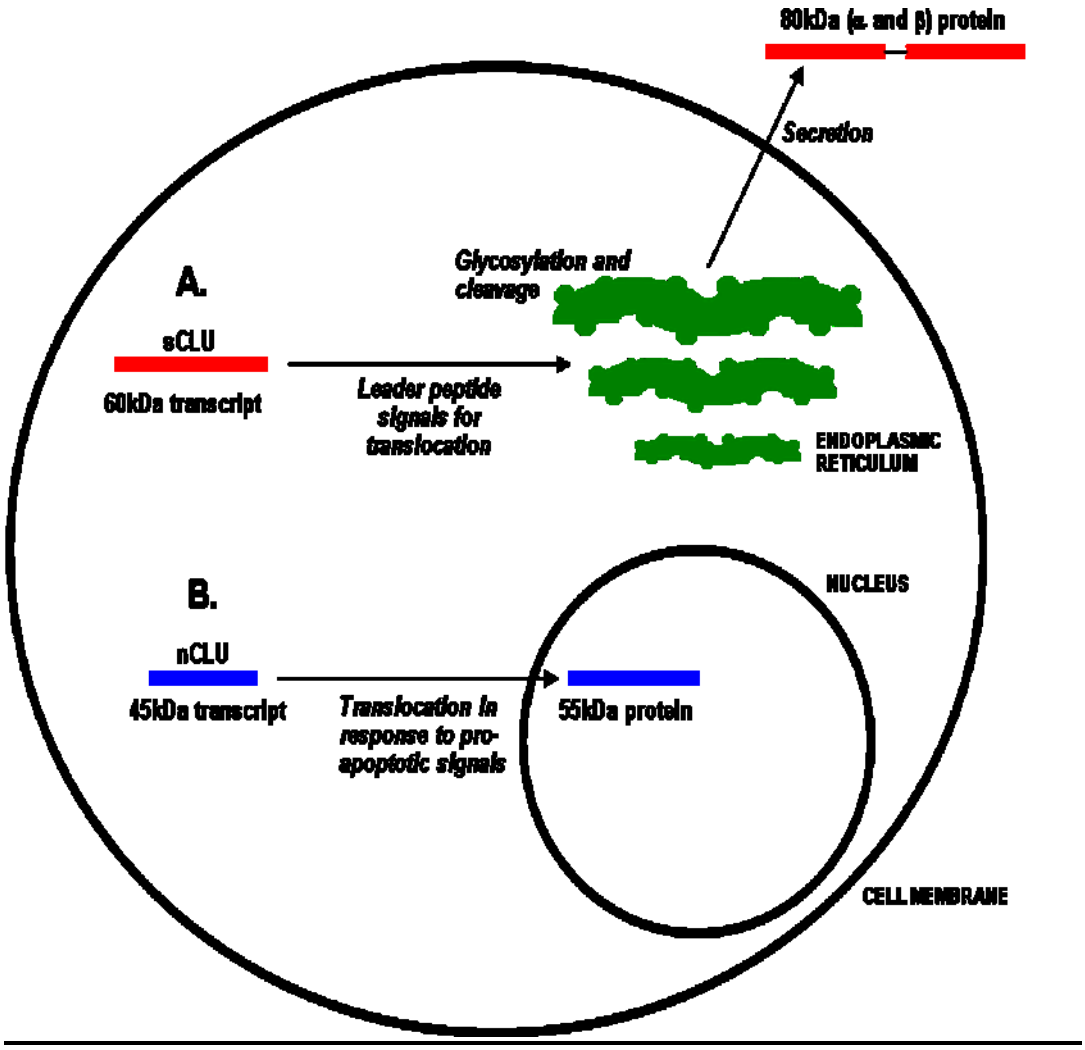


Figure 1.3. Generation of secreted CLU and nuclear CLU.
A. The full length CLU transcript (60kDa) is targeted to the endoplasmic reticulum (ER) by its 22mer leader peptide. This precursor protein is glycosylated and cleaved in the ER to generate α- and β-chains, held together by disulfide bonds. This mature protein is then secreted from the cell. **B.** The truncated 45kDa CLU transcript does not contain the protein export sequence in the leader peptide and therefore the protein does not enter the ER for glycosylation and α/β cleavage. Instead, the precursor protein resides in cytoplasm and translocates to the nucleus in response to pro-apoptotic signals.

1.6.3. Regulation of clusterin expression

As previously described, clusterin expression is found in a wide variety of normal tissues, as well as showing both overexpression and downregulation in cancerous tissue. CLU expression is responsive to a wide range of stimuli, both external and internal, and elucidation of how these pathways function and interact is clearly vital if the role of CLU in normal tissue and the carcinogenic process is to be fully understood.

1.6.3.1. Regulation of clusterin expression at the genomic level

There is a range of evidence that at a genomic level the regulation of CLU expression is likely to be affected through either epigenetic regulation or large-scale deletion of the gene.

1.6.3.1.1 Epigenetic regulation

Silencing of gene expression by CpG methylation of tumour suppressor genes is a frequent event in cancer (Esteller and Herman 2002; Worm and Guldberg 2002; Baylin 2005; Gronbaek, Hother et al. 2007; Palii and Robertson 2007; Esteller 2008). CLU is down regulated and hypermethylated within its promoter in a CpG island 14.5kb up-stream of the transcription start site after induction of oncogenic HRAS in immortalized rat fibroblasts (Lund, Weisshaupt et al. 2006). Following treatment with the commonly used demethylating agent 5'-Aza-2'deoxycitine and a MEK1,2 inhibitor (as previously described, CLU is a MEK/ERC target gene) up-regulation of CLU was observed. It is also possible that CLU is epigenetically regulated in tumour-

conditioned endothelial cells, as CLU was re-expressed following administration of the demethylating agent 5'-Aza-2'-deoxycytidine and trichostatin A (Hellebrekers, Melotte et al. 2007).

1.6.3.1.2. Genomic alterations at the CLU locus

Alteration of oncogene and tumour suppressor gene transcription has been examined in terms of loss of heterozygosity (LOH) and DNA copy number in microdissected colorectal cancers. 67% of the tumours showed both LOH and reduced DNA copy number, suggesting loss of one CLU allele (Andersen, Schepeler et al. 2007).

1.6.3.2. Alteration of CLU expression in response to external stimuli

Alteration of CLU expression is associated with a variety of cytotoxic and oncogenic stimuli, and is known to have a range of effects dependent upon the cellular environment it is altered in.

1.6.3.2.1. Androgen ablation

Androgens are known to stimulate prostate cancer cells to grow. Treatment for prostatic cancer has therefore included the use of drugs which reduce the levels of androgens (anti-androgens such as flutamide) and cause tumours to slow down their growth rate and shrink. The use of drugs, hormones or even castration to reduce levels of androgens has proved useful in the short

term to slow tumour growth; however it is not a cure as the tumour eventually finds a way to grow independent of androgens. The progression to androgen independence has been extensively studied using the androgen responsive mammary models. CLU was initially thought to be an androgen-repressed gene in the rat prostate, with a loss of apoptotic potential in the progression of an androgen-dependent tumour to an androgen-independent state (Bettuzzi, Hiipakka et al. 1989).

Shionogi Carcinoma 115 (SC115) is an androgen-sensitive transplantable mouse tumour. CLU expression is sensitive to androgen withdrawal in androgen-dependent and-independent Shionogi carcinomas and was up-regulated after androgen withdrawal in regressing SC115 (Rennie, Bruchovsky et al. 1990; Rennie, Bruchovsky et al. 1994; Akakura, Bruchovsky et al. 1996). Androgen withdrawal appears to send an apoptotic signal that causes the up-regulation of CLU in order for cells to adapt and survive this change. This would explain the difficulties in treating prostate cancer, which becomes androgen-independent following up-regulation of anti-apoptotic genes such as CLU (July, Akbari et al. 2002).

Androgen withdrawal has been further investigated by a number of other groups who also saw the up-regulation of CLU after castration in mice bearing PC-82 xenografts (Kyprianou, English et al. 1990), Shionogi carcinomas (Rennie, Bruchovsky et al. 1988; Miyake, Nelson et al. 2000), Dunning R3327 PAP prostatic adenocarcinoma (Brandstrom, Westin et al. 1994) and in a rat tumour progression model (Asamoto, Hokaiwado et al. 2001). Increasing testosterone levels

caused a rise in CLU expression levels via increased basal reactive oxygen species levels; this effect was reversed by androgen withdrawal (Pinthus, Bryskin et al. 2007). These studies all highlight the anti-apoptotic nature of CLU that confers resistance to androgen ablation and thereby helps accelerate the progression to androgen independence. This is further strengthened by the observation that CLU isoform 1 (which can generate nCLU) is down regulated by androgens, while isoform 2 (which appears to be retained in the cytoplasm) increases as the LNCaP xenograft tumour progresses to androgen-independence (Cochrane, Wang et al. 2007).

1.6.3.2.2. Oestrogen ablation

Oestrogen stimulates the growth of breast cancer cells in pre-menopausal women with oestrogen receptor positive breast cancer which accounts for round 80% of breast cancers. Oestrogen ablation, be it by surgical removal of the ovaries, radiotherapy or hormone manipulation has been shown to improve pre-menopausal women's chance of long-term survival (Cochrane *et al*, 1998(2000). In healthy women, oestrogen is involved in regulation of the menstrual cycle and in particular the thickening of the endometrium and growth of the uterus. Anti-apoptotic CLU was found to be repressed by oestrogens in normal uterus (Wunsche, Tenniswood et al. 1998) and may be required for the shedding of the endometrium in the menstrual cycle. CLU has been shown to be up-regulated in DMBA-induced rat mammary carcinoma by the selective oestrogen receptor modulator Toremifene, opposing the effects of oestrogen (Huovinen, Warri et al. 1993) and by another selective oestrogen receptor modulator, tamoxifen in the absence of the oestrogen oestradiol in RUCA-1 cells *in vitro* and *in vivo* (Zierau, O'Sullivan et al. 2004). Tamoxifen was

found to increase the expression of CLU both at the RNA and protein level in both the primary tumours and lymph metastases in intact and ovariectomized animals, perhaps explaining why there is an increased incidence of endometrial cancer in post-menopausal patients treated with tamoxifen (Lhomme, Pautier et al. 2003).

Anti-oestrogens such as vitamin D have been shown to inhibit breast cancer growth *in vitro* and *in vivo* through reduced proliferation and increased apoptosis, although the mechanism by which this occurs is unclear. CLU is modulated by treatment with 1,25(OH)2D3 in vitamin D-responsive but not in vitamin D-resistant melanoma cell lines (Shannan, Seifert et al. 2006). Up-regulation of CLU has been observed following oestrogen ablation in mice with MCF-7 tumours (Kyprianou, English et al. 1991) and following treatment with the anti-oestrogens 4-hydroxytamoxifen (Welsh 1994), toremifene (Warri, Huovinen et al. 1993), 1,25(OH)2D3 (James, Mackay et al. 1996; Simboli-Campbell, Narvaez et al. 1996; Simboli-Campbell, Narvaez et al. 1997), tamoxifen (Chen, Tritton et al. 1996) and paclitaxel (2005). This is interesting given that up-regulation of secreted or cytoplasmic CLU has an anti-apoptotic effect. In these studies anti-oestrogens induced apoptosis and showed increased secretion of active TGF-beta. It is known that TGF-beta is able to stimulate translocation of CLU to the nucleus (Reddy, Jin et al. 1996), which would explain the pro-apoptotic effect in these studies. Combination of current neo-adjuvant therapy and screening for deregulated genes such as CLU that could be targeted to overcome resistance to treatment has promising potential as a new therapeutic strategy (Cappelletti, Gariboldi et al. 2008).

1.6.3.2.3. Human chorionic gonadotrophin

The glycoprotein hormone human chorionic gonadotropin (hCG) inhibits mammary tumorigenesis through induction of differentiation and inhibition of proliferation of human breast epithelial cells *in vitro*. This inhibition may be a bystander effect or indeed mediated by CLU given that it is up regulated post treatment with hCG in Sprague-Dawley rats (Srivastava, Russo et al. 1997) and in MCF-10F, a normal human immortalized bronchial epithelial cell line (Srivastava, Russo et al. 1998).

1.6.3.2.4. Chemotherapy

Resistance to apoptosis caused by CLU overexpression leads to a variety of cellular phenotypes, including increased chemoresistance in response to a range of drugs including etopiside, camptothecin, paclitaxel, cisplatin and taxol (Miyake, Nelson et al. 2000; Hara, Miyake et al. 2001; Hoeller, Pratscher et al. 2005; Zhang, Kim et al. 2005).

A number of drugs commonly used in chemotherapy regimens to treat cancers such as prostate and breast have been shown to up-regulate cell survival genes such as CLU. This provides a means by which cancer cells are then able to resist death ligand-induced apoptosis by expressing anti-apoptotic proteins. Pre-treatment of breast cancer cell lines with the glucocorticoid dexamethasone has been shown to inhibit chemotherapy-induced cytotoxicity and is associated with CLU induction. Anti-sense oligonucleotides targeted to CLU reverses this effect, even in the

presence of glucocorticoids, and play a role in attenuation of the inflammatory response (Redondo, Tellez et al. 2007). Glucocorticoids have been shown to inhibit chemotherapy induced apoptosis by conferring protection from the pro-apoptotic signals of cytokines and tumour suppressors through modulation of survival genes such as NF-kB, Bcl-2 and Bcl-xL. This suggests a possible role for CLU in glucocorticoid-mediated survival. Given that glucocorticoids are frequently used prior to treatment with chemotherapy to prevent any adverse effects, it is possible that they might actually be hindering the effectiveness of chemotherapy (Wu, Chaudhuri et al. 2004).

As described previously, androgen ablation leads to apoptosis in prostate cancer cells, but androgen-independent human prostate cancer cells may not show the same effects. Non-androgen ablative cytotoxic drugs such as 5-fluorodeoxyuridine have been used to induce proliferation-dependent death. While androgen-independent human prostate cancer cells retain the ability to activate the apoptotic cascade, this is only achievable after treatment with cytotoxic compounds. CLU is up-regulated in androgen-independent Dunning R-3327 AT-3, PC-3 and DU-145 prostatic cancer cells (Kyprianou and Isaacs 1989; Furuya and Isaacs 1994; Kyprianou, Bains et al. 1994) and mouse mammary tumour cells treated with 5-fluorodeoxyuridine (Furuya and Shimazaki 1996). CLU is also up-regulated in response to irinotecan in colon cancer cell lines (Chen, Turner et al. 2004). These studies suggest that CLU is associated with initiation of proliferation-dependent programmed cell death.

Cisplatin also has been observed to up regulate CLU in the human bladder cancer KoTCC-1 model (Miyake, Hara et al. 2001), in the renal cell carcinoma cell line Caki-1 (Lee, Jin et al. 2002) and in a cisplatin-sensitive parental human ovarian carcinoma cell line but not its cisplatin-resistant variant (Deng, Parekh et al. 2002). However, this effect was only transient and down regulation of CLU actually increased the cytotoxic effect of cisplatin. This evidence therefore suggests that knockdown of CLU to counteract its up-regulation by cisplatin could be used to improve chemotherapy. This is of importance since CLU up-regulation has been seen after chemotherapy in primary peripheral blood leukemic cells (Chow, Nowak et al. 2006), by paclitaxel in the renal cell carcinoma cell line Caki-2 (Zellweger, Miyake et al. 2001), by glucocorticoids in an *in vitro* haemangioma model (Hasan, Tan et al. 2003) and post treatment of breast cancer cells with Trastuzumab, a HER2-targeted monoclonal antibody. Biroccio *et al* also investigated knockdown of CLU by anti-sense oligonucleotide in combination with Trastuzumab to counteract CLU up-regulation by the antibody and found a significantly enhanced sensitivity of cells to Trastuzumab (Biroccio, D'Angelo et al. 2005).

Up-regulation of CLU by the ribonucleotide reductase inhibitor MDL 101,731 was observed in human prostate tumour xenografts (Wright, Cross-Doersen et al. 1996) and the prostate cancer cell line PC-3 but not in LNCaP cells after treatment with camptothecin (CPT), a topoisomerase I inhibitor (Mizutani, Matsumoto et al. 2006). Two groups demonstrated up-regulation of nuclear CLU by ethylenediaminetetraacetic acid in COLO 205 cells (Pajak and Orzechowski 2007) and significant up regulation in PC-3 cells after treatment with doxazosin accumulating first in cytoplasm, then in nucleus (Youm, Yang et al. 2007). Contrary to the rest of the literature,

Artwohl et al showed a down-regulation of CLU by levamisole in cultured human micro- and macrovascular endothelial cells and fibroblasts (Artwohl, Holzenbein et al. 2000).

CLU is down regulated in both docetaxel resistant PC3 and DU145 prostate cancer cells by resveratrol, which functions as a tyrosine kinase inhibitor, sensitising tumour cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors. This effect is thought to be mediated through inhibition of Src and Jak kinases, resulting in loss of STAT1 activation essential for CLU transcription (Sallman, Chen et al. 2007).

In conclusion, it can be seen that the effects of CLU expression on chemotherapy outcome is obstructive conferring chemoresistance in cancer cells that would otherwise undergo programmed cell death, an effect which is overcome by treatment of cells with anti-sense oligonucleotides to CLU in combination with current chemotherapeutic regimes. Recent publications have also suggested that CLU is able to directly bind to chemotherapeutic drugs such as paclitaxel to prevent their interaction with microtubules which would otherwise lead to apoptosis (Chi, Siu et al. 2008; Park, Yeo et al. 2008).

1.6.3.2.5. Radiation

Following exposure to ionizing radiation, DNA repair pathways are activated conferring delays in cell cycle checkpoints leading to either repair or cell death if cells are too badly damaged

(Leskov, Criswell et al. 2001). A number of IR-inducible proteins are also activated, such as protein-tyrosine kinases, which may affect cell fate of not just irradiated cells but may also have a bystander effect on nearby cells. A potential role for CLU has been suggested in low dose ionizing radiation (IR)-inducible adaptive response and genomic instability if damaged cells that would normally undergo apoptosis are allowed to survive and continue to proliferate. The potential mechanism is thought to be that secreted CLU allows cells to escape growth arrest via binding to TGF-beta (Klokov, Criswell et al. 2004).

Basal CLU expression increased in MCF-7 and RKO colon cancer cells after exposure to small non-toxic doses of irradiation and by can be increased by HPV E6; however expression was repressed by p53 in HCT116 colon cancer cell line (Criswell, Klokov et al. 2003). This repression of the sCLU protein may be important for the cascade of p53-mediated events leading to cell death after IR or other cytotoxic agent exposure.

CLU up-regulation by ionizing radiation has been demonstrated in the prostate cancer cell lines DU-145 and PC-3 (Sklar, Eddy et al. 1993), in a dose dependent manner in PC-3 cells (Kyprianou and Rock 1998; Zellweger, Chi et al. 2002) and in MCF-7 breast cancer cells (Yang, Leskov et al. 2000). *In vitro* 8-12 Gy of radiation over 3 days led to an over-expression of CLU and increased cell survival with cells becoming less sensitive to radiation; this was also the case *in vivo* when radiation dose was increased up to 30 Gy. Irradiation of LNCaP tumours which do not express CLU resulted in small amount of CLU being expressed, presumably in a cell survival

response. When CLU was transfected into this cell line there was a more than a three-fold increase in its expression in response to radiation, with decreased radiosensitivity and a 54% reduction in cell cycle arrest and apoptosis (Zellweger, Kiyama et al. 2003). Knockdown of CLU in prostate cancer patients may therefore help reduce radioresistance with cells losing their protection from apoptosis.

Criswell *et al.* showed that this up regulation of CLU by ionizing radiation is dependent on stress-induced activation of IGF-1R-Src-Mek-Erk-Egr-1 signalling (Criswell, Beman et al. 2005). Minutes after ionising radiation, MAPK signalling can be detected, with re-activation of Src/MAPK signalling 24–72 hours after radiation leading to up-regulation of insulin-like growth factor-1 (IGF-1) and phosphorylation-dependent activation of its receptor (IGF-1R). Activated IGF-1R then stimulates the downstream Src-Mek-Erk signal transduction cascade to finally transactivate the early growth response-1 (Egr-1) transcription factor, required for CLU promoter activation leading to sCLU expression. Activation of Erk1/2 is of importance given that it has been shown to cause EGFR activation, whose over-expression has been demonstrated at a number of tumour sites such as breast cancer may contribute to radio-resistance in various tumours, including breast cancer cells by activation of Erk1/2 (Zhou, Kim et al. 2004).

CLU induction has been previously identified as a marker of apoptosis (CLU induction correlates with internuceosomal DNA fragmentation), and overexpression of anti-apoptotic BCL2 after radiation is able to delay induction of CLU expression (Kyprianou, King et al. 1997).

In addition to this, nCLU is known to prevent DNA-PK-mediated end joining in DNA repair and therefore contribute to induction of apoptosis in response to IR (Leskov, Criswell et al. 2001). It is probable that the effect of sCLU is a late response to IR in providing an adaptive survival response. This further underlines the dichotomy of its role as both a pro-apoptotic and anti-apoptotic protein. In summary, ionising radiation up-regulates CLU which leads to increased radioresistance in tumour cells, and to increased radiosensitivity in tumour cells. It would be worth exploring knockdown of CLU in combination with radiation therapy.

1.6.3.2.6. Heat shock

Clusterin has been described as a molecular chaperone given its ability to mimic the cytoprotective nature of heat shock proteins in binding to unfolded proteins (Humphreys, Carver et al. 1999). A number of groups have demonstrated the up-regulation of CLU by heat shock in the human epithelial carcinoma cell line, A431, specifically recognized by the heat shock transcription factor, HSF1 (Michel, Chatelain et al. 1997). Rat primary culture hepatocytes also showed elevation of CLU levels by heat shock treatment (Kimura, Asami et al. 1997). CLU expression did not significantly increase in mouse Sertoli cells until 12 hours after the initial stimulus (Clark and Griswold 1997). In these cell types expression of the stress regulated gene HSP70 was induced 4 hours post heat shock, whereas CLU expression was increasingly elevated between 12 and 48 hours with sustained heat shock indicating that this was a late stress response with a cytoprotective effect. Other cellular stresses such as hydrogen peroxide, superoxide anion,

hyperoxia and UVA exposure also led to elevated levels of CLU in these cell types (Viard, Wehrli et al. 1999).

CLU secretion in PC-3 prostate cells is inhibited by a sub-lethal heat shock leading to increased cytoplasmic CLU; however lethal heat stress caused accumulation of nCLU, degradation of HSP70 and caspase-3-dependent anoikis-death (Caccamo, Desenzani et al. 2006). The degradation of HSP70 is important because over-expression of heat shock proteins acts as anti-apoptotic signal in damaged cells, thereby increasing the tumourigenic potential of cancer cells (Garrido, Schmitt et al. 2003). This finding is mirrored by another study demonstrating that CLU is up regulated and then down-regulated by a lethal dose of heat shock in prostate cancer line, PC-3, and the bladder cancer line TSU-Pr1 (Wu, Park et al. 2002). It therefore seems that in response to non-lethal heat shock, via the heat shock element in its promoter, sCLU is able to act as a molecular chaperone, preventing cells from undergoing apoptosis. Additionally, in response to sub-lethal heat shock secretion of the CLU protein is blocked resulting in its accumulation in the cytoplasm and correlating with cell survival. In protecting partly denatured protein from caspase 3 mediated apoptosis, cytoplasmic CLU acts as an intracellular chaperone, supporting HSP70. However, when cells receive a lethal dose of heat shock, CLU translocates to the nucleus to signal programmed cell death. Addition of exogenous nCLU might therefore increase the effectiveness of heat shock therapy in ablation of prostate cancer.

1.6.3.2.7. Immunosuppressants and carcinogens

Up-regulation of CLU has also been associated with immunosuppressants and carcinogens. Mycophenolic acid is an immunosuppressant drug used to prevent rejection in organ transplantation. In cancer cells it induces terminal differentiation by inhibiting inosine 5'-monophosphate dehydrogenase, causing replication arrest and accumulation of cells in the S-phase of the cell cycle (Floryk and Huberman 2006). This can be explained given that CLU is up regulated following mycophenolic acid-induced differentiation in the androgen-independent prostate cancer cell line DU145 and is known to be anti-proliferative. Sprague-Dawley rats have shown an up-regulation of CLU by phenol II, a cystogenic chemical (Rosenberg, Manivel et al. 1995), an effect which has also been observed in rat prostate following initiation with N-nitroso-N-methylurea and promotion with testosterone propionate (Kadomatsu, Anzano et al. 1993). CLU expression increased as cysts developed, although this was reversible by withdrawal of the drugs.

1.6.3.2.8. Serum deprivation

Serum deprivation *in vitro* causes cell stress and leads to apoptosis. CLU has been shown to be up-regulated by serum deprivation in P19 embryonic carcinoma cells (Fratelli, Galli et al. 1996) and a study by Caccamo et al found that the 70-80 kDa precursor protein of sCLU was down-regulated by serum deprivation and the 45kDa nuclear isoform up regulated in PNT1a cells (Caccamo, Scaltriti et al. 2003). This is consistent with serum deprivation inducing CLU

translocation to the nucleus and resulted in anoikis. Therefore, serum deprivation induces expression of pro-apoptotic nuclear CLU as a stress response.

1.6.3.2.9. Response to other external cellular stimuli

Due to their anti-proliferative activity, retinoids have shown promise as anti-cancer agents. Bayon *et al.* demonstrated that CLU expression could be induced by retinoid antagonist MX781 in PC-3 and DU-145 prostate cells via HSF1 and possibly AP-1 (Bayon, Ortiz et al. 2004). There was a significant increase in HSF-1 and to a lesser extent AP-1 DNA binding activity; mutation of the AP-1 site and the heat shock element site in the CLU promoter completely abolishes MX781-induced transcriptional activation in PC3 and DU145 prostate cancer cells. In response to retinoid antagonists HSF-1 participates in the regulation of anti-apoptotic CLU.

Oxidative stress by photodynamic therapy is associated with the induction of apoptosis in many cancer cells *in vitro* and *in vivo*, which could be explained by an induction of nCLU in cancer cells (Kalka, Ahmad et al. 2000). TNF alpha has been shown to induce cell death in LNCaP cells via a transient increase in levels of pro-apoptotic nuclear CLU and a sustained decrease in the pro-survival secreted isoform (Sensibar, Sutkowski et al. 1995). LNCaP cells gain protection against the cytotoxic TNF-induced cell death following stable over-expression of sCLU.

Other factors such as anchorage, UVB irradiation and cAMP have all been shown to down-regulate the expression of CLU. CLU is suppressed by anchorage, however anchorage-independent growth is a characteristic of tumour cells and so could provide a means of preventing programmed cell death of MCF-7 cells by re-expression of the pro-survival protein CLU (Goldberg, Jin et al. 2001). CLU is reversibly down regulated by cyclic adenosine 3',5'-monophosphate (cAMP) in testicular Leydig tumour cell lines through activation of the protein kinase A pathway (Pignataro, Feng et al. 1992). CLU has been found to be down-regulated following programmed cell death induced by UVB irradiation of human U937, HeLa, and A431 cell lines and dexamethasone-induced cell death of the human lymphoblastoid cell line CEM-C7 (French, Wohlwend et al. 1994). Furthermore, CLU expression was found to be up-regulated only in normal cells and led to their survival despite induction of programmed cell death pathways.

CLU has been shown to be down-regulated in rapidly dividing human keratinocytes infected with a Myc-encoding adenovirus, with knockdown enhancing proliferation (Thomas-Tikhonenko, Viard-Leveugle et al. 2004). Following overexpression of CLU DNA replication was suppressed in keratinocytes and other cells of epithelial origin, suggesting that CLU is an inhibitor of cell proliferation in epithelial cells. This observation has been confirmed *in vivo* where squamous cell carcinomas were more differentiated with increased skin papillomas in CLU deficient mice.

In melanoma cell lines the down-regulation of secreted protein, acidic and rich in cysteines (SPARC) is associated with increased tumour aggression and resulted in reversible CLU down-regulation and inhibition of melanoma growth *in vivo* (Sosa, Girotti et al. 2007). Interestingly decreased SPARC expression also resulted in decreased N-Cadherin and increased levels of HSP27, it has previously been demonstrated by Poon *et al* that CLU and HSP70 have a conflicting relationship., with CLU stabilizing stressed proteins in a state competent for refolding by heat shock protein 70 (Poon, Easterbrook-Smith et al. 2000).

1.6.3.2.10. Regulation of nuclear clusterin expression by external stimuli

The expression of pro-apoptotic nuclear CLU is inducible by Ca++ depletion (Caccamo, Scaltriti et al. 2005); (Pajak and Orzechowski 2007). CLU is localised to the nucleus following TGF-beta stimulation of two epithelial cell lines, HepG2 and CCL64 (Reddy, Jin et al. 1996). Nuclear expression of CLU increased in MCF-7 cells after initiation of apoptosis with anti-estrogens and TNF (O'Sullivan, Whyte et al. 2003). These studies indicate that it is possible to induce expression of the nuclear isoform of CLU in response to cellular stresses, which results in apoptosis. CLU isoforms may be modulated by somostatin, a cytostatic hormone in colon cancer where translocation of CLU from the nucleus to the cytoplasm is indicative of increased tumour aggressiveness, and where nCLU is predominantly expressed in normal cells (Pucci, Bonanno et al. 2004).

1.6.3.3. Regulation of clusterin expression in response to internal cellular signals

Intracellular CLU has been shown to interact with Bax, inhibiting its oligomerization and the subsequent release of cytochrome c and caspase activation, which results in inhibition of apoptosis in cells (Zhang, Kim et al. 2005). This interaction also leads to inhibition of oncogenic c-Myc-mediated apoptosis, where CLU promotes c-Myc-mediated transformation *in vitro* and tumour progression *in vivo*. CLU expression has been associated with tumorigenesis and the progression of various malignancies, possibly through interfering with Bax pro-apoptotic activities. Indeed, there is a large range of evidence in the literature which describes CLU as a cell survival protein, however as previously described this is dependent on the cellular localisation of the protein.

Cellular senescence and restricted proliferative ability in normal cells could potentially be explained by the up-regulation of cytoprotective CLU, which is found in normal rat embryonic fibroblasts and human osteoblasts cell cultures undergoing aging *in vitro* (Gonos, Derventzi et al. 1998). A study by French et al found CLU to be down-regulated in *in vitro* aging of human peripheral blood neutrophils undergoing programmed cell death (French, Wohlwend et al. 1994). In undifferentiated Leydig cells expressing anti-apoptotic Bcl-2 and proapoptotic Bax in tumours nCLU, along with Bax, was up-regulated during regression of the tumourigenic rat Leydig cells, with subsequent elevation of Fas-R and Fas-L (Woolveridge, Taylor et al. 2001).

B-MYB is a universally expressed transcription factor, which like CLU is involved in the regulation of cell survival, proliferation, and differentiation. CLU expression is induced in mammalian cell lines following transient transfection of B-MYB by interaction with a MYB binding site in the CLU 5' flanking region (Cervellera, Raschella et al. 2000).

Cellular CLU overexpression is a common and permanent feature shared by all trefoil factor 1 (TFF1) null antropyloric tumours, and it has be suggested that this deficiency causes the accumulation of misfolded proteins in the endoplasmic reticulum by TFF1 interacting with the ER machinery affecting protein folding and secretion (Torres, Karam et al. 2002). Mutations in the von Hippel-Lindau (VHL) tumour suppressor gene are known to predispose people to cancer. In tumours it is able to target the transcription factor hypoxia induced factor (HIF) for destruction by polyubiquitination and in doing so down-regulates CLU and its secretion (Nakamura, Abreu-e-Lima et al. 2006). CLU has been shown to be up regulated by STAT1 up-regulation by docetaxel in docetaxel-resistant prostate tumour cells (Patterson, Wei et al. 2006). Inhibition of STAT1 leading to inhibition of CLU led to re-sensitisation of DU145-DR prostate cancer cells to docetaxel. Combined therapies targeting the STAT1 pathway could therefore reduce chemo-resistance through down-regulation of CLU.

1.6.4. Clusterin regulation of NF-κB

The regulation of transcriptional targets of CLU can also give us insight into its role in normal and malignant tissue. One particularly pertinent example of this is its regulation of NF-kB. With about 300 genes under its control, NF-κB is a key regulator of the transcription of stress proteins as well as the control of apoptosis (Karin 2006; Papa, Bubici et al. 2006; Basak and Hoffmann 2008). NF-κB function is regulated through its interaction with inhibitory molecules, IkBs, which are able to suppress its activity by keeping it in an inactive state. Phosphorylation and ubiquitination of IkBs by kinases (IKK) abolishes IkBs allowing for the release and translocation to the nucleus of an active NF-κB (Karin 2006). The up-regulation of NF-κB is a common trigger mechanism for the cell propagation essential for their transformation and tumourigenesis, with its expression contributing significantly to the pathogenesis of inflammatory disease (Grossmann, Nakamura et al. 1999; Chen, Castranova et al. 2001; Ghosh and Hayden 2008).

In 2003 Santilli *et al.* proposed an explanation for the pro-apoptotic activity of CLU through an involvement in the regulation of NF-κB activity. Transfection of CLU into both normal and tumourigenic cells caused a stabilisation of inhibitors of NF-kB and consequently inhibition of NF-κB activity. CLU has been shown to modulate both signal dependent and independent IkB turnover downstream of IKK kinase (Santilli, Aronow et al. 2003). Later studies revealed that CLU interacted with phosphorylated IkBa to prevent E3 ubiquitin ligase binding which induced IkBa stabilisation and prevented NF-kB translocation to the nucleus (Devauchelle, Essabbani et al. 2006). NF-κB is important for cell invasive properties and CLU inhibition of it may therefore

suppress cell invasion and result in increased responsiveness of cancer cells to apoptotic stimuli. Deletion of the CLU export signal in the N-terminus of the protein did not affect CLU ability to down-regulate NF-κB which would suggest that intracellular CLU, be it cytoplasmic or nuclear, is involved in this regulation, and not the secreted form of the protein (Pajak and Orzechowski 2006).

Conversely, down-regulation of CLU results in loss of IkB stability and a TNF dependent increase in NF-κB activity, with a consequential effect on NF-kB target genes (Savkovic, Gantzer et al. 2007). siRNA knockdown of CLU promoted the production of cytokines IL6 and IL8, of which NF-κB is a key transcriptional regulator (Devauchelle, Essabbani et al. 2006). The anti-apoptotic function of CLU may perhaps be explained by reduced NF-κB activation and subsequent reduced expression of pro-apoptotic cytokines such a TNFα. It is possible that CLU is part of an NF-κB feedback mechanism regulating stress response mechanisms (Savkovic, Gantzer et al. 2007). When cells get stressed NF-κB is activated which then activates NF-κB target genes including pro-inflammatory and pro-apoptotic cytokines such as TNFα, resulting in cell death. CLU is an NF-κB target gene which when activated translocates to the nucleus, reducing the levels of NF-κB. It therefore acts as a pro-survival protein to limit the effects of pro-apoptotic cytokines.

Having already demonstrated that CLU down regulates NF-κB and IL-6, Zhang *et al.* investigated this further in cells over-expressing phospholipase A2 (PLA2)-activating protein

(PLAA), which has been shown to regulate the production of prostaglandins (PGE2) and tumour necrosis factor (TNF)-α (Ribardo, Kuhl et al. 2002). In these cells there is an induction in NF-κB activation and in the production of IL-6, with a concurrent reduction in CLU expression. Transfection of CLU into these cells reduced TNF- α dependent production of the pro-inflammatory lipid PGE_2 (Zhang, Sha et al. 2008). It is thought that by inhibiting NF-κB that CLU is able to reduce inflammation by limiting expression of pro-inflammatory cytokines.

By exposing cells to high serum concentrations, similar to those in disease states, it is possible to induce apoptosis in cell culture. In response to BSA induced apoptosis ERK and AP1 are activated, which have been linked with NF-κB activation (Hsu, Young et al. 2000; Lombardi, Cantini et al. 2008) CLU is also induced by BSA which serves as a negative feedback loop stabilising IkBα and in doing so prevents continued NF-κB activation and its production of anti-apoptotic Bcl-xL, thus switching from inflammation to AP-1 induced apoptosis (Takase, Marumo et al. 2008; Takase, Minto et al. 2008). These findings are in line with the data produced by other groups who have already shown that CLU significantly suppresses NF-κB but not AP1 dependent transactivation (Santilli, Aronow et al. 2003).

These data suggest that CLU could be a tumour suppressor gene that is essential for controlling NF-κB pathway derived signals and that loss of CLU in cells that depend on NF-κB activity for chemoresistance, proliferation, or invasion could lead to tumour progression. A conflicting paper by Nuutinen *et al.* suggested that CLU induction is NF-κB independent and unrelated to the

inflammatory response in N9 microglia (Nuutinen, Suuronen et al. 2005). A possible reason for this may be that in the murine microglial cell line there does not appear to be any non-glycosylated nCLU by Western blot analysis. It is therefore possible that it is indeed nuclear CLU that interacts with NF-κB and has a role in its regulation. This finding is mirrored in a very recent study showing that over-expression of the sCLU does not interfere with IkB stability or NF-κB activity (Ammar and Closset 2008).

1.6.5. Clusterin knockdown: a potential therapeutic strategy in cancer

A phosphorothioate antisense oligonucleotide, OXG-011, targeted to the first ATG codon attenuates full length CLU expression with unknown effect on nCLU is currently being validated in phase II clinical trials at a number of sites of cancer including breast and prostate. It has been shown to strongly inhibit clusterin expression in *in vitro* and *in vivo* laboratory models, with an increase in apoptosis. This possibly suggests that there is an up-regulation of pro-apoptotic nCLU given that OGX-011 inhibits translation of full-length secreted clusterin but not the alternatively spliced nuclear clusterin.

CLU repression by antisense oligonucleotides is presently in early stage clinical trials for prostate cancer given that it causes sensitization of cancer cells to chemotherapeutic drug-mediated apoptosis. One of the most commonly used treatments for prostate cancer is docetaxel and it has been found that in blocking CLU expression the phytoalexin resveratrol is able sensitise the docetaxel-resistant tumour cells PC-3 and DU145 to TRAIL mediated apoptosis (Sallman, Chen

et al. 2007). In the prostate cancer cell line PC-3M siRNA targeted to CLU rendered cells sensitive to apoptosis and promoted Bax oligomerization, and subsequent caspase activation (Zhang, Kim et al. 2005). Since the anti-apoptotic genes Bcl-2 and Bcl-xL have been implicated in chemo-resistance and tumour progression a Bcl-2/Bcl-xL bispecific ASO has been used in combination with CLU siRNA in LNCaP cells, which resulted in chemo-sensitisation and the up-regulation of Bax (Yamanaka, Rocchi et al. 2006).

Treatment of prostate cancer cells with CLU ASO enhanced their chemo-sensitivity to paclitaxel, mitoxantrone and docetaxel in vitro and vivo, resulting in induced tumour regression and a reduction in mean tumour volume.(Miyake, Chi et al. 2000; Gleave, Miyake et al. 2001; Zellweger, Miyake et al. 2001; Miyake, Hara et al. 2003; Springate, Jackson et al. 2005). Since CLU mediates radioresistance through the inhibition of apoptosis, inactivation of clusterin using ASO technology might offer an innovative line of attack to increase the efficacy of radiation therapy for prostate cancer patients. CLU antisense oligodeoxynucleotide has also been shown to enhance the efficacy of adenoviral-mediated p53 gene transfer in the treatment of orthoptic PC-3 tumours. (Zellweger, Chi et al. 2002; Yamanaka, Gleave et al. 2005)

Clusterin offers a protection to PC-3 cells against heat shock and plays an important role in the cascade of events initiated by heat shock. Prostate cancer cells treated with CLU ASO lose their protection against heat shock and thus have an increase in apoptosis, which can be reversed by addition of exogenous CLU (Sensibar, Sutkowski et al. 1995; Wu, Park et al. 2002).

siRNA knockdown of CLU in breast cancer cell lines resulted in a decrease in cell survival after doxorubicin treatment and a 3-fold increase in IR-induced lethality (Mallory, Crudden et al. 2005; Sutton, Kim et al. 2006). CLU ASO enhanced the sensitivity of breast cancer cells to a HER2-targeted monoclonal antibody and increases chemotherapy-induced cytotoxicity, even in the presence of glucocorticoids which regulate cell survival signalling in breast cancer (Biroccio et al., 2005; Redondo et al., 2007). ASO treatment *in vivo* enhanced paclitaxel induced delay of MCF-7 tumour growth (Biroccio, D'Angelo et al. 2005; So, Sinnemann et al. 2005; Redondo, Tellez et al. 2007). Therefore, therapies which combine CLU ASO or perhaps antibodies to CLU with current chemotherapy regimens could provide a means of inhibiting breast cancer progression.

In bladder cancer CLU ASO in combination with cisplatin or gemcitabine decreased KoTCC-1 tumour volume and enhanced radiation sensitivity and chemosensitivity of bladder cancer cells to cisplatin (Miyake, Hara et al. 2001; Chung, Kwak et al. 2004; Miyake, Eto et al. 2004; Yamanaka, Gleave et al. 2005). Combined treatment with ASO, adenoviral-mediated p53 gene transfer and cisplatin eradicated KoTCC-1 tumours and lymph node metastases in 60% and 100% of mice, respectively (Miyake, Yamanaka et al. 2005). In cervical cancer cell lines, the expression of CLU was concurrent with paclitaxel resistance. Post siRNA treatment of HeLa cell line, chemosensitivity to paclitaxel was significantly reduced, suggesting that CLU may indeed cause paclitaxel resistance in cervical cancer cells (Park, Yeo et al. 2006).

The effectiveness of antisense oligonucleotides targeted to CLU has also been shown in number of other cancer cell lines. For example, in a lung cancer cell line treated with the ASO showed enhanced chemosensitivity to paclitaxel *in vitro* and *in vivo* and decreased cell survival in H460 xenografts treated with OGX-011 plus radiotherapy (July, Beraldi et al. 2004; Cao, Shinohara et al. 2005). Treatment of the renal cancer cell lines Caki-1 and Caki-2 with CLU ASO enhanced chemosensitivity to paclitaxel *in vitro* and delayed tumour growth *in vivo* with an increase in chemosensitivity lost 48 hours after cisplatin treatment (Zellweger, Miyake et al. 2001; Lee, Jin et al. 2002). Chemosensitisation has also been observed in Melanoma cell lines which show high levels of CLU expression post treatment with CLU ASO. This finding was also replicated *in vivo* in CLU ASO treated SCID-mouse/human-melanoma xenotransplantation model, showing improved tumour response to the chemotherapy drug dacarbazine (Hoeller, Pratscher et al. 2005). ASO treatment of HT29, HCT116, HT29 APC led to decreased chemotherapy or APC induced apoptosis (Chen, Turner et al. 2004). ASO treated A431 cells showed increased proliferation (Thomas-Tikhonenko, Viard-Leveugle et al. 2004).

CLU provides a cytoprotective function in osteosarcoma cell lines, where its knockdown has been shown to reduce cell growth and induce endogenous apoptosis. Cells were sensitized to genotoxic and oxidative stress aggravated by chemotherapeutic drugs and H_2O_2 (Trougakos, So et al. 2004). CLU ASO strongly inhibited angiogenesis and caused a significant apoptotic response in the endothelial cell line HUVEC (Jackson, Gleave et al. 2005). This is of particular interest given that the use of anti-angiogenic ASOs is progressing well in cancer clinical trials. The anti-proliferative effect of CLU has also be shown to be eradicated post treatment of the human

epidermoid line A431 with CLU ASO leading to increased proliferation (Thomas-Tikhonenko, Viard-Leveugle et al. 2004). In colon cancer cell lines nuclear CLU has been shown to be affected by CLU ASO and was found to be highly dependent on p21 but not p53 expression, resulting in decreased chemotherapeutic or APC induced apoptosis (Chen, Turner et al. 2004).

In conclusion the use of antisense oligonucleotides targeted to full length cyoplasmic and secreted CLU show very promising results in trials so far. However, at sites of cancer where CLU has been demonstrated to be down-regulated it would be of use to introduce the nuclear isoform of CLU to increase the sensitivity of cancer cells to chemo- and radio-therapies and result in programmed cell death of cancer cells.

Aims and objectives

1. To define the pattern of expression of CLU in cervical, oral and nasopharyngeal cancer compared with normal epithelium
2. To determine the contribution of epigenetic regulation, in terms of DNA methylation, to the transcriptional control of CLU
3. To determine the phenotypic consequences of re-expression of CLU in an NPC cell line model
4. To investigate the contribution of CLU to the regulation of NF-kB activity

Chapter 2

Materials and Methods

2.1. Search for candidate genes

I used several sources of information to identify candidate tumour suppressor genes which may be inactivated by promoter methylation. The first, based on the results of a demethylation experiment, utilised 4 cervical cell lines, 2 of which are infected with HPV16, one with HPV18 and one HPV negative cell line, which were treated with 5-Aza,2-deoxycytidine and Trichostatin A. Gene expression profiles were compared before and after demethylation treatment. Genes which were found to be up-regulated following treatment were matched against a list of tumour suppressor genes identified from a literature review. This exercise revealed that a number of tumour suppressor genes not previously associated with cervical cancer had been up-regulated following demethylation. Secondly, the literature was reviewed to identify those studies which reported changes in gene expression in cervical keratinocyte cell lines in response to transfection with the viral oncogenes E6 & E7. I included in this review one study which described changes in gene expression following integration of HPV16 in the W12 cell line since it has been suggested that viral integration may play a role in expression of tumour suppressor genes such as p16INK4a (Wentzensen, Vinokurova et al. 2004; Vinokurova, Wentzensen et al. 2005).

2.2. Natural history cohorts

Sections were taken under Bolton cohort study ethical committee reference number 28/93/9. The study population for this investigation is drawn from a randomised controlled trial completed some years ago. All women referred for evaluation of cytological abnormality to the colposcopy clinics in the Birmingham and Midlands Hospital for Women were included in the trial if: (a) the

colposcopist considered them suitable for out-patient laser vaporisation and (b) histological examination of a colpscopically directed punch biopsy revealed changes consistent with cervical HPV infection, either alone or in association with CIN 1 or CIN 2. Eligible women were randomised into treatment and non-treatment groups. Untreated patients were monitored by regular cytological and colposcopic examination at intervals of four months and further histological samples removed when the cytological or colposcopic findings suggested progression of disease. Progression was defined as histological evidence of a change from HPV infection alone to CIN, or an increase in grade of CIN. All patients were assessed in a dedicated research clinic by one observer, but histological material removed during the course of the trial was processed in one of two laboratories, dependent on the consultant to whom the patient had been initially referred. One laboratory routinely fixed specimens in formal saline and the other in Bouin's fluid, which has been shown to inhibit the detection of HPV DNA sequences; these cases have been discarded from this investigation. Baseline material from the remaining 93 cases has previously been tested for the presence of HPV 16 and HPV 18 DNA. Seven women progressed to CIN 1, 9 to CIN 2 and 25 to CIN3 after a median follow-up of 36 months (range 4-84); the relative risk of progression associated with the presence of HPV 16 or HPV 18 DNA sequences was 2.3 (95% CI 1.23 to 4.27).

2.3. CELL LINES AND TISSUES

2.3.1. Growth media and supplements for cell culture

All cell lines used were anchorage dependent and subclones of the parental cell lines were grown in complete growth media which consisted of RPMI 1640 (GIBICO, Invitrogen, US) media supplied ready supplemented with L-glutamine (0.2M) and adjusted to pH 7. This was further supplemented with 10% foetal calf serum (GIBCO), 1% penicillin-streptomycin solution (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich, US) and 200mg/ml ciproxin (GIBCO); which was stored at 4°C for up to 1 month. Genticin G418 (GIBCO) was used to supplement the growth media of cells transfected with neomycin resistance genes, this was added to media at 1mg/ml.

2.3.2. Cultivation of cell lines

All cell lines were maintained in incubators at 37°C supplied with 5% CO_2. Cells were grown in a variety of tissue culture plasticware depending on the nature of the experiment. Cell stocks were maintained in 75cm^2 tissue culture flasks (IWAKI, Japan) and fed twice weekly. When the cell lines reached 90% confluency cells were washed with PBS and then incubated for ~5 minutes at 37°C with 3-4mls of 1x trypsin solution (GIBCO). When cells became separated from the flask they were recovered in access media supplemented with 5% foetal calf serum, pelleted at 1500rpm for 5 minutes and then resuspended in fresh complete media. Cells were then plated out in new culture flasks for further cultivation.

Cell line	Cell origin	Basal Media	Supplemented with	Plate Coating / drug selection
NHFK	Normal human foreskin Keratinocytes	Defined keratinocyte SFM	Defined keratinocyte SFM growth supplement	Gelatin coated plates
NHCK	Normal human cervical Keratinocytes	Defined keratinocyte SFM	Defined keratinocyte SFM growth supplement	Gelatin coated plates
HeLa	HPV18 cervical cancer	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
CaSki	HPV16 cervical cancer	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
SiHa	HPV16 cervical cancer	DMEM	10% FCS, 2% L-glutamine, 2% pen/strep	
C33a	HPV negative cervical cancer	DMEM	10% FCS, 2% L-glutamine, 2% pen/strep	
W12	Low grade cervical intraepithelial neoplasia	DMEM	10% FCS, 2% L-glutamine, 2% pen/strep	3T3 feeder layer
HEK293	Human embryonic kidney	DMEM	10% FCS, 2% L-glutamine, 2% pen/strep	
H103	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
H157	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
H376	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
SCC4	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
SCC9	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
SCC15	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
SCC27	Oral squamous cell carcinoma	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
NP69	SV40 immortilised nasopharyngeal cells	DMEM/F12 (1:1)	5% FCS, 2% L-glutamine, 1% pen/strep, 0.5% Ciproxin	
C666-1	EBV positive undifferentiated nasopharyngeal carcinoma	RPMI	10% B cell serum, 2% L-glutamine, 2% pen/strep, 0.5% ciproxin	1mg/ml fibronectin coated plates
Ad-AH parental	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
Ad-AH neo	EBV negative nasopharyngeal carcinoma + neomycin resistance cassette	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH EBV	Nasopharyngeal carcinoma infected with recombinant EBV	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH -LMP2A	Nasopharyngeal carcinoma infected with mutant rEBV in which LMP2A is deleted	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH EBNA1	Nasopharyngeal carcinoma transfected with EBNA1	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH LMP2A	Nasopharyngeal carcinoma transfected withLMP2A	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH LMP2B	Nasopharyngeal carcinoma transfected withLMP2B	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
Ad-AH LMP1	Nasopharyngeal carcinoma transfected with LMP1	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	Geneticin G418
NPC TWO 1	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
NPC TWO 4	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
HONE1	Nasopharyngeal carcinoma latency infected with EBV	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
CNE 1	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
CNE 2	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
SUNE1	EBV negative nasopharyngeal carcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	
AGS	Gastric adenocarcinoma	RPMI	10% FCS, 2% L-glutamine, 2% pen/strep	

Table 2.1: Growth media for cell lines used in this thesis (GIBCO)

2.3.3. Cell counting

Many experiments require a set number of cells so it is important to have a method of determining cell numbers. After harvesting cells in the same manner as for subculture a sample of cell suspension was drawn into the main square on a haemocytometer grid (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich Ltd, Gillingham, Dorset, UK) overlain with a cover slip. The volume that can be contained in a large grid is 10^{-4}ml. Light microscopy was used to count the number of cells in four large grids on the haemocytometer, and the number divided by four to give an average number per 10^{4}ml. This number is them multiplied by the total volume (mls) of the cell suspension to give the overall number of cells present.

2.3.4. Preparation for mycoplasma testing

Mycoplasma infections are a common problem with cultured cells that can affect cell behaviour and obscure results. All cell lines were periodically tested for the presence of mycoplasma, using the MycoAlert mycoplasma Detection Kit (Lonza, Switzerland) according to the manufacturer's protocol, and were found to be consistently negative for mycoplasma.

2.3.5. Cryopreservation

The day prior to cryopreservation cells were washed in complete media by centrifugation at 1500rpm for 5 minutes and then resuspened at a density of $5x10^{5}$ cells/ml of complete media and cultivated in tissue culture flask. On the day of cyropreservation cells were pelleted as described

previously. Typically, $5x10^6$ cells were re-suspended in 1ml ampoules of freezing media containing 10% DMSO and transferred to cryotubes (Nunc, Denmark). These were placed in cyropreservation boxes (Mr Frosty, Nalgene, US) and stored for 24hrs at -80°C. The cells were then transferred from the cyropreseravtion boxes to liquid nitrogen freezers (-140°C) for long-term storage. Frozen cells were recovered by warming rapidly in a 37°C water bath and then transferring to a 15ml tube (Falcon). 5ml of pre-warmed complete media (37°C) was then added to the cells drop-wise, and the cells were then pelleted at 4530rcf for 5 minutes. The supernatant was subsequently discarded and the cells resuspended in 8ml complete media and transferred to a $25cm^2$ culture flask (IWAKI).

2.3.6. Tissue section preparation

2.3.6.1. Section preparation for immunohistochemistry

Parffin-embedded tissues from the cervix, oral cavity and nasopharynx were cut at 5μm on to adhesive-coated slides (Vectorbond reagent, Novocastra Laboratories Ltd, UK) using a rotary microtome. Slides were incubated at 37°C overnight and stored at room temperature prior to staining. Immediately prior to immunhistochemical (IHC) staining, paraffin embedded tissue sections were de-waxed by incubating in xylene (2-times 5 min) and then industrial methylated spirits (IMS) (2-times 5 min). A standard cryostat was used to prepare frozen sections at 5μm. Sections were fixed in formal-saline, washed in PBS (pH 7.6), dried at room temperature and then individually wrapped in aluminium foil prior to storage at -20°C.

2.3.6.2. Section preparation for quantitative-reverse transcriptase-PCR and cell lysis

A standard cryostat was used to cut 20-30 frozen 5μm sections directly in to a pre-chilled sterile 1.5ml eppendorf. The tube was then sealed and placed immediately into liquid nitrogen for storage prior to protein or RNA extraction.

2.3.7. Cytospin preparation for immunohistochemistry and immunofluorescent staining

Between 10^5-10^6 cells were washed in warm PBS by centrifuging at 1500rpm 3-times. After the final wash the pellet was resuspended in 5-15ml of warm PBS. 1-3 drops of cell suspension was loaded on to an adhesive-coated slide (Novocastra) through a cytospin funnel. The cells were then centrifuged on the slide using a standard Cytospin at 4530rcf for 5 minutes. Slides were then fixed in formal-saline (for IHC) or 4% paraformaldehyde (for IF staining) for 20 minutes, washed in PBS for 5 minutes, water for 5 minutes, air-dried and stored at room temperature (RT) prior to staining.

2.4. TRANSFECTION OF PLASMID DNA INTO MAMMALIAN CELLS

2.4.1. Transient transfection of cells

The lipid-based transfection reagent, Lipofectamine (Invitrogen) was used in conjunction with Plus reagent (Invitrogen) to transfect cells. The day prior to transfection $1x10^6$ cells were seeded in 60mm tissue culture plates (IWAKI) with 2ml of complete RMPI media and cultivated overnight. To transfect the cells complete RPMI media was aspirated from the cells and they were washed by gently over-laying with serum-free Optimem media (GIBCO). This was done to remove serum, which would otherwise reduce the transfection efficiency. 2ml of fresh Optimem media was then added to the cells and the Plus reagent mastermix prepared (6µl Plus reagent, 133µl Optimem media, 1µg DNA), gently mixed and incubated in the dark for 15 minutes. The Lipofectamine mastermix was then prepared (4µl Lipofectamine, 133µl Optimem media) with gently mixing by pipette. The two master mixes were then combined and mixed by pipetting, and the solution incubated for a further 15 minutes in the dark. Following this, 276µl of the combined mastermix was added drop-wise to the cells. After 8hrs incubation with the combined mastermix 2ml of complete RPMI media was added to the cells. 24hrs post-transfection all media was aspirated from the cells and replaced with 2ml fresh complete RPMI media. The transfected cells were harvested 48hrs-post transfection.

2.4.2. Small interfering RNA (siRNA) transfection

RNA interference describes a phenomenon that occurs naturally in cells in which the expression of individual genes is inhibited by interference of the mRNA being transcribed. When double stranded RNAs are made, the enzyme Dicer can snip them to form short interfering RNAs (siRNAs). The short siRNA sections unwind into single stranded RNAs which then combine with proteins to form a complex called RNA-Induced Silencing Complex (RISC). The RISC complex captures a native mRNA molecule complementary to the short siRNA sequence and digests it into RNA fragments which are not translated. By using synthetic 21bp siRNA duplexes this process can be mimicked to achieve specific gene knock-down at the transcriptional level in mammalian cells during culture. Small interfering RNA (siRNA) and scrambles siRNA were designed according to the criteria determined by (Reynolds, Anderson et al. 2006), to the coding region of human CLU, with no homology to any known human sequence. The siRNA duplexes were made to working stocks of 20 µM in RNase-free water.

For transfecting HeLa/293 cells in 6 well plates, $2x10^5$ cells were seeded one day before transfection. The following day, the transfection mix was prepared by mixing 20µM of siRNA or scrambled siRNA with 167.5µl of optiMEM. In a separate tube, 3µl of lipofectamine RNAiMAX (Invitrogen) was diluted in 27µl of optiMEM (0.3%). After mixing gently, both mixtures were incubated at room temperature for 10 minutes. Then the two mixtures were combined, gently flicked and incubated for a further 10 minutes at room temperature. Meanwhile, the cells were washed twice with phosphate-buffered saline (PBS) before adding 800µl optiMEM to the 6 well

dishes. The final transfection mix was then added to the plate and tilted to ensure even distribution. Mock cells were transfected using the method above but with no siRNA included. After 4 hours of incubation at 37 °C with 5% CO_2, the transfection mix was replaced with normal RPMI media containing no antibiotics. Cells were assayed 24 and 48 hours after the transfection and knockdown assessed by RT-PCR, real-time PCR and Western blot.

Gene	Strand	Sequence	application
CLU	sense	5'-AGG AAG AAC CCU AAA UUU A99-3'	siRNA
	anti-sense	5'-UAA AUU UAG GGU UCU UCC U99-3'	
CLU	sense	5'-GGU UUA UAU GAU CUU CAU A99-3'	SCRAMBLE
	anti-sense	5'-UAU GAA GAU CAU AUA AAC C99-3'	

Table 2.2. siRNA duplexes used for knockdown of CLU *in vitro*

2.5. PROTEIN ANALYSIS

2.5.1. Protein extraction

The day prior to harvesting, cultured cells were fed with new complete media and seeded at a density of $5x10^5$cells/ml. Usually, ~$5x10^6$ cells (roughly 10ml of cell suspension) were harvested and washed three times in 10ml of cold PBS, centrifuging for 5 minutes at 1500rpm and 4°C. The final wash was carried out in a 1.5ml eppendorf. After the last wash all supernatant was aspirated on ice. The cell pellet was then either snap-frozen in liquid nitrogen and stored at -80°C for future use or lysed in between 50-500µl of lysis buffer (9M urea, 50mM Tris pH7.5, 15mM β-mercaptoethanol), unless otherwise stated. Following addition of lysis buffer samples were vortexed vigorously for 1 minute and then clarified by centrifugation at 15000rpm for 20 minutes. The supernatant was transferred to a new 1.5ml eppendorf and stored at -20°C.

Protein concentration was determined using the Dc protein assay (Bio-Rad, Hercules, CA). Five standards were prepared by solublising bovine serum albumin (BSA) in a 1:10 lysis buffer/water solution (BSA standards at 2mg/ml, 1mg/ml, 0.5mg/ml, 0.2mg/ml and 0.1mg/ml). These were used to generate a standard curve to quantify test samples by. 5µl of each standard was added in triplicate to a 96 well flat-bottomed plate (Falcon). An aliquot of each sample was then diluted 1:10 in water and 5µl added in triplicate to the plate. 50µl of Reagent A (Bio-rad) was added to each well and the plate gently agitated. Then 200µl of reagent B was added and the plate gently agitated for a further 5 minutes at RT before the absorbance at 630nm was read on a spectrometer plate reader and the protein concentrations calculated.

2.5.2. Immunoblotting

2.5.2.1 Solutions

4x Laemmli loading buffer

250mM Tris pH6.8
40% Glycerol
5% SDS
0.005% Bromophenol blue
Water
10% β-mercaptoethanol added prior to use.

Running Buffer

0.25M Tris base
1.92M Glycine
1% SDS
water

Blocking buffer

5% non-fat milk powder
0.01% Tween 20
PBS

Transfer Buffer

25mM Tris base
0.2M Glycine
20% Methanol
water

Stripping Buffer

62.5mM Tris-HCl (pH6.8)
2% SDS
Water
0.1M β-mercaptoethanol added prior to use.

2.5.2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Typically 30µg of protein lysate was separated by SDS-PAGE using 10% gradient pre-cast polyacrylamide gels (PAG) (Invitrogen). Laemmli loading buffer was added to each sample and then samples were heated at 95°C for 10 minutes, cooled on ice and briefly pulsed before loading onto the gel. Weight markers were prepared and run alongside the protein samples (Kaleidoscope

marker, Bio-Rad). Gels were run (in running buffer) at 90mV for the first 20 minutes and then 125mV for a further 90 minutes, or until the gel front had migrated to the bottom of the gel. Proteins were transferred (in transfer buffer) to a nitrocellulose membrane (Invitrogen) at 28mV for 2.5 hrs using the Novex immunoblotting apparatus. During transfer the transfer tank was packed with ice to prevent heat disruption of the gel or membrane.

2.5.2.3. Immunoblotting

Following blotting the membrane was washed in 0.1% Tween-20 PBS with gentle agitation for 10 minutes. It was then stained for 1 minute with poncaeu S (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich) to confirm successful protein transfer. The membrane was then washed a further three times for 5 minutes in 0.1% Tween-20 PBS. The final wash was replaced with blocking buffer and the membrane incubated for 1hr at RT with gently agitation. Membranes were then probed overnight with primary antibody, diluted to the appropriate concentration with blocking buffer. The following day membranes were washed in 0.1% Tween-20 PBS for 1hr; washes were changed at regular intervals. Secondary peroxidase-labelled antibody diluted in blocking solution was used to incubate the membranes in for 1hr. Following further washing for 1hr, proteins were visualised with the enhanced chemiluminescence technique (ECL) (Amersham, UK) as per manufacture's instructions.

Gene	Species	Supplier	Epitope	Applications	Dilution
CLU	Mouse monoclonal	Vector	7D1	IHC	1:200
CLU	Mouse monoclonal	Upstate	41D	WB, IHC, IF	1:1000
CLU	Mouse monoclonal	Santa Cruz	B-5	WB, IHC, IF	1:1000
DKK3	Goat polyclonal	Santa Cruz	C19	IHC	1:600
DKK3	Goat polyclonal	Santa Cruz	N17	IHC	1:600
AKAP12	Mouse monoclonal	abcam	JP74	IHC	1:500
TIMP1	Mouse monoclonal	Chemicon	8E8.2	IHC	1:25

Table 2.3. Primary antibodies used for Western blotting, Immunohistochemistry and Immunofluorescence

2.5.2.4. Stripping membranes for re-probing

To facilitate re-probing of membranes with different primary antibodies, previous blotting reagents were removed. Initially membranes were washed in 0.1% Tween-20 PBS for 30 minutes, followed by 20 minute incubation in stripping buffer. The membranes were then washed in 0.1% Tween-20 PBS for a period of 4hrs, with wash buffer being exchanged every 30 minutes. Following stripping membranes was re-probed as described in section 2.5.2.3.

2.5.3. Immunohistochemistry

2.5.3.1. Solutions

Antigen retrieval buffer

1mM EDTA
0.1% Tween-20
Water

Tris buffered saline (TBS) pH 7.6

6.05g Tris
8.76g NaCl
1L Water
pH adjust to pH 7.6 with 1M HCl.

2.5.3.2. Immunohistochemical staining with low temperature antigen retrieval

All slides were incubated for 10 minutes in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in running tap water. Sections were incubated in antigen retrieval buffer on a hotplate stirrer at 600rpm, 65°C for 16hrs. Immunostaining was carried out in the Shandon Sequenza Immunostaining Station (Thermo Electron Corp. US) to ensure consistent staining. Slides were mounted in to coverplates (Thermo Electron) and washed in TBS for 5 minutes. Next, 100µl of primary antibody was applied at the appropriate dilution in TBS and incubated at RT for 1hr. The antibody solution was then removed and the slide washed in 0.1% Tween-20 TBS for 5 minutes. 2 drops of Dakochemate Envision Secondary (Dako) was then placed on each section and incubated for 30 minutes. However, for rat primary antibodies a rabbit anti-rat secondary antibody (Dako) was used diluted 1:200 in TBS. Slides were then washed with 0.1% Tween-20 TBS for 5 minutes. The coverplate was then removed from the slide and the slide placed on to a staining rack. Slides were then rinsed with TBS. Staining was visualised by incubating with chromagen (DAB, Dako) for 5 minutes or Novared (Dako) for 30secs. Following visualisation, slides were placed in a coplin jar and washed in water. The

slides were then counterstained in haematoxylin (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich) for 30secs and rinsed in hot water for 2 minutes, followed by cold for 5 minutes. The slides were then dehydrated by sequential incubation for 5 minutes in IMS and xylene. Following dehydration, after the xylene had evaporated from the slides, coverslips were applied with DPX mountant (BDH chemicals, Merck, US).

2.5.3.4. Haematoxylin and Eosin (H&E) staining

H&E staining of sections was carried out by the Queen Elizabeth Hospital's Department of Pathology. The department's automated system was used to ensure consistency of staining.

2.5.3.5. Scoring of staining

Immunohistochemical staining was evaluated subjectively using light microscopy blinded to the data on at least 2 separate occasions by both an observer and a pathologist, either Dr Terry Rollason or Dr Maizaton Abdullah. In the event of intra-observer or inter-observer error a consensus score was decided on after examination by both observers at a multi-headed microscope. Staining was assessed semi-quantitatively in terms of candidate gene expression on a scale of 0 to 3+ according to the intensity of staining, where;

- denotes a complete absence of staining

+/- denotes faint/equivocal staining

1+ denotes weak staining intensity

2+ denotes moderate staining intensity

3+ denotes a strong staining intensity

2.5.4. Immunofluorescent (IF) staining

Cytospins were subjected to heat-activated antigen retrieval prior to IF staining. Slides were submerged in 1L of citric acid buffer pH 6.0 (0.1M citric acid, 0.1M sodium citrate) and microwaved at full power for 20 minutes. After microwaving slides were air-cooled for 5mins then rinsed under a running tap for 10 minutes. Following that the slides were washed in 1% tween-20 PBS for 5 minutes. Cytospins were permeablised in 0.5% Triton X-100 for 5 minutes and then rinsed with 1% tween-20 PBS. The cells were then blocked with 100µl of 20% HINGS for 1hr. The blocking solution was then removed and the cells incubated for 1.5hrs with 50µl of primary antibody diluted in 20% HINGS at 1:100. Following this cells were washed by repeatedly passing 1% tween-20 PBS over the slides (6 passes in total). Excess PBS was then removed from the cells and replaced with 50µl of fluorophore-conjugated secondary antibody diluted in 20% HINGS at 1:1000 (Alexa Fluor 594 anti-mouse, Molecular Probes). Slides were then incubated in the dark for 1.5hrs and washed as described previously. DAPI nucleic acid stain (Molecular Probes, Invitrogen) was used to counterstain the cell nuclei. 150nM of DAPI diluted in PBS was applied to the cytospin for 1 minute and then washed in 1% tween-20 PBS for 10 minutes. Slides were then mounted with coverslides using the ProLong Antifade kit (Molecular Probes, Invitrogen) and viewed using a fluorescence microscope.

2.6. RNA ANALYSIS

2.6.1. RNA Extraction

RNA was extracted from cells using the RNA Easy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and quality of RNA was assessed using the Nanodrop spectrometer (NanoDrop Technologies, US). The $A_{260/280}$ of RNA samples was required to be between 1.9 and 2.1. RNA stocks were diluted to 1mg/ml for routine use. All RNA samples were stored at -80°C.

2.6.2. cDNA synthesis

cDNA synthesis was carried out under RNase-free conditions, on ice unless otherwise stated. 1µl of Random Primers (Promega, 20µg vial) and 1µl dNTPs (Invitrogen, 10mM) were added to 500ng of RNA in 0.5ml thin walled PCR tubes (Eppendorf) and the volume made up to 13µl with DEPC-treated water. The samples were then incubated at 65°C for 5 minutes and cooled rapidly on ice to denature RNA and prohibit re-annealing. cDNA mastermix was prepared (Table 2.4) and 7µl added to each RNA sample. Reaction mixtures were then subject to incubation at 25°C for 5 mins (annealing step), 50°C for 60 mins (extension step), 70°C for 15 mins (inactivation/denaturation step) and then held at 4°C or kept at -20°C for longer term storage. Incubations carried out in a Thermal Cycler.

Reagent	Volume (µl)	Supplier
5x First strand buffer	4	Invitrogen
DTT (0.1M)	1	
Superscript III reverse transcriptase	1	
RNase Out	1	

Table 2.4. cDNA mastermix

2.6.3 Semi-quantitative-reverse transcriptase polymerase chain reaction

1µg of cDNA was then amplified by addition of 12.5µl of 2x PCR mastermix (Promega) and 5µl of a 2.5pmole/µl mix of 5'sense primers and 3'anti-sense (1:1 ratio). Reaction mixes were then subject to appropriate thermal cycling in a Thermal Cycler. In semi-Q-RT-PCR 5 reaction mixtures were prepared for each sample, and these were subjected to either 15, 20, 25, 30 or 35 thermal cycles.

Step number	Cycle step	Time (mins)	Temperature (°C)
1	Initial denaturing	5	95
2	Denaturing	0.75	95
3	Annealing	1	x
4	Extension	2	72
5	Final extension	5	72
6	Hold/terminate	HOLD	4

Table 2.5. RT-PCR thermal cycle program

Annealing temperatures (x) and cycles are given in table 2.6.

Gene	Strand	Sequence	application	Annealing temperature (°C)	cycles	Product size (bp)
DKK3	sense	5'-TTC ATC CAG CAG TGT TGC TC-3'	RT-PCR	60	28	238
	anti-sense	5'-GGT GTG GGG TAG TGG AGA GA-3'				
KLF4	sense	5'-CCC ACA CAG GTG AGA AAC CT-3'	RT-PCR	60	25	169
	anti-sense	5'-ATG TGT AAG GCG AGG TGG TC-3'				
RNASET2	sense	5'-ATT CAC TCG TTT CCC AAT CG'3'	RT-PCR	60	33	225
	anti-sense	5'-TAC TCT GGC AAG GGC ATC TT-3'				
CADM1	sense	5'-CCC CAG CCT GTG ATG GTA A-3'	RT-PCR	43	30	198
	anti-sense	5'-GGA TAG TTG TGG GGG GAT CGT A-3'				
AKAP12	sense	5'-GTA GCT GAG CAA GAT GAG CTC-3'	RT-PCR	60	28	191
	anti-sense	5'-CCG CTG ACT TAG TAG CCA TCT-3'				
TIMP1	sense	5'-AAT TCC GAC CTC GTC ATC AG-3'	RT-PCR	44	25	297
	anti-sense	5'-TGC AGT TTT CCA GCA ATG AG-3'				
CLU V1	sense	5'-GCG AGC AGA GCG CTA TAA AT-3'	RT-PCR	60	35	232
	anti-sense	5'-CCC TGA TTG GAC ATT TCC TG-3'				
CLU V2	sense	5'-AGA TGG ATT CGG TGT GAA GG-3'	RT-PCR	60	35	203
	anti-sense	5'-CCC TGA TTG GAC ATT TCC TG-3'				
GAPDH	sense	5'-CCA CCC ATG GCA ATT CCA TGG CA-3'	RT-PCR	60	25	187
	anti-sense	5'-TCT AGA CGG CAG GTC AGG TCC AC-3'				

Table 2.6. Primers used for RT-PCR

All primers supplied by Alta Bioscience, UK. Stocks prepared at 100pM by dilution in RNase-free water and stored at -20°C. Where possible oligonucleotides for RT-PCR were designed to span an exon junction, have a GC content of between 40-60%, 3'-end content of <40%, low self or cross annealing potential and minimal hairpin structure.

2.6.4. Agarose gel electrophoresis

Agarose gel electrophoresis is commonly used to separate both DNA and RNA molecules on the basis of size. DNA and RNA molecules are negatively charged so migrate through gels towards the cathode. Agarose gels generally have relatively large pore sizes compared to other gel matrixes so are suitable for large molecules such as DNA or RNA. To some degree pore size can be determined by agarose concentration, with low concentrations of agarose providing better separation of large molecules than high concentrations and vice versa. A 0.8% gel will show good separation (resolution) of large DNA fragments (1–10kb) and a 2% gel will show good resolution for small fragments (0.2–1kb). In order to visualise DNA/RNA molecules once separated, ethidium bromide is incorporated into the gel matrix as this intercalates with the DNA/RNA molecules. Under ultraviolet illumination (UV light) the intercalated ethidium bromide emits light showing the position of the RNA/DNA molecules on the gel, and the use of a marker containing fragments on known size, allows estimations of band size to be made.

2.6.4.1. Solutions

Tris-boric acid-EDTA (TBE) buffer

108g	Tris base
55g	Boric acid
9.3g	EDTA
1L	water

6x Gel loading dye

25mg	bromophenol blue
25mg	xylene cyanol FF
3ml	glycerol
Water to a final volume 10ml	

2.6.4.2. Agarose gel electrophoresis of amplified products

Agarose gels were prepared by dissolving agarose powder (Invitrogen, Paisley, UK) in 1xTBE (Sigma-Aldrich Ltd, Gillingham, Dorset, UK) by heating in a microwave on medium power for 2-5 minutes. As described above the percentage agarose used depended on the application, molecules below 1kb were analysed on 2% (w/v) gels and molecules above 1kb were visualised on 0.8% (w/v) gels. Once cooled ethidium bromide was added at a final concentration of 1μg/ml (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich). The solution was then poured into a prepared gel tray and an appropriate sample comb inserted. Once set, the comb and tray barriers were removed and the gel and tray placed into the electrophoresis tank previously filled with 1x TBE buffer. Bromophenol blue loading dye was added to each PCR sample at a 1:5 dilution. Samples were then loaded into the gel and run at 90V using a Powerpac (Bio-rad laboratories Inc., Hercules, California, USA), until the dye front had migrated ~2cm from the end of the gel. A molecular weight marker (100bp ladder, Promega) was run alongside the samples. Intercalated DNA was visualised under UV light using a transilluminator (Bio-rad laboratories Inc., Hercules, California, USA).

2.6.5. Quantitative-RT-PCR (Q-RT-PCR)

Q-RT-PCR uses a fluorogenic probe to enable the detection of a specific PCR. As in standard PCR, Q-RT-PCR amplifies a specific region of DNA located between two primers. A probe, which is specific to a sequence in the region flanked by the two PCR primers and that is labelled at the 5'-end with a fluorophore (FAM or VIC) and at the 3'-end with a quencher (TAMRA or MGB), is also included in the reaction. The Taq polymerase used possesses a 5'-3' exonucleases activity. Thus, amplification of the target DNA sequence results in digestion of the fluorogenic probe and in turn release of the fluorophore from the quencher. Consequently, the amount of fluorescence is directly proportional to the amount of DNA amplification. The accumulation of fluorophores is monitored digitally at every thermal cycle. Relative quantitation is enabled by monitoring at which thermal cycle each different sample reaches a specific level of fluorescence; the cycle threshold (Ct).

RNA was extracted for Q-RT-PCR as described in section 2.6.1 and cDNA generated as described in section 2.6.2. Prior to proceeding all equipment to be used was irradiated under UV light to remove any DNA contaminations. PCR reactions were performed in 96-well reaction plates (Applied Biosystems, US) and set-up on ice. All samples were run in triplicate to allow analysis using the delta CT method. 50ng of cDNA were used per well (1µl). In addition 19µl of mastermix was added to each well (Table 2.20). Pre-mixed primer sets and probe (Assays-on-demand, Applied Biosystems) were used to amplify endogenous control and target gene (Table 2.21). All primer sets amplify a region crossing an exon junction.

Reagent	Volume (µl)	Supplier
Taqman universal mastermix	10	Applied Biosystems
20x GAPDH endogenous control	1	
20x (target gene) primer and probe mix	1	
DEPC-treated water	7	Ambion

Table 2.7. Q-RT-PCR Mastermix

Target gene	Probe fluorescent reporter dye	Product details
CLU	FAM	Hs00156548_m1
CADM1	FAM	Hs00204937
GAPDH	VIC	Human endogenous control 4352934E (VIC/TAMRA)
B2M	VIC	Human endogenous control 4333766T (VIC/MGB)
PGK1	VIC	Human endogenous control 4333765T (VIC/MGB)
18S	VIC	Human endogenous control 4310879E (VIC/TAMRA)

Table 2.8. Q-RT-PCR TaqMan® primer and probe kits (Applied Biosystems)

After the mastermix was added to all samples the wells were sealed with optically clear caps (Applied Biosystems) and the 96 well plate centrifuged at 1200rpm for 5 minutes at 4°C to remove air bubbles. The plate was then placed in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) and subjected to 40 thermal cycles.

Step number	Cycle step	Time (secs)	Temperature (°C)
1	Enzyme activation	120	50
2	Denature	600	95
3*	Denature	15	95
4*	Anneal/extend	60	60

Table 2.9. Q-RT-PCR thermal cycle program

*Steps 3 and 4 were sequentially repeated 40-times.

2.6.5.1 The comparative Ct method (ddCt) for relative quantitation of gene expression

The delta-delta (dd)Ct method (Livak and Schmittgen, 2001) enables relative quantitation of transcripts without the need for standard curves when looking at expression levels of a target gene relative to an endogenous control (e.g.GAPDH). The ddCt was used to calculate the n-fold change in the amount of target gene transcripts (CLU, CADM1) present in one cell type relative to another, for example the n-fold change in CLU mRNA transcripts in HeLa cells relative to the level of CLU mRNA transcripts in CaSki cells. In order to calculate this the difference (dCt) between the Ct values of the target gene and the endogenous gene was calculated for each sample studied (dCt = target Ct – endogenous Ct). The sample selected as the 'baseline' sample for expression of the target gene is referred to as reference sample. The difference between each of the samples dCT and the reference samples dCT was calculated, generating the ddCt value for each sample (ddCt = reference dCt – target dCt). The ddCt for each sample was then converted to an absolute value using the following equation: n-fold change in expression level = $2^{-ddCt.}$ The n-fold change in expression of target genes studied by Q-RT-PCR was calculated in this way.

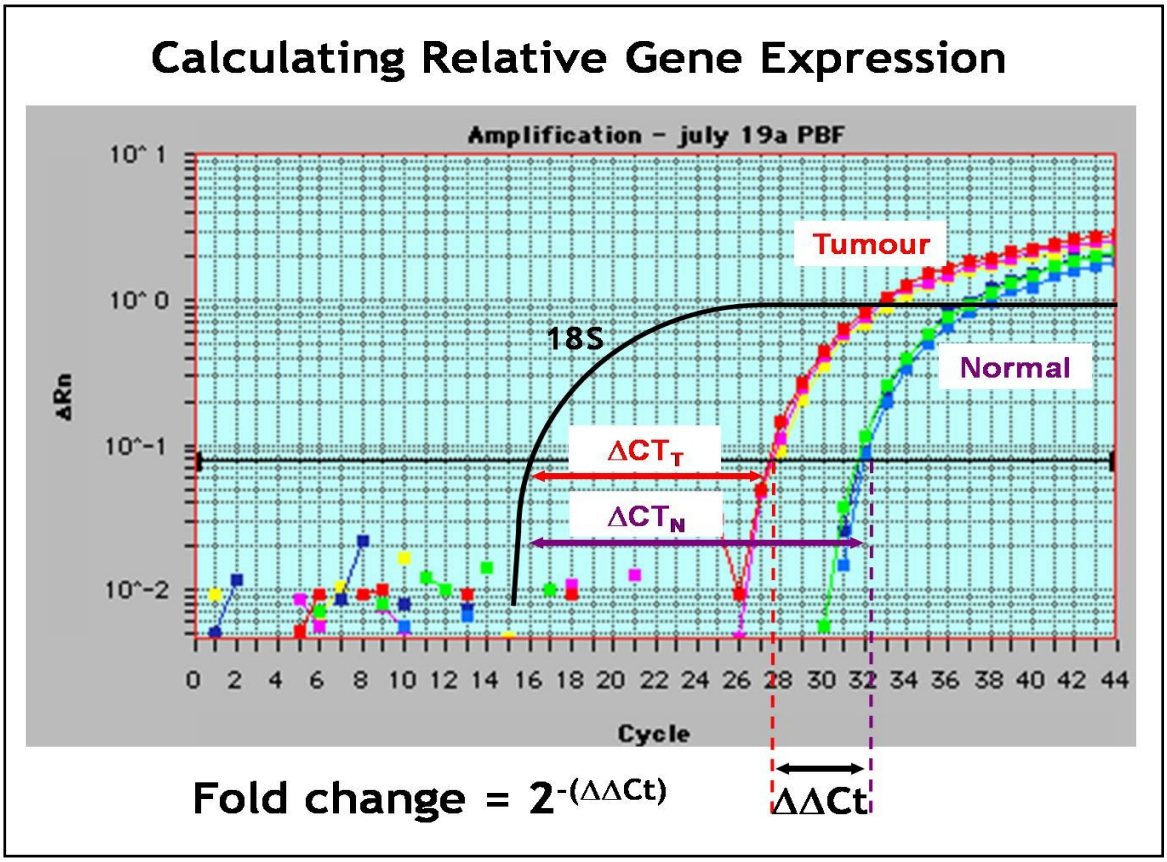


Figure 2.1: Calculation of relative gene expression using ddCt method with the ABI Prism 7700 Sequence Detection System (Applied Biosystems)

2.7. *IN VITRO* FUNCTIONAL STUDIES

2.7.1. Cell Viability

Following cell culture media was removed and cells were washed with PBS, detached with trypsin–EDTA, neutralized with complete medium, centrifuged and re-suspended in PBS. An aliquot of cell suspension was diluted with 0.4% trypan blue (Sigma-Aldrich Ltd, Gillingham, Dorset, UK–Aldrich), pipetted onto a hemocytometer and counted under a microscope at 40x magnification. Live cells excluded the dye, whereas dead cells admitted the dye and consequently stained intensely with trypan blue. The number of viable cells for each experimental condition was recorded. Trypan blue was used as rapid initial screen to check if cells were viable for subsequent transfection and apoptosis studies. PI was used as part of the dual staining procedure to measure apoptosis with Annexin V post transfection quantitatively.

2.7.2. Apoptosis assay

Multiple lipofectamine transfections (section 2.4.2.1) were performed to produce sufficient cells for the viability study. 24hrs post-transfection cells were pooled and washed 3-times by centrifugation at 1500rpm for 5 minutes in RPMI media supplemented with either 10% or 1% foetal calf serum depending on study requirements. Cells were then resuspended at $3x10^5$ cells/ml in RPMI media supplemented with the appropriate amount of foetal calf serum. In 48-well tissue culture plates 500µl of cell suspension was added to 5 wells in triplicate for each time point to be studied (0, 24, 48, 72 and 96 hrs post-transfection). Cells were then cultivated as normal without

additional feeding. At each time point cells were harvested and viability and apoptosis studied by annexin-V FITC/PI staining.

Loss of plasma membrane is one of the earliest features of apoptosis. Annexin V has a high affinity for phospholipid phosphatidylserine, which is exposed to the external cellular environment in apoptotic cells permitting binding. Annexin-V specifically binds to the fluorochrome FITC and is therefore used to stain for early stage apoptotic cells. Later stages of apoptosis and necrosis include the loss of membrane integrity and so Annexin V is used in combination with propidium iodide, which is a vitality dye that is only taken up by dead cells due to their increased membrane permeability. Therefore, cells which are Annexin V positive but PI negative are in the early stages of apoptosis with the cell membrane still intact, with an absence of necrosis.

In order to measure cell viability and apoptosis cells were stained with FITC-labelled annexin-V and PI using the Annexin V-FITC apoptosis detection kit I (BD Biosciences) following manufacturers instructions. Cells were stained in a volume of 100 µl 1 x binding buffer which contained 5 µl Annexin V-FITC and propidium iodide at 50 ng/ml. Cells were stained in the dark for 15 minutes before being analysed by FACS-Becton Dickson FACS-Calibur to detect apoptosis. An aliquot of 10,000 cells were analysed per condition. Green fluorescence was detected using FL1 channel and red fluorescence was detected using FL3 channel. Cell Quest Software (version 3.1f) displayed the results as a bivariate dot plot of Annexin V (FL1) and PI

(FL3) fluorescence intensity. A two-dimensional dot plot was generated of annexin V-PE versus PI fluorescence allowing the differentiation of viable, apoptotic and necrotic cells.

2.7.3. Proliferation assay

The MTS cell proliferation assay (Promega, Madison, WI) was used to measure cell growth over time according to the manufacturer's instructions. The MTS dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, is converted to a formazan product that is soluble in tissue culture medium and quantified by its absorbance at 492 nm. Cell numbers were counted after the desired incubation time using a Beckmann Coulter counter. The cell number was proportional to MTS dye conversion within the range used in these experiments.

The day prior to the experiment cells were fed with new complete media and seeded at a density of $5x10^5$cells/ml. The following day, cells were washed three times in RPMI media supplemented with either 1% or 10% foetal calf serum, centrifuging for 5 minutes at 1500prm. Cells were then resuspended at $3x10^5$ cells/ml. A separate 96-well tissue culture plate was used for each time point (0, 24, 48 and 72hrs) and 300μL of cell suspension ($9x10^5$ cells/well) was added to wells in triplicate. Cells were then cultivated as normal without feeding.

2.7.4 TNFα stimulation

To establish whether expression of CLU in HeLa cells regulated NF-kB in a TNF-dependent or independent manner, cells were transfected with CLU siRNA or scrambled siRNA (section 2.4.2). At 24, 48 and 72 hours post-transfection the cells were stimulated with 20mg/ml TNFα for 3 hours. Subsequently luciferase reporter assays for NF-kB reporter activity were carried out (section 2.7.5).

2.7.5. Luciferase reporter assays

The Dual-Luciferase® Reporter (DLR™) Assay System provides an efficient means of performing two reporter assays. In the DLR™ Assay, the activities of firefly and *Renilla* luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a luminescent signal lasting at least one minute. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding Stop & Glo® Reagent to the same sample. Luciferase reporter assays for NF-κB activity were carried out according to the manufacturer's instructions.

2.8. DNA METHYLATION

2.8.1. DNA extraction

Solutions

DNA lysis buffer	Precipitating solution
0.2 ml 10X PCR buffer	1 ml absolute ethanol
1.8 ml D/R water	40 µl 3M sodium acetate (pH 5.2)
10µl Tween 20	1 µl glycogen

2ml of DNA lysis buffer was made up immediately prior to extractions. Pelleted cells were washed with 1xPBS and pelleted at 3000rpm for 5 minutes. The supernatant was discarded and cell pellets resuspended in 400ul of prepared lysis buffer with 13µl Proteinase K (Sigma-Aldrich Ltd, Gillingham, Dorset, UK Aldrich) and incubated overnight at 55°C. The following day 400µl phenol (Sigma-Aldrich Ltd, Gillingham, Dorset, UK Aldrich) was added to each sample and vortex mixed until the solution appeared milky. After centrifugation at 13000rpm for 5 minutes the upper phase was aspirated and transferred to a fresh tube containing 400µl phenol and 400µl chloroform (Sigma-Aldrich Ltd, Gillingham, Dorset, UK Aldrich). Samples were vortex mixed, centrifuged for 5 minutes at 13000rpm and the upper phase aspirated and transferred into a fresh tube containing 400µl chloroform. This was followed by a final vortex mix and centrifugation at 13000rpm for 5 minutes. The upper phase was very carefully transferred into a fresh tube containing precipitating solution, ensuring that no lower phase was transferred. Samples were precipitated at -80°C for a minimum of 24 hours.

The following day samples were centrifuged at 13000rpm, 0°C for 20 minutes and the resulting supernatant discarded. 800µl of ice cold 70% ethanol was added, samples were vortex mixed and centrifuged at 13000rpm, 4°C for 20 minutes. The resulting supernatant was discarded and 400µl room temperature 70% ethanol added to pellets, proceeded by a vortex mix and centrifugation at 13000rpm, 4°C for 10 minutes. After discarding the final supernatant samples were dried at 37°C for up to 30 minutes until all ethanol had evaporated. Finally, DNA pellets were reconstituted in 30µl of pre-warmed Dnase/Rnase free water and stored at -20° C.

2.8.2. MSP primer design

A source database search was carried for the gene of interest, e.g.CLU (http://genome-www5.stanford.edu/cgi-bin/source/sourceResult). The upstream genomic sequence plus the first exon was retrieved and copied into the Methprimer design program (http://www.urogene.org/methprimer/index1.html). The desired characteristics of MSP primer sequences were a length of 15-30bp, G+C content of 40-60%, no stretches of sequence complementary within or between primers and a G or C base at the 3' end. The options 'pick MSP primers' and 'use CpG island prediction' were selected to generate a graphical representation of the CpG island(s) and a list of suitable unmethylated and methylated primers given. Where possible primers were designed to meet these criteria and basic local alignment search tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed in all cases to ensure that primers were not complimentary to other regions of the genome and bound specifically to target sequences.

2.8.3. Sodium Bisulphite modification of DNA

In this procedure, all unmethylated cytosines are deaminated and sulfonated, converting them to uracils, while 5-methylcytosines are protected from this modification. Bisulphite conversion of DNA results in uncomplimentary single-stranded DNA molecules, and subsequent PCR analysis results in incorporation of thymines in the place of the converted uracils. 3M NaOH (Sigma-Aldrich Ltd, Gillingham, Dorset, UK) was added to 1-10µg DNA and incubated at 37°C for 15 minutes. During this incubation 3M NaOH was added to Sodium Bisulphite (Sigma-Aldrich Ltd, Gillingham, Dorset, UK) to give a 3.12M solution which was warmed to 37°C and kept in the dark. After 15 minutes pre-warmed hydroquinone (Sigma-Aldrich Ltd, Gillingham, Dorset, UK) was added to the sodium bisulphite and vortex mixed vigorously. 200 l of this was added to DNA samples, which were placed in a thermocycle and subject to 20 cycles of heating to 99°C for 15 seconds followed by 50°C for 15 minutes and subsequently held at 20°C for a maximum of 16 hours to avoid precipitation of the sodium bisulphite. Following this, samples were purified using the Wizard® DNA Clean-Up System (Promega) according to the manufacturer's protocol.

2.8.4. Methylation Specific PCR

10µl of modified DNA was amplified by addition of 12.5µl of 2x PCR mastermix (Promega) and 5µl of a 2.5pmole/µl mix of 5'sense primers and 3'anti-sense (1:1 ratio). Reaction mixes were then subject to appropriate thermal cycling in a Thermal Cycler, with unmethylatd and methylated reactions carried out in parallel. Resulting amplified products were analysed by agarose gel electrophoresis a described in section 2.6.4.

Step number	Cycle step	Time (mins)	Temperature (°C)
1	Initial denaturing	5	95
2	Denaturing	0.75	95
3	Annealing	1	x
4	Extension	2	72
5	Final extension	5	72
6	Hold/terminate	HOLD	4

Table 2.10. MSP thermal cycle program

Annealing temperatures (x) and cycles are given in table 2.11.

	Gene	Strand	Sequence	Application	Annealing temperature (°C)	cycles	Product size (bp)
U	DKK3	sense	5'-TTT TGG TTT TTT TTT GTT TTT GGG-3'	MSP	60	40	179
		anti-sense	5'-CCA AAC CAC TAC ATC TCC ACT-3'				
M		sense	5'-CGG TTT TTT TTC GTT TTC GGG-3'	MSP	60	40	175
		anti-sense	5'-CAA ACC GCT ACA TCT CCG CT-3'				
U	DKK3	sense	5'-GGT TAA TGG TTG GGT TGT GG-3'	MSP	61	40	215
		anti-sense	5'-AAA ACC ACC AAA CTA CAA ATA CAA ATA-3'				
M		sense	5'-TTG GTT AAT GGT AGG GTT GC-3'	MSP	62	40	211
		anti-sense	5'-GCC GAA CTA CGA ATA CAA ATA CGA A-3'				
U	KLF4	sense	5'-ATT AGT TTG TAG TTT TGT GTT ATG-3'	MSP	57	40	187
		anti-sense	5'-TCT TTA AAT TAA ATA TAA CTT AAA AAC ATC-3'				
M		sense	5'-GAT TAG TTC GTA GTT TCG CGT TAC-3'	MSP	57	40	185
		anti-sense	5'-AAA TTA AAT ATA ACT TAA AAA CGT-3'				
U	KLF4	sense	5'-GAA TTT AGG GAG TTG ATA ATG GTG G-3'	MSP	60	40	151
		anti-sense	5'-ACA ACT AAC AAA ACT AAA ACC AAA CTA ACA-3'				
M		sense	5-GAA TTT AGG GAG TCG ATA ATG GC-3'	MSP	60	40	155
		anti-sense	5'-CTA ACG AAC TAA AAC CGA ACT AAC G-3'				
U	RNASET2	sense	5'-GGT GTT TGT GTG GTG AAG GAA TGT AGT TGT T-3'	MSP	59	40	180
		anti-sense	5'-TCC ACT ACA ACA ACA ACC ACC AAA TAC ACC C-3'				
M		sense	5'-TTT GCG TGG CGA AGG AAC GTA GTC GTT-3'	MSP	61	40	183
		anti-sense	5'-TAC AAC AAC GAC CAC CGA ATA CGC CCG-3'				
U	RNASET2	sense	5'-TAT GAT TTT GAA GGG GAG TTT TGA-3'	MSP	61.5	40	192
		anti-sense	5'-CCT ACT ACC CAA AAA AAA CTT CCA A-3'				
M		sense	5'-GTA CGA TTT TGA AGG GGA GTT TC-3'	MSP	60	40	187
		anti-sense	5'-CTA CCC GAA AAA AAC TTC CGA-3'				
U	CADM1	sense	5'-TAG GTT AGA TGT ATT TGG TGT GTG G-3'	MSP	60	40	204
		anti-sense	5'-AAA TTC AAA TTT TAC TTT CCC CAA A-3'				
M		sense	5'-GTT AGG TTA GAT GTA TTC GGT GTG C-3'	MSP	60	40	204
		anti-sense	5'-ATT CGA ATT TTA CTT TCC CCG A-3'				
U	AKAP12	sense	5'-TTG GGT TGT TTT TGT AGT TTT AGT TG-3'	MSP	60	40	130
		anti-sense	5'-AAC CAA AAA CAC TAC AAC ACA CC-3'				
M		sense	5'-GGG TCG TTT TCG TAG TTT TAG TCG-3'	MSP	60	40	133
		anti-sense	5'-CAA AAA CGC TAC GAC GCG CC-3'				
U	TIMP1	sense	5'-GGT GTA TTT TTT TTT GTT GGT TT-3'	MSP	60	40	187
		anti-sense	5'-CTC TAA ACA CTA AAT CTA CAA CA-3'				
M		sense	5-GTG TAT TTT TTT TCG TCG GTT C-3'	MSP	60	40	183
		anti-sense	5'-CTA AAC GCT AAA TCT ACG ACG-3'				
U	TIMP1	sense	5'-TTT ATT TAG TTT GGT TTG TGG-3'	MSP	51.5	40	234
		anti-sense	5'-TTA ACA ATA AAT ATT TAA AAA ACA AT-3'				
M		sense	5'-GTT TGT TTA TTT AGT TTG GTT TGC-3'	MSP	54.5	40	239
		anti-sense	5'-TAA CGA TAA ATA TTT AAA AAA CGA T-3'				
U	CLU	sense	5'-TGT TTT GGA TTG GGA TAG ATA GTT G-3'	MSP	60	40	210
		anti-sense	5'-CTT TCT AAA AAC CAA AAA ATA AAA ACC A-3'				
M		sense	5'-GTT TTG GAT TGG GAT AGA TAG TCG-3'	MSP	60	40	204
		anti-sense	5'-TAA AAA CCG AAA AAT AAA AAC CGA T-3'				

Table 2.11. Primers used for MSP

All primers supplied by Alta Bioscience, UK. Stocks prepared at 100pM by dilution in RNase-free water and stored at -20°C

2.8.5. Bisulphite genomic sequencing

DNA was bisulphite modified as in section 2.8.3 and amplified as in section 2.8.4 using the primers and cycling conditions below.

Gene	Strand	Sequence	Application	Annealing temperature (°C)	Product size (bp)
CLU 1	sense	5'-GGA TAG TGA TTG TTT AAG TT-3'	BGS	60	317
	anti-sense	5'-ACA CAA TCT CAC AAT TCC CTA ACC CTA C-3'			
CLU 2	sense	5'-GTA GGG TTA GGG AAT TGT GAG ATT GTG T-3'	BGS	60	483
	anti-sense	5'-TCT CTA CCT AAC TAC CAT CCC CTA C-3'			
CLU 3	sense	5'-GTA GGG GAT GGT AGT TAG GTA GAG A-3'	BGS	60	254
	anti-sense	5'-ACA AAT CTC CAA ATC TCA ATT TCC C-3'			
CLU 4	sense	5'-GGG AAA TTG AGA TTT GGA GAT TTG T-3'	BGS	60	367
	anti-sense	5'-CCT CTC CAA ACC TCA AAT TAT CAT CTA T-3'			
CLU promoter	sense	5'-TGT GTT TTG GAT TGG GAT AGA TAG T-3'	BGS	60	282
	anti-sense	5'-CAC CCC CTT TAA AAC TAA CTA CAA AC-3'			
CLU p3	sense	5'-GTT TGT AGT TAG TTT TAA AGG GGG TG-3'	BGS	60	204
	anti-sense	5'-TCT CTA CCT AAC TAC CAT CCC CTA C-3'			
CLU promoter A	sense	5'-TGT AGT TAG TTT TAA AGG GGG TGT G-3'	BGS	60	282
	anti-sense	5'-CTC TAC CTA ACT ACC ATC CCC TAC C-3'			
CLU promoter B	sense	5'-ATT GTG TTT TGG ATT GGG ATA GAT A-3'	BGS	60	200
	anti-sense	5'-CAC ACC CCC TTT AAA ACT AAC TAC A-3'			
M13	sense	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	BGS	60	286
	anti-sense	5'-TCA CAC AGG AAA CAG CTA TGA C-3'			

Table 2.12. Primers used for BGS

All primers supplied by Alta Bioscience, UK. Stocks prepared at 100pM by dilution in RNase-free water and stored at -20°C

The resulting PCR products were subsequently cloned using the pGem T Easy Vector system II (Promega) according to the manufacturer's guidelines. A minimum of 10 colonies were then amplified by PCR using cycling condition as in section 2.7.4 and 5µl analysed by agarose gel electrophoresis (section 2.6.4) to confirm inserts. The remaining amplified product was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

Sequencing was performed using the PE Applied Biosystems 3100 ABI prism™ capillary sequencer. The labelling reactions were set up using 30-50ng of PCR product, made up to 11µl with sterile distilled water, 1µl (80ng) of primer, 2µl of Big-Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and 6µl of 2.5x sequencing buffer (200mM Tris HCl, 5mM $MgCl_2$, pH9.0). The labelling PCR was run for 30 cycles at 95°C for 10s, 50°C for 5s and 60°C for 4 minutes. The labelled products were then precipitated with 50µl absolute ethanol and 2µl 3M sodium acetate (pH4.6) in 0.5ml eppendorfs at room temperature for 15 minutes. The samples were then micro-centrifuged at 20,000 x *g* for 20 minutes. The supernatant was removed and the pellet washed with 250µl of 70% (v/v) ethanol, mixed by gentle flicking and spun at 20,000 x *g* for 5 minutes. The supernatant was then removed and pellets dried for at least 1hr. 10µl of 'Hi-Dye™' (Applied Biosystems) was then added to the dry tubes to dissolve the DNA pellet, and samples were denatured at 97°C for 5 minutes then put on ice immediately to prevent reduplexing.

The buffer chambers of the 3100 capillary sequencer were filled with 2.5x EDTA buffer (PE Applied Biosystems) and the water chambers filled accordingly. 3100 POP6™ Performance Optimised Polymer was used to fill the 50cm sequencer capillary array (PE Applied Biosystems). The 3100 data collection software version 1.0 was set for sequencing at: Dye Set, E; Mobility File, DT3100POP6(BD)v2.mob; BIOLIMS Project, 3100_Project 1; Run Module 1, StdSeq50_POP6DefaultModule and Analysis Module 1, BC-3100_SeqOff.saz. The samples were loaded into the appropriate 96 well plates and run for approximately 2.5hrs. Following the run,

the extracted data files were analysed using the ABI sequencing analysis programme version 3.6.1.

2.8.6.1. Pyrosequencing primer design

All primers were designed using Biotage PSQ primer design software. The genomic sequences were obtained from the UCSC genome browser (http://genome.ucsc.edu) and the sequences were then *in silico* bisulfite converted. The chosen sequence was used to generate both biotinylated and non- biotinylated primers under the allele quantification assay type settings. Primers were required to be no more than 200bp in length and between 18-24 bp in size. 4 sets of primers were designed to span the CLU promoter and after initial optimisation of the PCR reaction only set 1 taken forward.

Gene	Strand	Sequence	Application	Annealing temperature (°C)	Cycles	Product size (bp)
CLU 1	sense	5'-AGA TTG TGT TTT GGA TTG GG-3'	Pyrosequencing	55	50	158
	anti-sense	5'-CRC ATC ATC ACC ACR AAT AAC TAT AC-3'				
	Probe	5'-TGT TTT GGA TTG GGA T-3'				
CLU 2	sense	5'-GGG TTT TTT TTG GAG TTA GTA TAG-3'	Pyrosequencing	55	50	177
	anti-sense	5'-CCA CAC ACC CCC TTT AAA-3'				
	Probe	5'-TTT TTT GGA GTT AGT ATA GT-3'				
CLU 3	sense	5'-GGT ATT TTT TGG GAG TGA GTT ATG TAG GT-3'	Pyrosequencing	55	50	247
	anti-sense	5'-CCA ACT ATA TCA TCC CTC TCT ACC T-3'				
	Probe	5'-GTT TGT AGT TAG TTT TAA AG-3'				
CLU 4	sense	5'-TAG GGG ATG GTA GTT AGG TAG AGA-3'	Pyrosequencing	55	50	245
	anti-sense	5'-CCA AAT CTC AAT TTC CCC ATC TTT A-3'				
	Probe	5'-TAC CCC CCC ACC ACA-3'				

Table 2.13. Primers used for pyrosequencing

All primers supplied by Alta Bioscience, UK. Stocks prepared at 100pM by dilution in RNase-free water and stored at -20°C

2.8.6.2. Pyrosequencing

DNA was bisulphite modified for pyrosequencing using the EZ DNA methylation gold kit (Zymo research) according to the manufaturer's instructions. DNA was subsequently amplified using 25µl hotstart taq mastermix (Thermo Scientific), 5pmol biotinylated primer and 10pmol each of biotinylated primer and sequencing primer as in section 2.8.4 using the primers in table 2.13.

Step number	Cycle step	Time (mins)	Temperature (°C)
1	Initial denaturing	15	95
2	Denaturing	0.25	95
3	Annealing	0.5	55
4	Extension	0.5	72
5	Final extension	10	72
6	Hold/terminate	HOLD	4

Table 2.14. Pyrosequencing thermal cycle program

Steps 2-4 were repeated for 50 cycles of denaturation, annealing and extension.

A Pyromark ID system (Biotage) was used for pyrosequencing reactions according to the manufacturer's instructions. Briefly, 3 µl streptavidin beads (GE healthcare) in 37 µl of binding buffer (Biotage) had 40 µl of biotinylated PCR product added in a 96 well plate and were left shaking for 5 minutes at 1300 rpm. Next, 15 pmol sequencing primer and 38.5 µl annealing buffer were added to each well of a sequencing plate (Biotage). Beads were captured using a vacuum preparation tool, and lowered into each of the following solutions for 5 s: 70% ethanol, denaturation buffer (Biotage) and wash buffer (Biotage). Following this, beads were released onto the sequencing plate, placed on a heat block at 80º C for 2 minutes and left in the machine to cool. The Pyrogold SQA reagents (Biotage) were loaded into the reagent cartridge and the plate run.

2.8.7. 5-aza-2-deoxycytidine cell treatment

When cells were around 50% confluent 20μM of 5-Aza-2'-deoxycitidine (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich Ltd, Gillingham, Dorset, UK) was added to cell and incubated overnight at 37°C and 5% CO_2. At 24 hour intervals for a period of 3 days both media and 5-Aza-2'-deoxycitidine were replenished. On the 4th day media was removed and replaced with media containing 0.3mM Trichostatin A and cells incubated overnight at 37°C and 5% CO_2. The following day cells were harvested as in section 2.3.2 and RNA extracted as in section 2.5.1, followed by cDNA synthesis (section 2.6.2), and analysis by RT-PCR (section 2.6.3).

2.9. Statistical analysis

All statistical analyses carried out on data obtained for this research thesis (t-tests and ANOVAs) were performed using R and MS Excel functions.

Chapter 3: Results 1

Validation of candidate genes in cervical neoplasia

3.1 Identification of candidate tumour suppressor genes in cervical neoplasia

Promoter hypermethylation has emerged as an important mechanism for tumour suppressor gene inactivation. By inhibiting DNA methylation and/or HDAC activity in cells in culture, it is possible to reverse gene silencing and transcriptionally reactivate epigenetically silenced genes (Stresemann, Bokelmann et al. 2008; Stresemann and Lyko 2008). Identification of candidate TSG in cervical neoplasia had been previously initiated in our laboratory in an experiment in which the cervical cancer cell lines, HeLa, CaSki, SiHa and C33a had been treated with 5-Aza-2'-deoxycitidine and Trichostatin A. Gene expression profiling of these cell lines subsequently showed that this treatment resulted in the up-regulation of 364 genes. A literature search confirmed that a number of these up-regulated genes were already known to be tumour suppressor genes at other sites of cancer. I assisted in this process of identifying candidate genes by searching published micro-array datasets for genes which were up-regulated by the viral oncogenes HPV E6/E7; or up-regulated following HPV integration; or up-regulated in invasive cancer compared with normal tissue (Nees 2001, Duffy 2003, Alawazi 2002). Following these searches, a short list of seven candidate genes was selected. Six of these genes (DKK3, KLF4, RNASET2, TIMP1, AKAP12, CLU) were found to be increased in a post-treatment sample compared to at least one pre-treatment sample, following demethylation of cervical cancer cell lines.

DKK3 and AKAP12 had not previously been reported to be methylated in cervical tissue at the time of this study, but methylated forms have been reported at other sites (Jin, Hamilton et al.

2008; Lee, Jo et al. 2009); RNASET2 expression has been reported in cervical tissue but methylated forms have not been identified (Smirnoff, Roiz et al. 2006). A seventh gene, CADM1, was added to this list although it was not present on the array because it had recently been reported to be epigenetically silenced in cervical cancer (Steenbergen, 2004)

3.2. Confirmation of candidate gene expression in cervical cell lines

Before investigating whether candidate genes were up-regulated post-demethylation it was important to establish their baseline expression in cervical cell lines. Figure 3.1 shows, using RT-PCR, the expression of candidate genes in the cervical cancer cell lines HeLa, CaSki, SiHa and C33a, compared with their expression in a primary cell line normal human foreskin keratinocytes (NHFK) as a positive control. GAPDH was used as a loading control for gene expression levels. Single discrete bands of the expected size of amplified products were detected for all genes in at least one of the cervical cancer cell lines.

TIMP1 is equally expressed in all of the four cervical cell lines compared with NHFK. KLF4 is expressed at a lower level in SiHa and C33a compared with NHFK, HeLa and CaSki, possibly suggesting its expression is associated with HPV infection. RNASET2 is expressed at comparable levels in NHFK, in the HPV 16 positive cell lines CaSki and SiHa, but expression is not detectable in the HPV 18 cell line HeLa and very high levels of expression were detected in the HPV negative cell line C33a. It expression has been previously reported in cervical tissue (Smirnoff, Roiz et al. 2006), however its expression was induced in HeLa post demethylation and

so these results are not surprising. AKAP12 is expressed weakly but at a comparable level to that of NHFK in HeLa and SiHa, highly expressed in C33a and expression was not detectable in CaSki. It has been reported to be methylated at other sites (Jin, Hamilton et al. 2008) and so it is possible that it is epigenetically silenced in these HPV positive cell lines, but given that it is expressed in HPV 16 positive SiHa and weekly in HPV18 positive HeLa, but not in HPV 16 positive CaSki, its expression is unlikely to be due to HPV infection. Given that its expression levels are comparable in NHFK, it expression in cell lines does not seem to be significant.

DKK3 and CADM1 show expression in C33a comparable to that of NHFK and do not appear to be expressed in the three HPV positive cervical cell lines HeLa, CaSki and SiHa, which is consistent with the data suggesting that they are epigenetically silenced in HPV positive cervical cell lines (Steenbergen, Kramer et al. 2004; Lee, Jo et al. 2009), CLU on the other hand was expressed at a higher level in all of the cervical cell lines compared with NHFK, especially in C33a, which is suggested of an oncogene, not a tumour suppressor gene. Its expression is unlikely to be associated with HPV infection given that its expression was similar in HPV 18 positive HeLa, HPV 16 positive CaSki and SiHa and HPV negative C33a. Variability in expression of all candidate genes in these cell lines might have a range of explanations, including the fact that different cell lines have unique origins, acquire different mutations during culture, and as mentioned have different infecting HPV types.

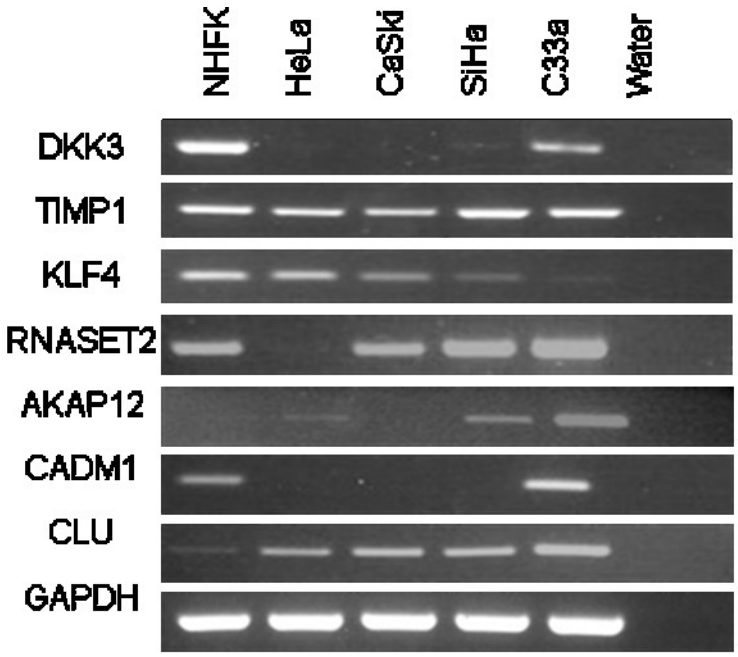


Figure 3.1: RNA expression in cervical cell lines compared with normal human foreskin keratinocytes (NHFK).

Expression of candidate genes in a range of cervical cancer cell lines was compared with that in normal foreskin keratinocytes to evaluate differential expression. Cell lines demonstrated a range of expression levels for all candidate genes tested.

3.2.1. Screening of candidate gene expression pre and post demethylation of the HeLa cell line using RT-PCR

Due to depletion of the RNA used in the initial demethylation experiment, and to confirm candidate genes, I repeated the demethylation experiment. I decided to restrict this experiment to the HeLa cell line because all of my candidate genes had been found to be up-regulated on the array in one or more of the treated replicates from this cell line.

Semi-quantitative RT-PCR was used to investigate changes in the expression of candidate genes following demethylation of HeLa. Figure 3.2 compares the expression of the candidate genes under consideration in untreated HeLa cells and in HeLa cells treated with increasing concentrations of the demethylating agent 5-Aza-2'-deoxycitidine. The results show up-regulation of DKK3, TIMP1, AKAP12, RNASET2, CADM1 and CLU post-demethylation. There is no evidence for the up-regulation of KLF4. These findings are consistent with the possibility that DKK3, TIMP1, AKAP12, CADM1 and CLU are epigenetically down-regulated in HeLa cells. The results also show that a concentration of 20μM of 5-Aza-2'-deoxycitidine is sufficient to increase the expression of these genes in HeLa cells.

3.2.2. Screening of candidate gene expression pre and post demethylation of HPV 18 transfected cervical and foreskin keratinocyte cell lines using RT-PCR

To establish whether epigenetic silencing of the candidate genes occurred in a wider panel of cell lines I extended these experiments to include the demethylation of untransformed cell lines. I examined the impact of demethylation on two human keratinocyte cell lines: one of these was a cervical keratinocyte cell line transfected with HPV 18; and the other, a foreskin keratinocyte cell line also transfected with HPV18. Figure 3.3 compares the expression of the candidate genes under consideration in untreated cells and in cells treated with 5-Aza-2'-deoxycitidine. The results show up-regulation of DKK3, TIMP1 and CADM1 following demethylation of both cell lines, and are consistent with these genes being epigenetically regulated. AKAP12 and CLU were up-regulated post demethylation of the HPV18 positive foreskin keratinocytes but not following demethylation of cervical keratinocytes. Furthermore, CLU was not expressed in pre or post treatment cervical keratinocytes.

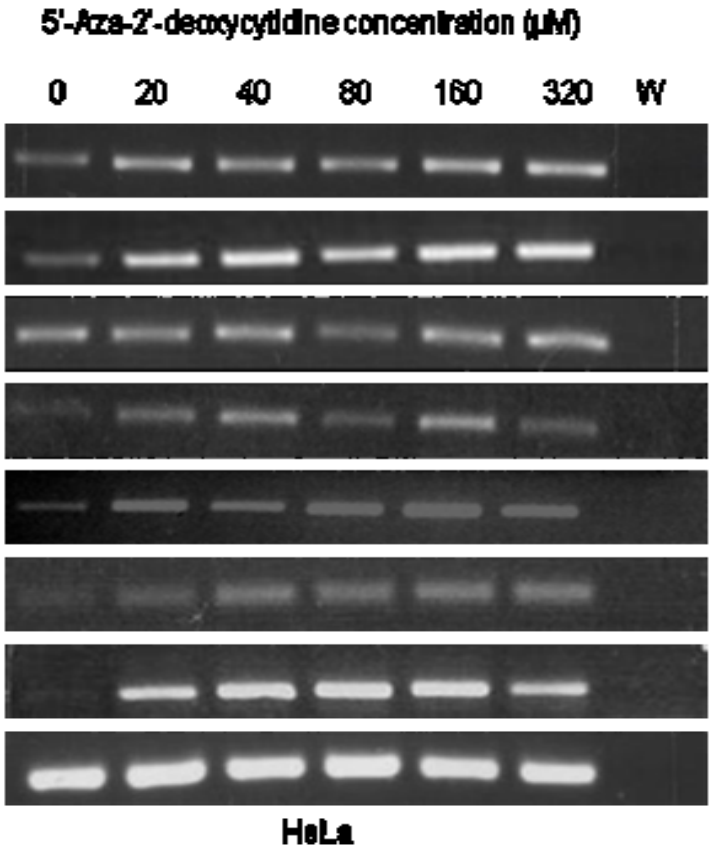


Figure 3.2: RNA expression pre- and post-demethylation of HeLa.

Expression levels of candidate genes were examined before (0) and at post-demethylation over a range of 5'-Aza-2'-deoxycytidine concentrations. DKK3, TIMP1, RNASET2, AKAP12, CADM1 and CLU were up-regulated following demethylation.

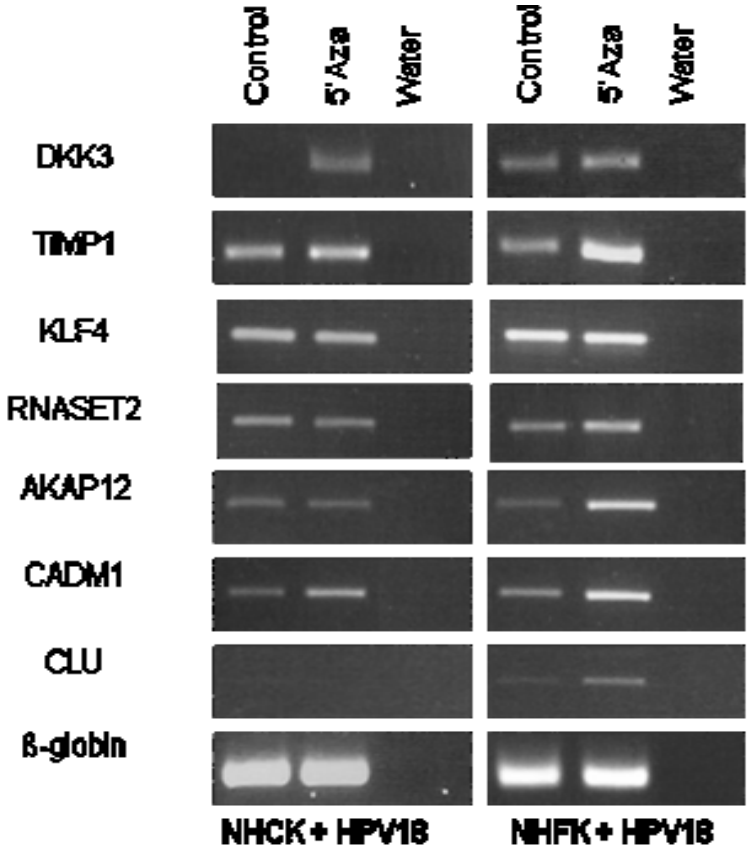


Figure 3.3: RNA expression pre- and post- demethylation of HPV 16 positive cervical and foreskin keratinocytes.

Expression levels of DKK3, TIMP1, CADM1, AKAP12 and CLU were examined pre and post demethylation of keratinocytes using 5'Aza2'deoxycytidine at 20μM. DKK3, TIMP1 and CADM1 were upregulated following demethylation in both cell lines, where as AKAP12 and CLU were only upregulated post demethylation of the foreskin keratinocytes.

3.3. Investigation of the presence of methylated forms of candidate tumour suppressor genes in cervical cancer cell lines

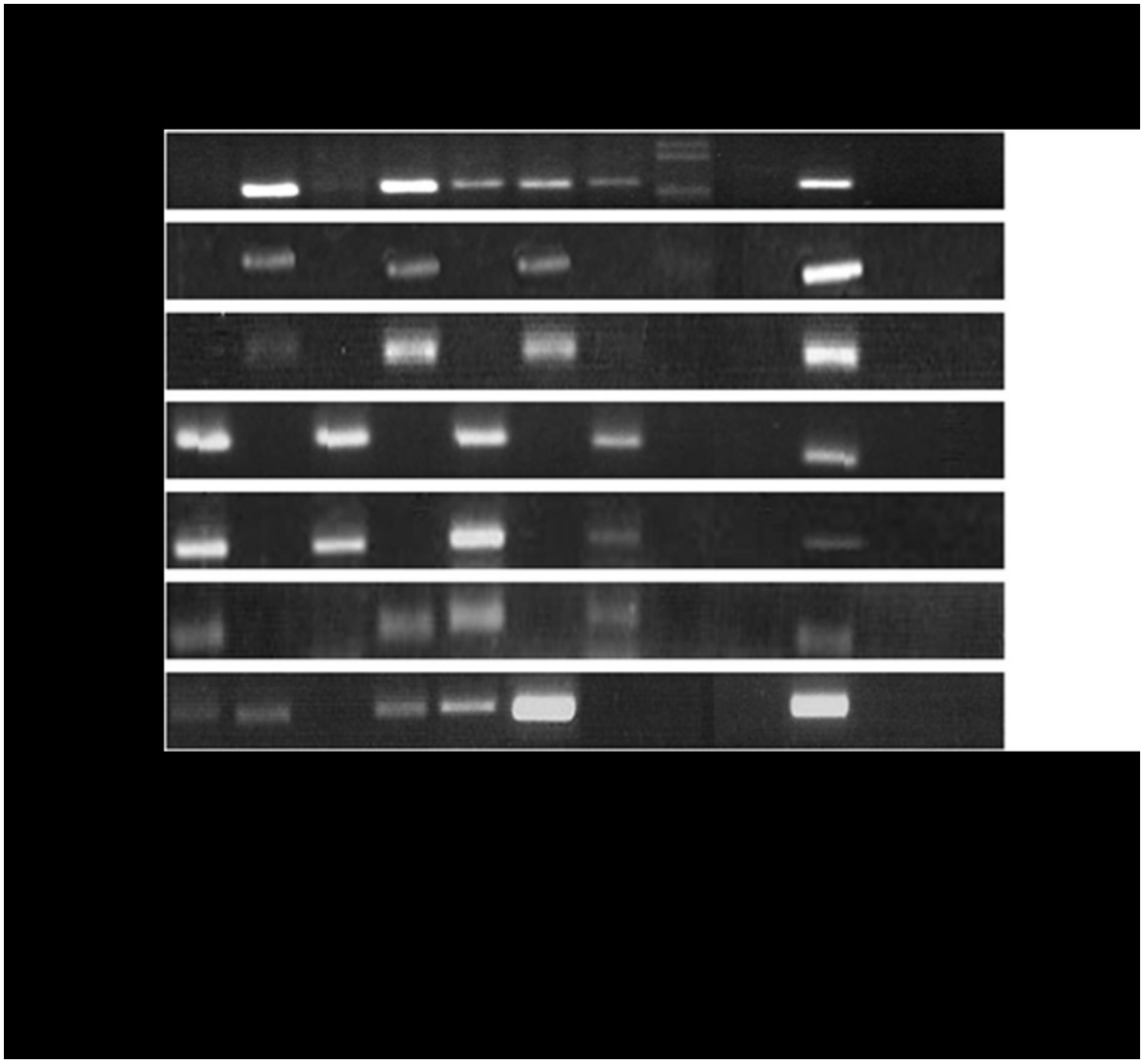
Methylation of epigenetically silenced genes often occurs at their promoters (Gronbaek, Hother et al. 2007). To establish the methylation status of the promoters of the candidate genes, I used methylation specific PCR (MSP) to determine the presence or absence of methylated and unmethylated DNA in the cervical cancer cell lines HeLa, CaSki and C33a (Figure 3.4). Single discrete bands of the expected size of amplified PCR products were detected for all primer sets and controls behaved as expected throughout. This is with the exception of the MSP for RNASET2 which infrequently produced a doublet of bands at the expected size of the amplified product. Where evidence of methylation was detected, results were repeated alongside unmethylated PCR reactions to confirm that the results were reproducible.

DKK3 appears almost entirely as methylated forms in HeLa and CaSki, with a weak unmethylated band also present in CaSki, whereas in C33a the presence of both methylated and unmethylated forms can be detected. TIMP1 and CADM1 are methylated in all three cell lines, but for KLF4 and RNASET2 only unmethylated forms can be detected. AKAP12 is unmethylated in HeLa and C33a, with detection of both methylated and unmethylated forms in CaSki. CLU is methylated in CaSki compared with the detection of unmethylated and methylated forms in HeLa and C33a; interestingly it is very heavily methylated in the HPV negative cell line C33a. The presence of both unmethylated and methylated products could be due to a mixed cell population with some cells being methylated while others are not, or mixture of methylated and

unmethylated alleles within these cell types reflecting that the population as a whole is heterogeneous. Of the six genes which showed a change in expression following demethylation of HeLa, four were found to be methylated in this cell line (DKK3, TIMP1, CADM1 and CLU), and two were not (AKAP12 and RNASET2). It is possible that AKAP12 and RNASET2 are regulated by acetylation since they were not methylated in HeLa yet showed transcriptional up-regulation post treatment with 5-Aza-2'-deoxycitidine and Trichostatin A. KLF4, which showed no change in expression following demethylation of HeLa, was found to be unmethylated in this cell line.

With the exception of KLF4, no change in methylation status was detected by MSP for any of the candidate genes post demethylation of HeLa cells (Figure 3.5). We would have expected to see an increase in the amount of unmethylated form and a decrease in methylated. However, the technique of MSP is naturally biased towards the detection of methylated forms and the PCR for unmethylated forms is not as sensitive as that for the methylated. MSP is not a quantitative method and small changes in methylation status will be easily missed, thus further analysis using a quantitative method such as bisulphite genomic sequencing is required before conclusions can be drawn. Human male DNA was used as a methylated control, and human foetal DNA as an unmethylated control. Whereas methylated control DNA always tested positive for methylated forms, a satisfactory unmethylated control was difficult to obtain. Both the commercially available unmethylated controls, and PBMCs isolated from laboratory donors, did not always test positive for unmethylated product, and when they did, they often yielded both methylated and unmethylated product. Therefore, the presence of a weak unmethylated product from any of the

genes tested cannot be ruled out since MSP is known to be biased towards detecting methylated DNA even when only 0.1% of alleles are methylated (Herman, Graff et al. 1996; Liu and Maekawa 2003).



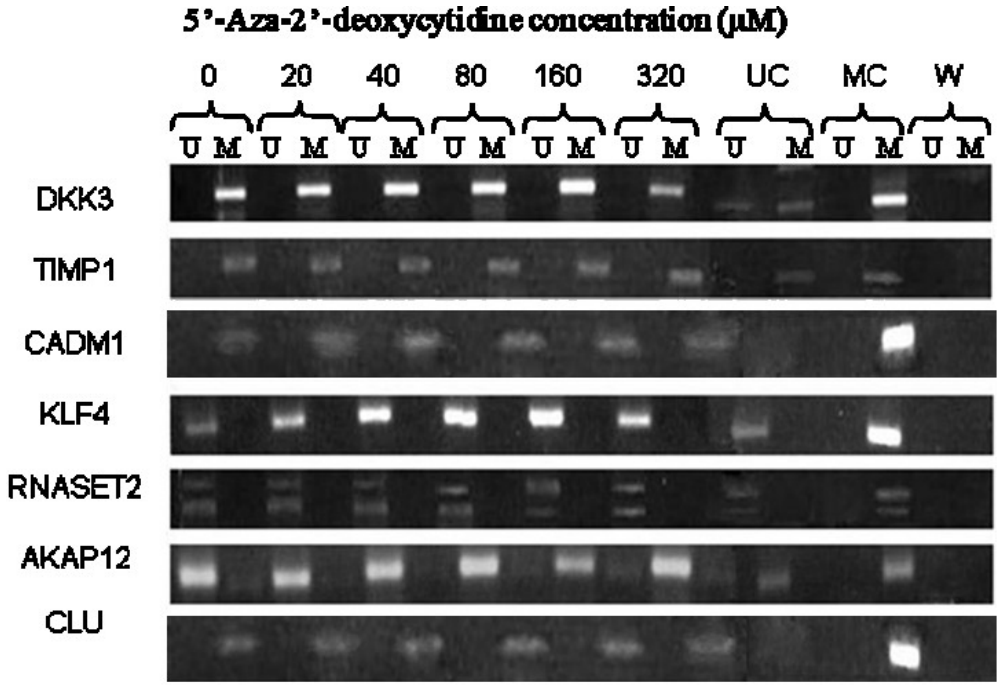


Figure 3.5: Methylation specific PCR (MSP) of candidate genes post demethylation of the HeLa cell line

No change in methylation status was detected by MSP for any of the candidate genes post demethylation of the cervical cell line HeLa. Controls used were placental DNA as an unmethylated contol and sperm DNA as a positive control for methylation.

3.4. A longitudinal investigation of epigenetic and transcriptional changes in candidate genes using the W12 cell line disease-progression model

The W12 cell line was originally generated from a woman with HPV 16 associated low-grade CIN (Stanley, Pett et al. 2007). At low passage, the cell line contains approximately 100 HPV episomes per cell and with serial passaging there is episomal loss and integrated clones emerge. Episomal W12 cells recapitulate the phenotypic characteristics of CIN I. When grown in organotypic raft culture, W12 cells have been shown to become progressively more dysplastic in successive passage. Therefore W12 cells can be used as an *in vitro* model for the longitudinal study of epigenetic changes contributing to disease progression.

Using MSP, I investigated the methylation status of candidate gene promoters in passage 11 of the W12 cell line, where there are still some episomal clones and the integrated passage 22. Figure 3.6 compares the methylation status of the candidate genes under consideration in these passages. Methylated forms for KLF4, RNASET2 and AKAP12 cannot be detected in either the pre- or post-integration passage of W12. DKK3 is unmethylated in passage 11 and becomes methylated by passage 22. TIMP1, CLU and CADM1 are weakly methylated at passage 11 and become heavily methylated by passage 22 of W12. This phenotype of progressive increase in methylation for DKK3, TIMP1, CADM1 and CLU was confirmed when the methylation status of these genes was examined in the intervening passages between 11 and 22 (Figure 3.7) However, these data are not quantitative, and changes in methylation status measured using pyrosequencing will be described in section 3.7.

Notwithstanding these changes in methylation status, there was no compelling evidence of transcriptional down-regulation on serial passage of W12 (Figure 3.8). However, there does appear to be some up-regulation of DKK3 and TIMP1 between passages 11 and 13 as there is episomal loss in W12. For other genes it is possible that transcriptional changes in any of the candidate genes are so slight that they cannot be detected by RT- PCR; this possibility is explored in a subsequent section by testing for transcriptional changes using Q RT-PCR (section 3.6) Alternatively, if the lack of transcriptional change is real, then it is possible that the increases in promoter methylation observed are insufficient to affect transcriptional change, or that methylation may precede transcriptional changes which will only become apparent in passages of the W12 beyond those tested here.

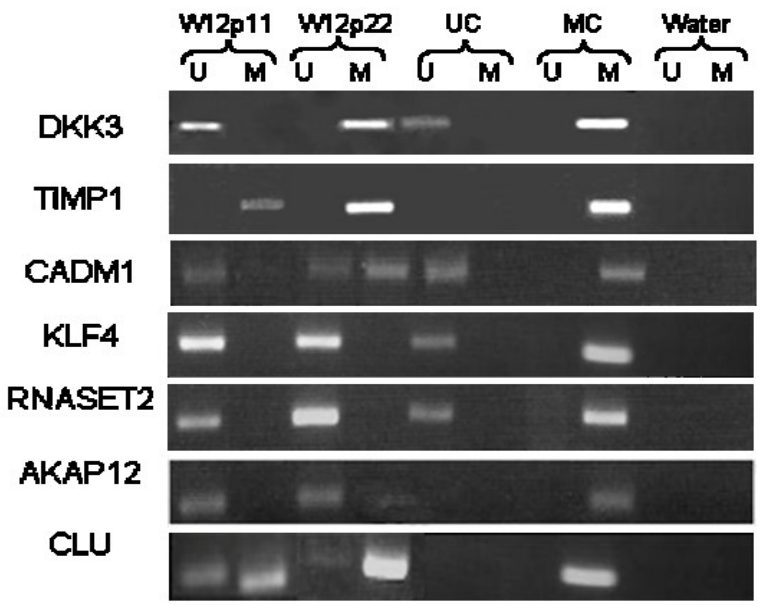


Figure 3.6: Methylation specific PCR (MSP) of candidate genes in W12 passages 11 and 22

Examination of the methylation status of candidate genes demonstrated that DKK3, TIMP1, CADM1 and CLU were methylated in W12 post integration passage 12. Of these, DKK3 and CADM1 were unmethylated at pre-integration passage 11, CLU was hemi-methylated and TIMP1 weakly methylated. KLF4, RNASET2 and AKAP12 are unmethylated in pre and post integration passages of W12.

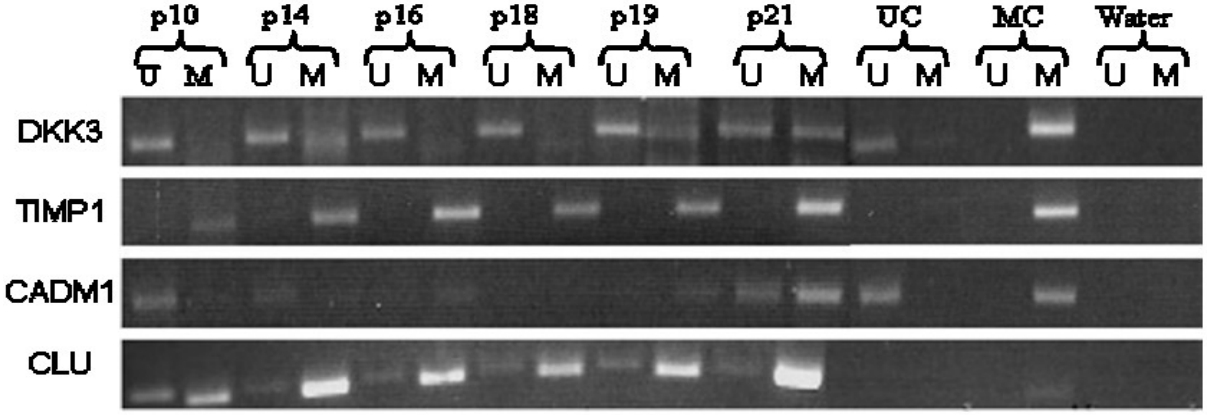


Figure 3.7: Methylation specific PCR (MSP) of candidate genes in W12 passages 10 to 21

Examination of the methylation status of candidate genes demonstrated that DKK3, CADM1 and CLU were increasingly methylated in serial passage of W12.

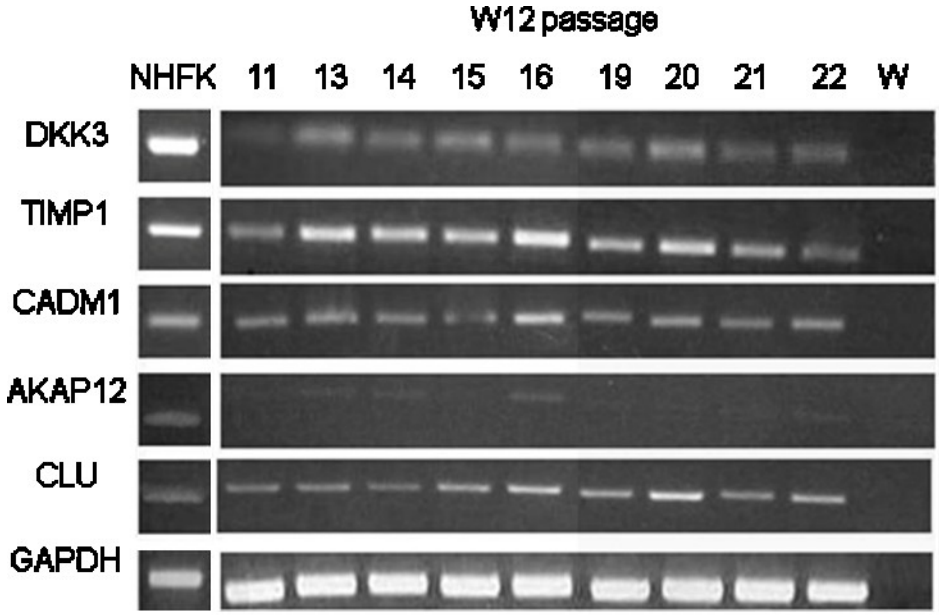


Figure 3.8: RNA expression of candidate genes in W12 passages 11 to 22

Expression of candidate genes in serial passage of the W12 cell line demonstrated a range of expression levels for all candidate genes tested. NHFK were amplified alongside samples as a positive control for the PCR.

3.5. Re-evaluation of candidate genes

At this stage, based on their expression and methylation status in cervical cancer cell lines and the W12 cell line model, the genes DKK3, TIMP1, CADM1, AKAP12 and CLU were taken forward for analysis *in vivo*. KLF4 was not pursued as methylated forms could not be detected in the cervical cancer cell line HeLa, CaSki and C33a or in the W12 model, nor was it transcriptionally up-regulated post demethylation. RNASET2 demonstrated transcriptional up-regulation post demethylation of HeLa and normal human foreskin keratinocytes transfected with HPV18 (NHFK), however was unmethylated by MSP in cervical cancer cell lines. DKK3, TIMP1, CADM1, AKAP12 and CLU were all up-regulated at the RNA level post demethylation of HPV18 positive HeLa, NHCK and NHFK. In addition, methylated forms could be detected in the three cervical cell lines, HeLa, CaSki and C33a for DKK3, TIMP1, CADM1 and CLU, and in CaSki for AKAP12. With serial passage of the W12 cell line model all five of these genes showed an increase in promoter methylation, coinciding with loss of episomes and viral integration. It has been shown by other groups that CADM1 gene silencing via promoter hypermethylation is a frequent event in the progression from high-risk HPV-containing, high-grade CIN lesions to invasive cervical cancer (Steenbergen, Kramer et al. 2004; Overmeer, Henken et al. 2008).

3.6. Pattern of candidate gene expression in cervical cancer, CIN and normal tissue

To evaluate the expression of the candidate genes DKK3, TIMP1, AKAP12, CADM1 and CLU in more detail, their expression at the RNA and protein level was investigated in cervical tissue. Two cohorts have been set up including one natural history cohort which included women who were referred for investigation of cytological abnormality; this cohort was be used for correlation of methylation of the CLU promoter to changes in CLU expression. A further study population included 37 paraffin fixed sections of cervical intraepithelial neoplasia, including CIN I, II and III, which were used to validate expression of candidate genes. Details of both cohorts are described in materials and methods chapter 2.2.

3.6.1. RNA expression in CIN

RT-PCR was used to investigate the pattern of expression of candidate genes in RNA extracted from fresh frozen biopsies taken from women with CIN as part of a cohort study (described above). Expression levels were compared with those seen in normal human foreskin keratinocytes (NHFK). The results show that all genes were expressed in NHFK (Figure 3.9). TIMP1 is expressed in all patients; DKK3 was weakly expressed in some samples, (patients 1, 2, 4 and 5) but not in others (3, 6, 7, 8, and 9). CADM1, AKAP12 and CLU are not expressed in any of the patient samples tested.

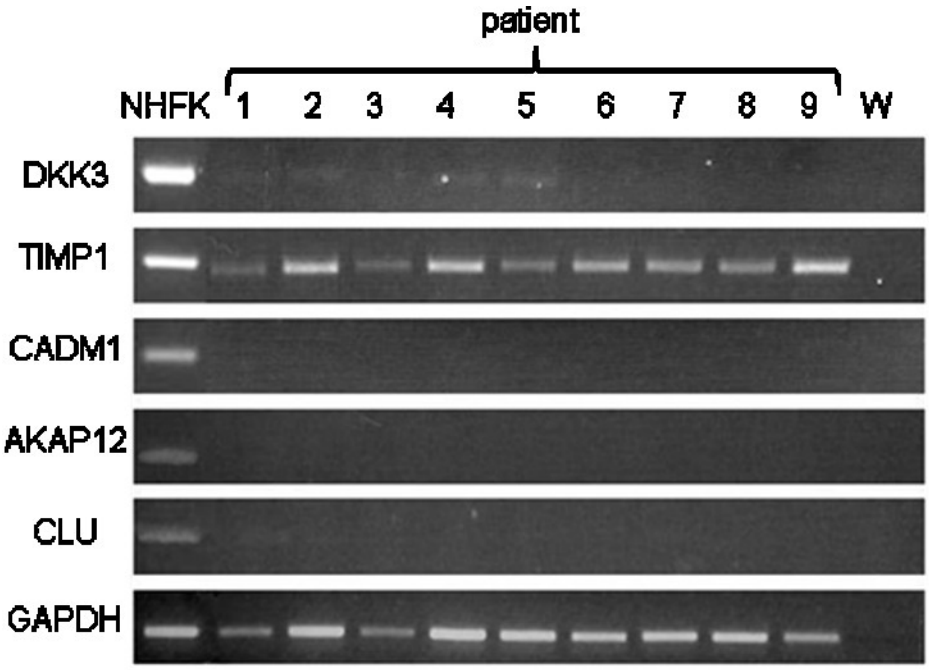


Figure 3.9: RNA expression in cervical tissue.

Expression of candidate genes in a cohort of primary cervical tissue. Expression of DKK3 , CADM1, AKAP12 and CLU was lower in primary tissue compared with normal human foreskin keratinocytes (NHFK).

3.6.2 Protein expression in cervical cancer, CIN and normal tissue

The pattern of protein expression of candidate genes was investigated in a panel of 37 biopsies taken from women with cervical intraepithelial neoplasia (CIN) and compared to biopsies taken from normal cervical epithelium. The pattern of expression of each of the candidate genes was first assessed in control tissue. The AKAP12 antibody stains outside of the germinal centre and in blood vessels with very few positive cells in the germinal centre. The DKK3 antibody stains kidney cells in the cytoplasm. TIMP1 is distinctly cytoplasmic in normal breast tissue. Strong clusterin expression in the germinal centre and a few positive staining in the mantle zone. The CADM1 antibody available at the time of this investigation was poor quality, very occasionally staining in the germinal centres, but on the whole was largely negative.

Prior to staining a panel of normal cervical epithelium and CIN, sections were stained with haematoxylin and eosin to examine the morphology of the cells and confirm their diagnosis (Figure 3.10). In normal squamous epithelium section shows normal keratinised squamous epithelium. In CIN I there are dysplastic squamous cells involving lower one third of the entire epithelium. The cells exhibit large hyperchromatic nuclei with abundant cytoplasm. In CIN II dysplastic squamous cells involving lower two third of the epithelium. The cells exhibit high nuclear-cytoplasmic ratio. In CIN III dysplastic cells involve the entire thickness of the squamous epithelium. Koilocytic changes are apparent.

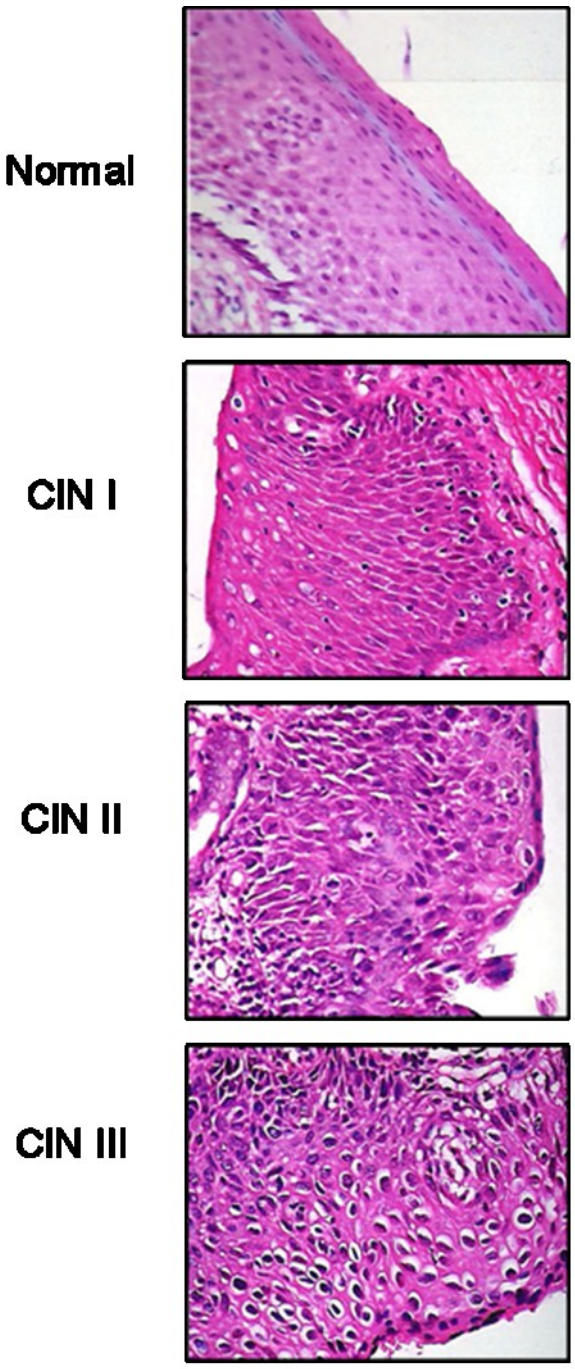


Figure 3.10 : Haematoxylin and Eosin staining of normal cervical tissue and cervical intraepithelial neoplasia (CIN).

Normal cervical epithelium and CIN sections were stained with haematoxylin and eosin to examine the morphology of the cells and confirm their diagnosis

Immunohistochemical staining of a panel of 37 cases of CIN compared with normal cervical tissue revealed an up-regulation of CLU and no difference in expression of DKK3, TIMP1 and AKAP12 between normal cervical epithelium and high grade CIN, as summarised in figure 3.11 and in table 3.1. AKAP12 staining in CIN III shows hyperchromatic nuclei with cytoplasmic and weak nuclear staining; nuclear in areas of necrosis which are immunoreactive. CADM1 results could not be interpreted as the antibody available at the time of this study was of poor quality and unsuitable for immunohistochemistry. The most striking pattern of staining was that of CLU, which demonstrated only sparse, weak expression in CIN I and was strongly positive in CIN III and there was an absence of staining in invasive tissue. In CIN a regular basal layer and underlying stroma is evident, with the differentiated squamous epithelium showing strong cytoplasmic staining of CLU. In contrast, in adenocarcinoma and squamous carcinoma of the cervix there is a complete absence of CLU staining. Where CLU staining was present in cervical tissue it was always cytoplasmic in localisation, not nuclear, which is in agreement with published data using these CLU antibodies for staining of paraffin fixed tissues (Ha and Califano 2006; Park, Yeo et al. 2006; He, Liu et al. 2009). Cytoplasmic expression of CLU is consistent with an anti-apoptotic function and since no necrotic tissue was present in these tissue sections of normal cervical epithelium and CIN no pro-apoptotic nuclear CLU was detected (Albert, Gonzalez et al. 2007).

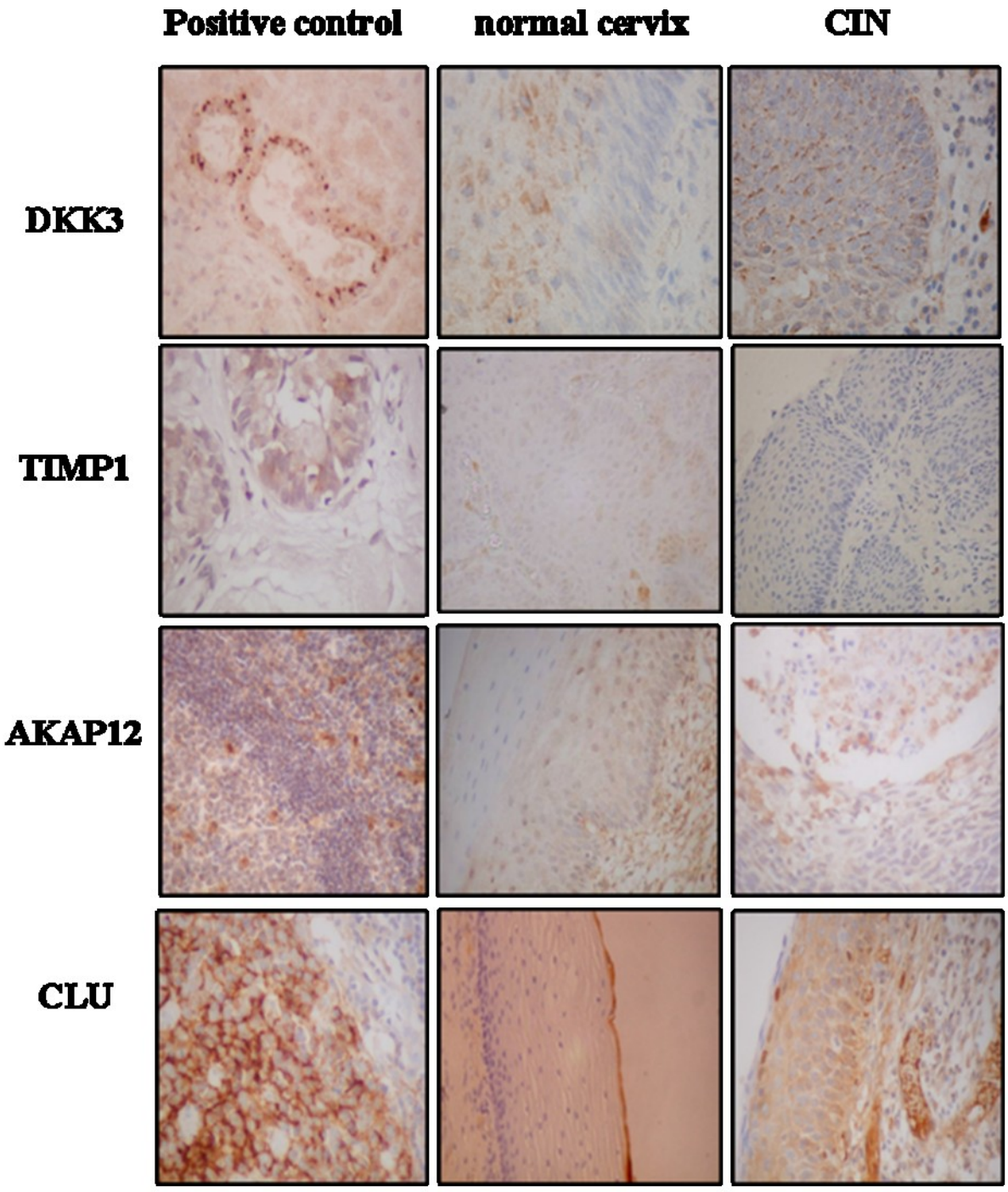


Figure 3.11: Immunohistochemical staining of normal cervical tissue and cervical intraepithelial neoplasia.

A comparison of staining for candidate genes demonstrated that DKK3,TIMP1 and AKAP12 show no difference in expression between normal cervix and CIN. CLU is up-regulated in CIN compared with normal cervical epithelium. Positive controls were kidney for DKK3, normal breast for TIMP1, tonsil for AKAP12 and CLU.

Cervical sample	Comment	Diagnosis	AKAP12	DKK3	TIMP1	CLU
3805	no squamous epithelium	Normal	1+/-	-		-
1783-93	no squamous epithelium, endothelial glands +ve	Normal		no squamous	no squamous	-
1783	endothelial glands +ve	Normal	-		no squamous	-
6429-4-93	no squamous epithelium, endo	Normal				-
4066	squamous epithelium	Normal	1+/-	-	-	-
4401	squamous epithelium	Normal	-	-	-	-
4546	HPV +ve koilocytes	CIN I	1+/-	-		1+
3671	HPV +ve koilocytes	CIN I	-	-	-	2+
6429	HPV +ve koilocytes	CIN I	-	-	-	-
6429-A-93	HPV +ve koilocytes	CIN I				-
6476	HPV +ve koilocytes	CIN I	-	-	-	1+
5591	HPV +ve koilocytes	CIN I	-	-	-	-
6379-2	HPV +ve koilocytes	CIN I	-	-	-	-
6379-6	HPV +ve koilocytes	CIN I	-	-		-
6030	HPV +ve koilocytes, fragmented	CIN I	no squamous	1+/-	no squamous	1+
0009-B8-93	HPV +ve koilocytes, little epithelium	CIN I	-	-	-	-
5758-3-93	HPV +ve koilocytes	CIN I/II	-	-	-	2+/3+
2545	HPV +ve koilocytes	CIN II	-	-	-	2+
6550-B1-93	HPV +ve koilocytes, squamous	CIN II			-	2+
6152	HPV +ve koilocytes, endo cervical glands +ve	CIN II	-	-	-	1+
6821	HPV +ve koilocytes, endo cervical glands +ve	CIN II				1+
6550	HPV +ve koilocytes, normal cells -ve, not squamous, dysplastic	CIN III	-	1+/-	-	1+
6235	HPV +ve koilocytes	CIN III	-	-	-	3+
6373	HPV +ve koilocytes	CIN III	-	-	-	-
6738	HPV +ve koilocytes	CIN III	-	-	-	-
5952	HPV +ve koilocytes	CIN III	-	-	-	2+
5588		CIN III	-	-	-	1+
6379-3	HPV +ve koilocytes	CIN III	-	-	-	1+
6378-5	HPV +ve koilocytes	CIN III			-	1+
6146	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	-	-	1+/-
6011	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	-	-	2+
6276	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	-	-	1+
5669	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	-	-	1+/-
6829-93	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	-	-	2+
6267-93		CIN III	-	-	-	2+
6233	staining at periphery of tissue	CIN III	-	-	-	2+
6615		CIN III	-	-	-	1+
6985		CIN III	-	-	-	-
6231	HPV +ve koilocytes	CIN III	-	-	-	1+
6750	HPV +ve koilocytes	CIN III	-		-	1+/-
6150		CIN III	-	-	-	1+/-
6218-2-93	patchy staining pattern	CIN III	-	-	-	2+
6211-2-93	HPV +ve koilocytes	CIN III	-	-	-	1+/-
6221-2-93	HPV +ve koilocytes	CIN III	-	-	-	1+/2+
15763		Adenocarcinoma	-	-	-	-
6293		Adenocarcinoma	-	-	2+	-
18091		Adenocarcinoma	-	-	1+/-	-
1355		Squamous carcinoma	-	-	-	-

Table 3.1: Immunohistochemical staining of normal cervical tissue and cervical intraepithelial neoplasia (CIN).

AKAP12, DKK3 and TIMP1 show little or no expression in cervical sections, however compared with normal cervical epithelium CLU is up-regulated in CIN and down-regulated in invasive disease compared to CIN

Diagnosis	AKAP12	DKK3	TIMP1	CLU
Normal	3/3	0/3	0/2	0/3
CIN I	1/6	1/9	0/6	4/10
CIN II	0/4	0/4	0/4	5/5
CIN III	0/22	1/21	0/23	20/23
Cancer	0/4	0/4	2/4	0/4

Table 3.2: Summary of immunohistochemical staining of normal cervical tissue and cervical intraepithelial neoplasia.

Numbers represent the number of tissues that were positive for the candidate gene expression. AKAP12, DKK3 and TIMP1 show little or no expression in cervical sections, however CLU is up-regulated in CIN and down-regulated in invasive disease

3.7. Selection of a final candidate gene

The validation of the 7 candidate genes was narrowed down to five genes for further validation *in vivo*. Although there was some down regulation of DKK3 at the RNA level in patient samples, this did not translate to the protein level with no difference detected between normal cervical epithelium and CIN, which could possibly be due to the poor quality of the antibody but a better commercial antibody was not available at the time of this study. TIMP1 was expressed in all patient samples at the RNA level at a comparable level to its expression in normal human foreskin keratinocytes, with no evidence of down regulation. At the protein level there appeared to be little difference in expression between normal cervical epithelium and CIN, with some up-regulation in 50% of invasive cancers. AKAP12 was down-regulated at the RNA level in patient samples compared with normal human foreskin keratinocytes but showed little difference in expression at the protein level between normal cervical epithelium and CIN. The antibody commercially available at the time of this study for CADM1 was of poor quality and not suitable for immunohistochemistry and so this gene could not be validated *in vivo.* Given the striking pattern of CLU expression in patient samples, being up-regulated in CIN and then down-regulated in invasive cancer this is the gene I took forward for further *in vitro* and *in vivo* validation.

To summarise at this stage, CLU was up-regulated in the initial demethylation experiment performed in my laboratory. Using MSP, the CLU promoter was found to be methylated in HeLa, Caski and C33a cell lines. Furthermore, when the demethylation experiment was repeated I was

able to confirm that CLU was up-regulated in the HeLa cell line following demethylation. Preliminary results, using MSP, also illustrated an increase in methylation of the CLU promoter in successive passage of the W12 cell line. However, I was not able to show a compelling change in CLU expression at the transcriptional level with increasing passage number.

An up-regulation of clusterin at the protein level using immunohistochemical staining has been confirmed by Dr Maizaton Abdullah. In normal tissue, a regular basal layer and underlying stroma is evident, with the differentiated squamous epithelium showing strong cytoplasmic staining of CLU. In CIN there is an up-regulation of CLU, but a complete absence of CLU staining in invasive cancer. CLU was therefore investigated in a larger number of cervical samples (section 3.7.2).

3.8. Further investigation of clusterin as a candidate tumour suppressor gene

Having identified CLU as potential tumour suppressor gene, it was necessary to confirm the results of previous RT-PCR using quantitative RT-PCR, confirm the changes in protein expression in a wider cohort of cervical samples and with different CLU antibodies, and to confirm MSP results using bisulphite genomic sequencing and pyrosequencing.

3.8.1. Evaluation of changes in expression of CLU by Q RT-PCR

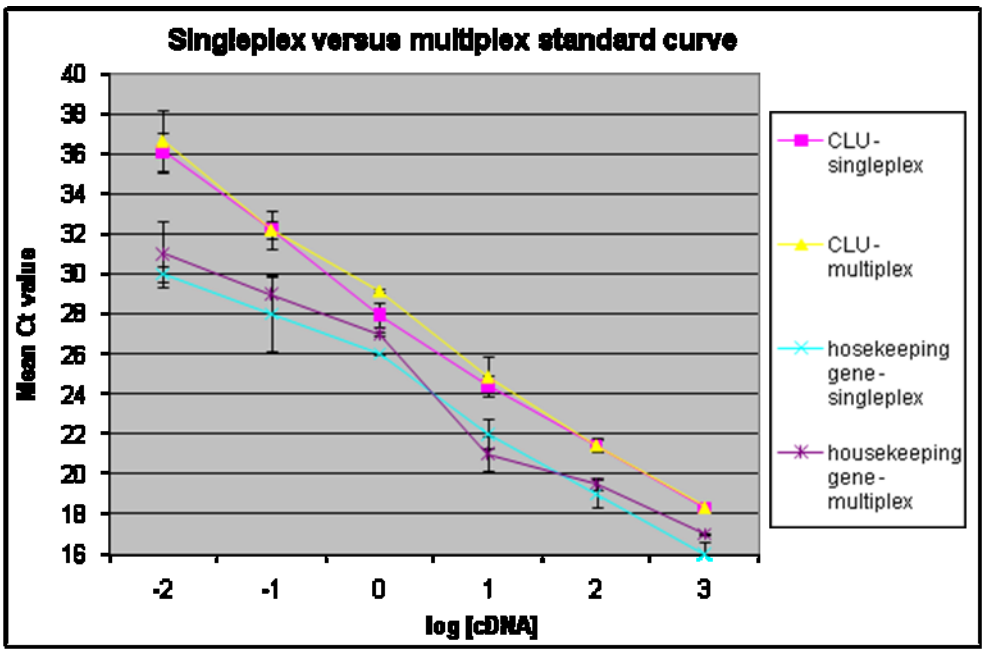
Before CLU expression could be validated by Q RT-PCR this technique itself needed to be validated by making sure that multiplexing the reaction did not result in competitive amplification, by optimising the amount of input cDNA for exponential amplification and by finding an appropriate house keeping gene.

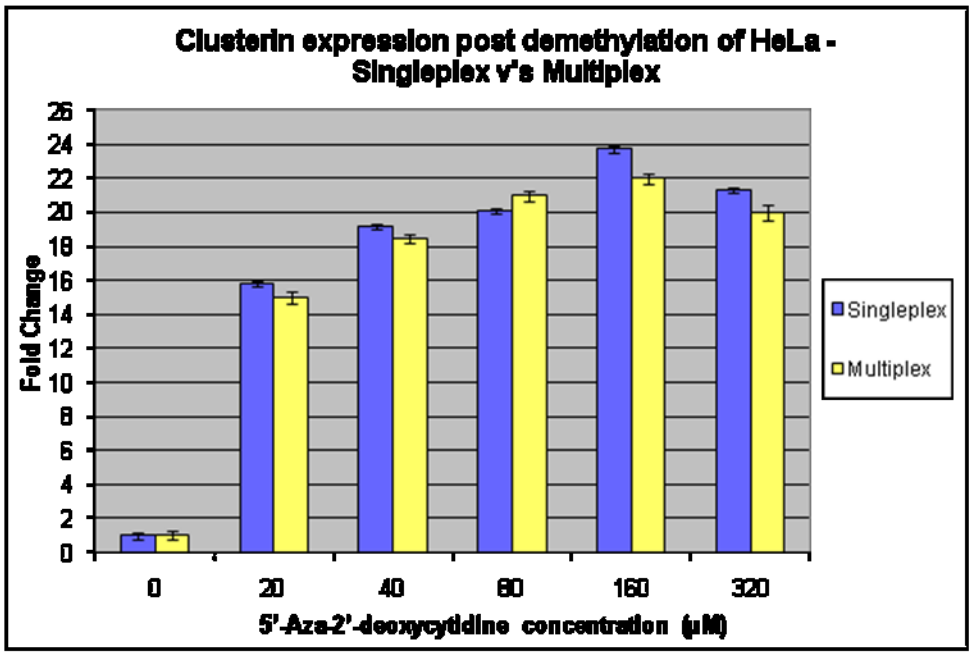
3.8.1.1 Optimisation of CLU Q RT-PCR

In multiplexing the reaction I needed to confirm that the two probes, one separate for the housekeeping gene and the other CLU, did not compete for amplification of the gene product and so not exhausting an entire reagent before the other has amplified. To address this issue, initial reactions were carried out in both singleplex and multiplex assays. Figure 3.12 shows no competitive amplification. Equally, ten-fold dilutions of cell line cDNA were used in titrations of 1ug, 100ng, 10ng, 1ng, 100pg, 10pg, to determine the exponential region for amplification of

both target and endogenous control; which was found to be between 100ng and 10ng, thus 50ng of cell line cDNA was used in all experiments.

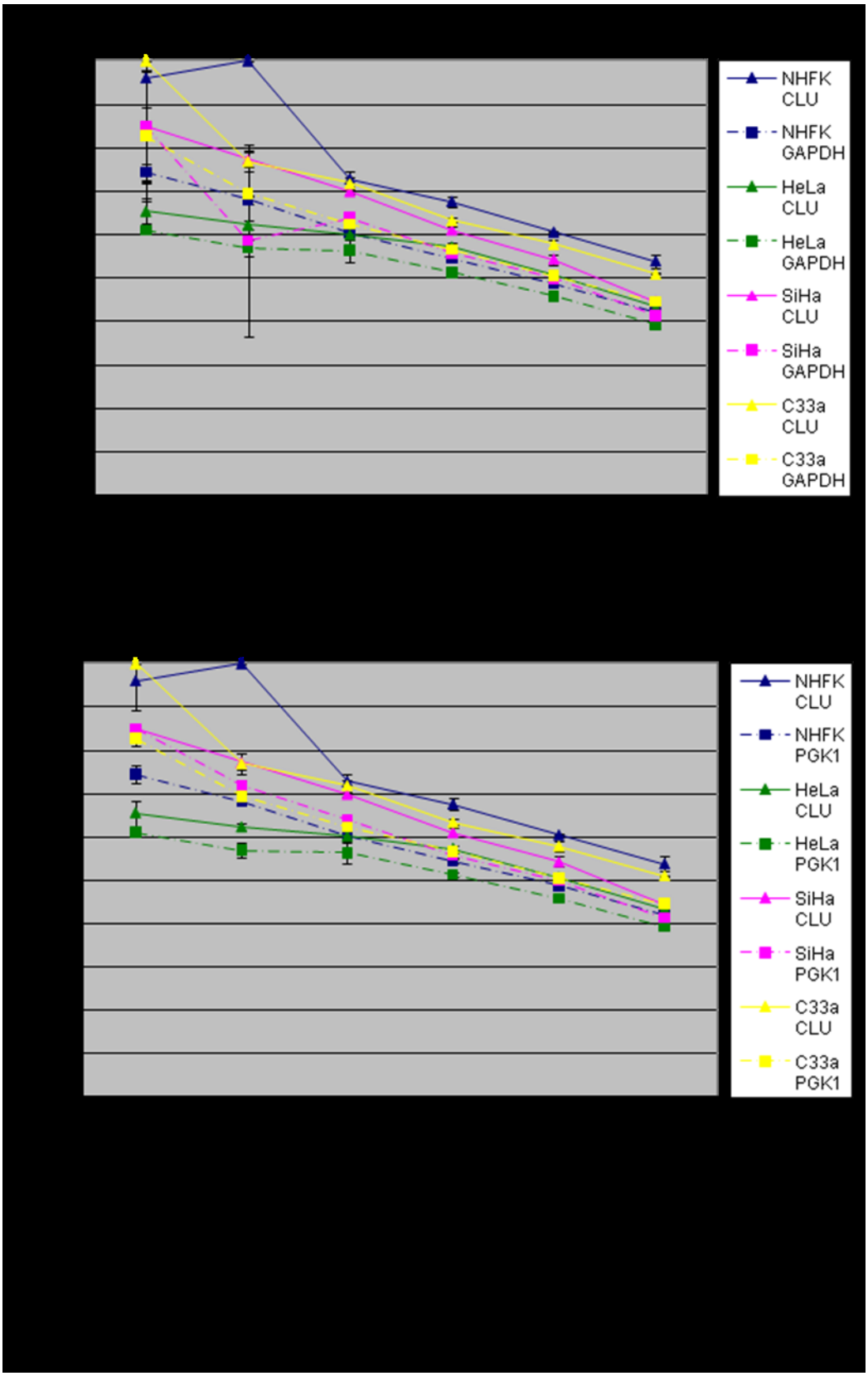
It is essential to test a number of housekeeping genes to determine which one shows least variation in cell types or data sets. Four commonly used housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Phosphoglycerate kinase 1 (PGK1), Beta-2 microglobulin (B2M) and 18S ribosomal RNA (18S) were validated as potential endogenous controls for expression of clusterin in cervical cell lines (Figures 3.13 and 3.14). Of the four housekeeping genes, B2M gave the most consistent amplification across all cell lines and figure 3.15 illustrates how variable the interpretation of gene expression data can be due to variation in housekeeping genes. There is a requirement to identify housekeeping type genes that show sample independent stability and studies have found that newly identified genes, that are not necessarily housekeeping genes, were more stably expressed in individual samples with similar ranges. Thus, statistical analysis of microarray data can be used to identify new candidate housekeeping genes showing consistent expression across tissues and diseases.

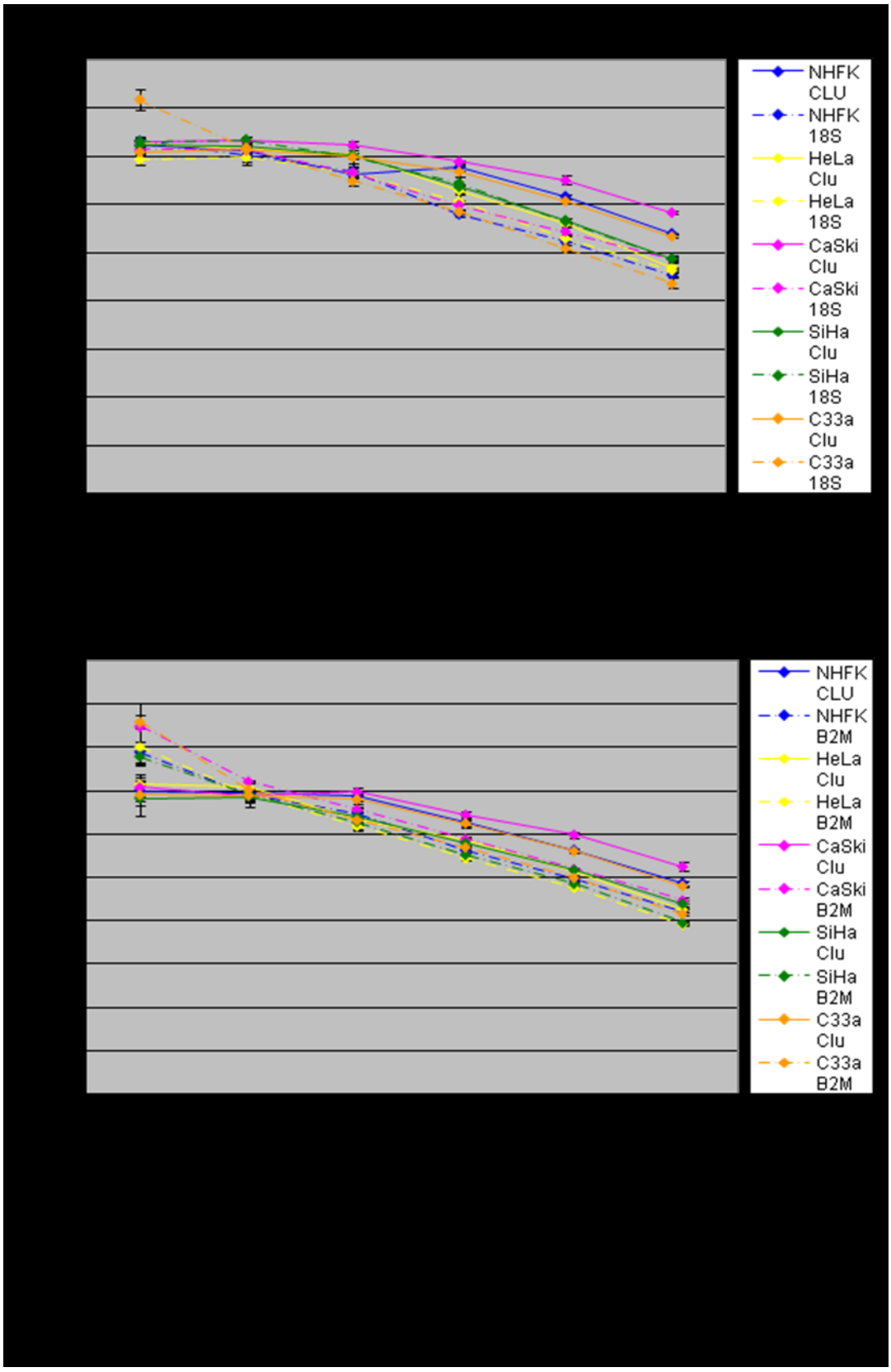




3.12: Clusterin and GAPDH expression in cervical cell lines across a range of concentrations of cDNA from 10pg to 1ug.

There was no competitive amplification for gene products when multiplexing Q RT-PCR reactions as there was a 2 cycle difference between target gene and housekeeping gene amplification. The exponential region of amplification was found to be between 100ng and 10ng input cDNA.


NHFK CLU
NHFK GAPDH
HeLa CLU
HeLa GAPDH
SiHa CLU
SiHa GAPDH
C33a CLU
C33a GAPDH
NHFK CLU
NHFK PGK1
HeLa CLU
HeLa PGK1
SiHa CLU
SiHa PGK1
C33a CLU
C33a PGK1


NHFK CLU
NHFK 18S
HeLa Clu
HeLa 18S
CaSki Clu
CaSki 18S
SiHa Clu
SiHa 18S
C33a Clu
C33a 18S
NHFK CLU
NHFK B2M
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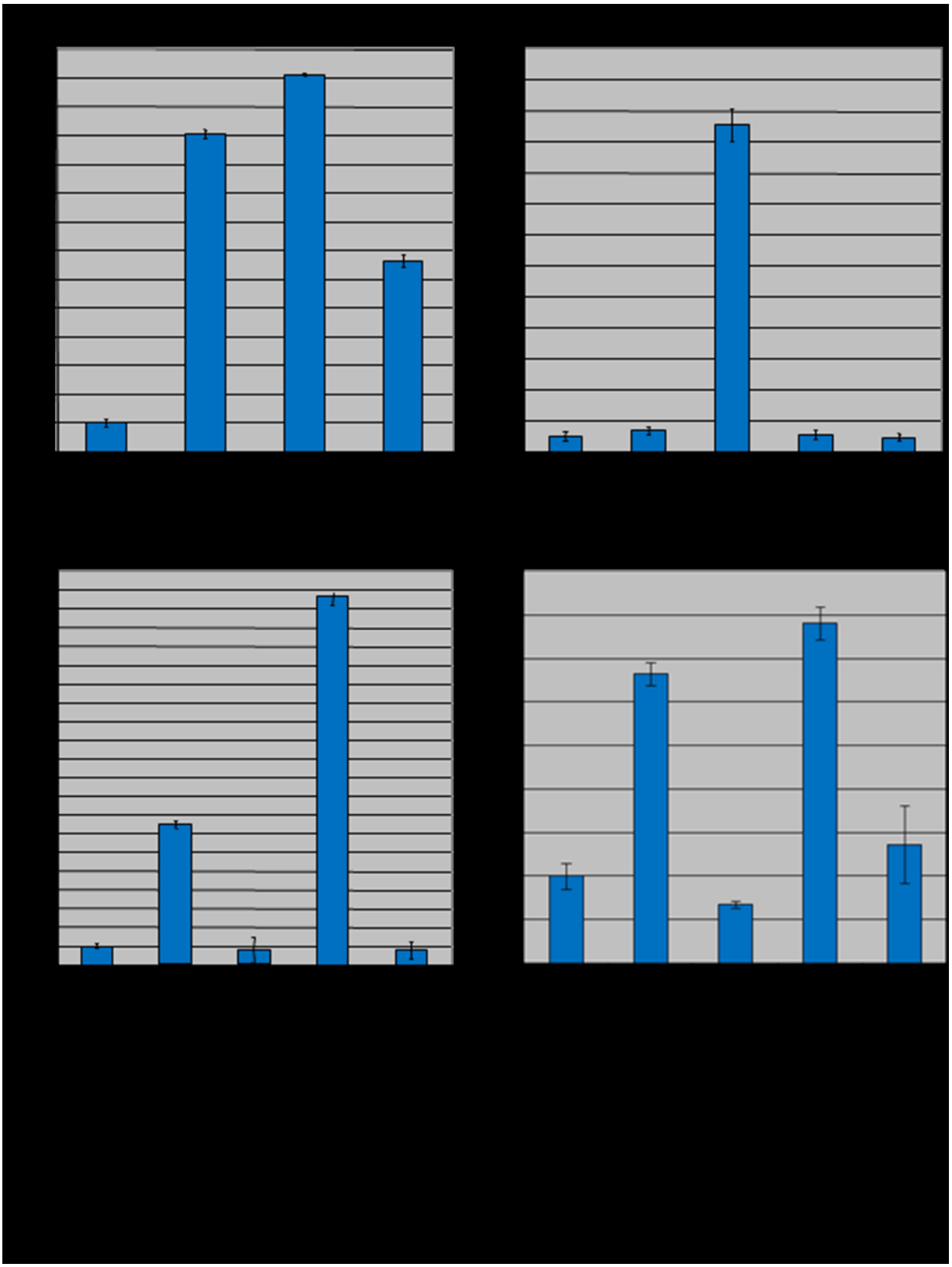
SiHa B2M
C33a Clu
C33a B2M



3.8.1.2. Q RT-PCR analysis of CLU expression in HeLa cell lines

Q RT-PCR using the housekeeping gene B2M verified the pattern of expression of CLU using RNA from the HeLa demethylation experiment. Figure 3.16 compares the expression of CLU in untreated HeLa cells and in HeLa cells treated with increasing concentrations of 5-Aza-2'-deoxycitidine. The results show up-regulation of CLU post demethylation and are consistent with those obtained using end point PCR (Figure 3.2).

3.8.1.3. Q RT-PCR analysis of CLU expression in the W12 cell line model

Transcriptional changes of CLU and CADM1 were validated in serial passage of the W12 cell line. CADM1 was included given that is a known tumour suppressor gene in cervical cancer, and hence was included for comparison in further validation of CLU. Figure 3.17 illustrates down regulation of CADM1 with serial passage as it becomes increasingly methylated. In contrast, CLU appeared not to be downregulated during serial passage of W12 cells.

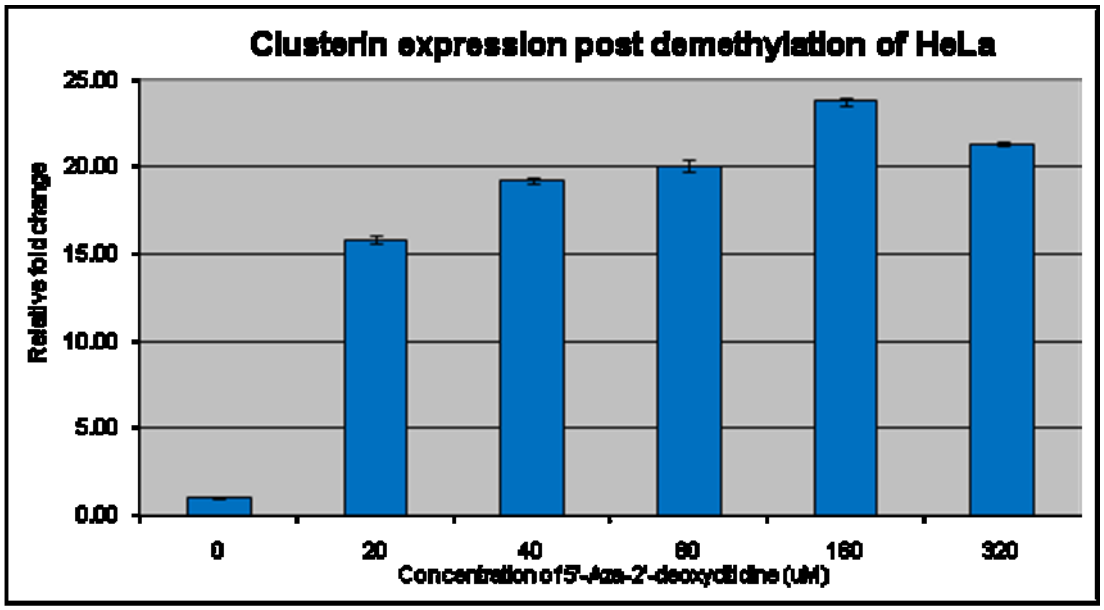
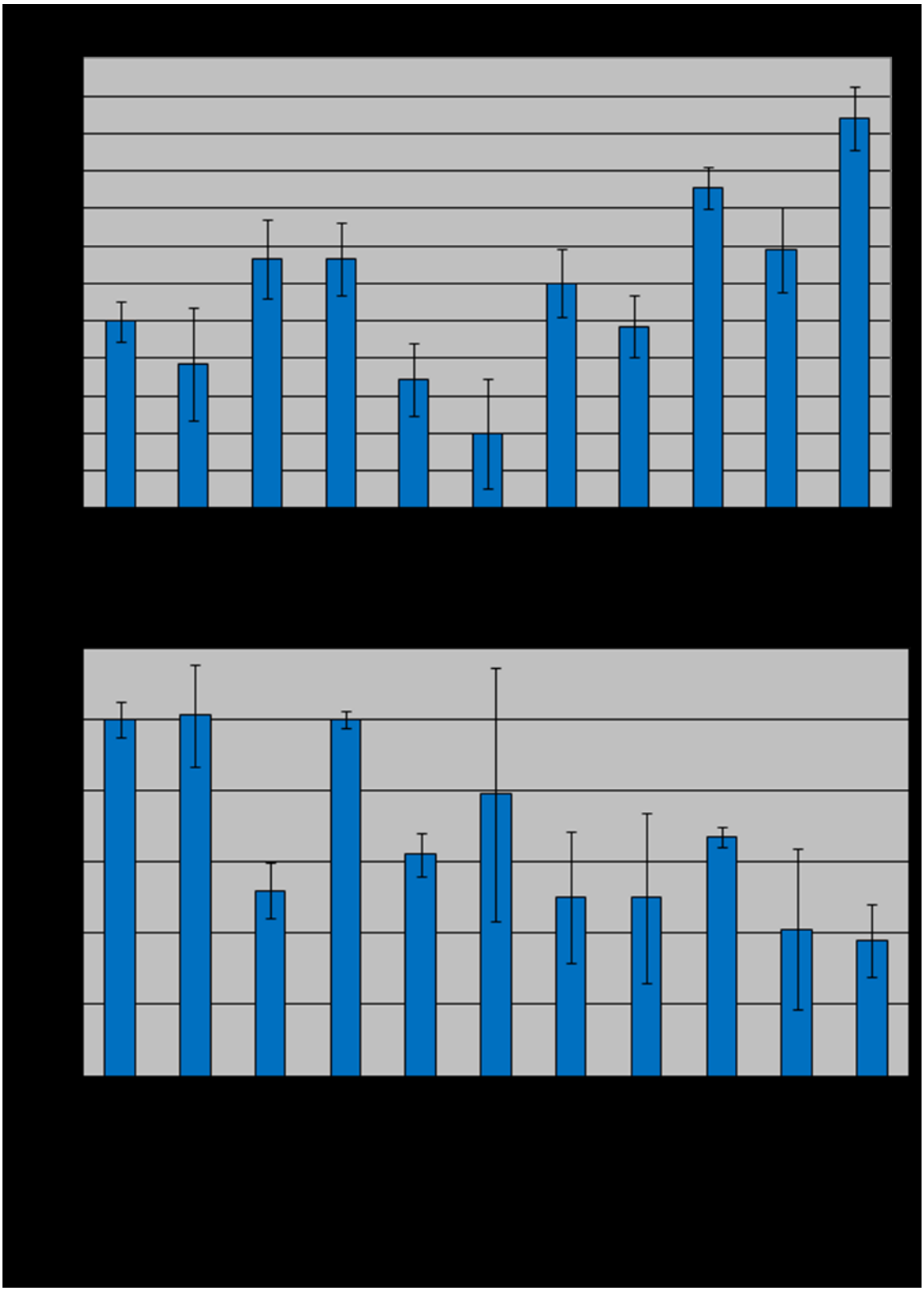


Figure 3.16: Q RT-PCR analysis of CLU expression in demethylated Hela cells compared with untreated cells
Expression levels of Clusterin was examined before (0) and post-demethylation over a range of 5'-Aza-2'-deoxycytidine concentrations. CLU was up-regulated following demethylation even at low drug concentrations.



3.8.2. Pattern of CLU protein expression in cervical tissue and cell lines

Having demonstrated that CLU was up-regulated in CIN compared with normal cervix using a commercially available antibody from Vector, the same cohort was examined with two further CLU antibodies. Both of these other antibodies when assessed by 2 pathologists, Dr Terry Rollason and Dr Maizaton Abdullah, who were blinded to the previous data confirmed the data obtained from the previous antibody, showing increased staining for CLU in high grade CIN compared with normal cervical epithelium (Table 3.3). Similarly, examination of a wider cohort of adenocarcinomas and squamous cell carcinomas of the cervix with 3 CLU antibodies confirmed its downregulation in invasive cancer (Table 3.4). Immunohistochemistry showed an up-regulation of CLU at the protein level in HeLa, CaSki and SiHa compared with NHFK (Table 3.5). With serial passage of the W12 cell line there was no apparent change in CLU protein expression.

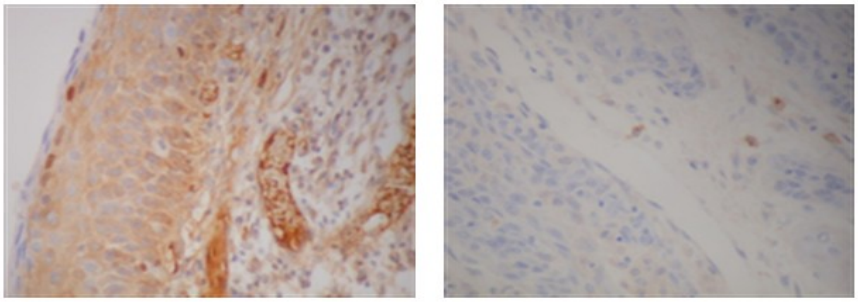
In order to validate the specificity of the CLU antibodies, the expression of CLU at the protein level in cell lines was investigated using western blotting. Figure 3.17 shows increased expression in HeLa, CaSki and SiHa when compared with C33a and normal human foreskin keratinocytes, as was seen in the cytospins. In HeLa and CaSki there is 45kDa band in addition to the 60kDa and 40kDa proteins observed in all the cell lines, which is the molecular weight corresponding to nuclear CLU. CLU transfected HEK 293 cell lysates were ran alongside as a positive control for CLU protein expression. Western blotting of W12 passage 15 and 56 cell lysates showed no change in CLU expression between the passages and confirmed results obtained by immunohistochemistry of cell lines.

Cervical sample	Comment	Diagnosis	Upstate	Santa Cruz	Vector
3905	no squamous epithelium	Normal	-	-	-
1783-93	no squamous epithelium, endothelial glands +ve	Normal	-	-	-
1783	endothelial glands +ve	Normal	-	-	-
6429-4-93	no squamous epithelium, endo	Normal	-	-	-
4646	HPV +ve koilocytes	CIN I	1+	1+	1+
3671	HPV +ve koilocytes	CIN I	1+/2+	2+	2+
6429	HPV +ve koilocytes	CIN I	-	-	-
6429-A-93	HPV +ve koilocytes	CIN I	-	-	-
6476	HPV +ve koilocytes	CIN I	1+	1+	1+
5591	HPV +ve koilocytes	CIN I	-	-	-
6379-2	HPV +ve koilocytes	CIN I	-	-	-
6379-9	HPV +ve koilocytes	CIN I	-	-	-
6030	HPV +ve koilocytes, fragmented	CIN I	1+/-	1+	1+
6009-B8-93	HPV +ve koilocytes, little epithelium	CIN I	-	-	-
5756-3-93	HPV +ve koilocytes	CIN I/II	2+	1+	2+/3+
2545	HPV +ve koilocytes	CIN II	2+	2+	2+
6550-B1-93	HPV +ve koilocytes, squamous	CIN II	2+	2+	2+
6152	HPV +ve koilocytes, endo cervical glands +ve	CIN II	1+	1+	1+
6821	HPV +ve koilocytes, endo cervical glands +ve	CIN II	1+	1+	1+
6550	HPV +ve koilocytes, normal cells -ve, not squamous, dysplastic	CIN III	1+	1+	1+
6235	HPV +ve koilocytes	CIN III	2+/3+	3+	3+
6373	HPV +ve koilocytes	CIN III	-	1+	-
6738	HPV +ve koilocytes	CIN III	-	-	-
5952	HPV +ve koilocytes	CIN III	2+	2+	2+
5596		CIN III	1+	1+	1+
6379-3	HPV +ve koilocytes	CIN III	1+	1+	1+
6378-5	HPV +ve koilocytes	CIN III	1+	1+	1+
6146	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	1+	1+/-
6011	HPV +ve koilocytes, endo cervical glands +ve	CIN III	2+	2+	2+
6276	HPV +ve koilocytes, endo cervical glands +ve	CIN III	1+	1+	1+
5669	HPV +ve koilocytes, endo cervical glands +ve	CIN III	1+	1+	1+/-
6929-93	HPV +ve koilocytes, endo cervical glands +ve	CIN III	-	1+/-	2+
6267-93		CIN III	-	1+	2+
6233	staining at periphery of tissue	CIN III	1+/2+	2+	2+
6615		CIN III	1+	1+	1+
6695		CIN III	-	1+/-	-
6231	HPV +ve koilocytes	CIN III	1+	1+	1+
6750	HPV +ve koilocytes	CIN III	-	1+	1+/-
6150		CIN III	-	1+	1+/-
6218-2-93	patchy staining pattern	CIN III	2+	2+	2+
6211-2-93	HPV +ve koilocytes	CIN III	-	1+/-	1+/-
62211-293	HPV +ve koilocytes	CIN III	1+	1+	1+/2+

Table 3.3: Immunohistochemical staining of normal cervical tissue and cervical intraepithelial neoplasia with a panel of CLU antibodies

CLU is up-regulated in CIN compared with normal cervicalepithelium with all three antibodies

	Cervical sample	Comment	Upstate	Santa Cruz	Vector
T	96/4436A15	moderately to poorly differentiated squamous cell carcinoma	-	-	-
J	95/5531A1	high grade CGIN	-	-	-
W	95/2500A1	well differentiated adenocarcinoma	-	1+/-	1+/2+
O	95/50401A30	poorly differentiated squamous cell carcinoma	-	-	-
I	95/2871A20	well differentiated adenocarcinoma	-	-	-
M	96/3043A13	moderately differentiated squamous cell carcinoma	-	-	-
N	95/2909B2	high grade CGIN	-	-	-
H	96/6308A15	moderately differentiated squamous cell carcinoma	-	-	-
V	95/4246A8	well differentiated adenocarcinoma	-	-	-
S	96/2840A15	well differentiated adenocarcinoma	-	1+/-	-
Q	96/4667A18	moderately differentiated squamous cell carcinoma	-	-	-
R	96/781A12	moderately differentiated squamous cell carcinoma	-	-	-
L	96/6441B4	high grade CGIN with focal invasion	-	-	-
A	95/2924A47	well differentiated adenocarcinoma	-	-	-
B	95/2871A30	well differentiated adenocarcinoma	-	-	-
K	96/68A14	moderately differentiated squamous cell carcinoma	-	-	-
P	95/2400A30	well differentiated adenocarcinoma, endometrial tumour	-	1+	1+/-
G	96/3574A5	invasive well differentiated squamous cell carcinoma	-	-	-
C	95/4920A6	poorly differentiated squamous cell carcinoma	-	-	-
E	96/6940E22	adenocarcinoma + squamous+ adenosquamous	-	-	-
D	95/2599A6	high grade CGIN with focal invasion	-	-	1+/-
F	96/6525A10	moderately differentiated squamous cell carcinoma	-	-	-



CIN Cancer

Table 3.4: Immunohistochemical staining of cervical intraepithelial neoplasia adeno and squamous carinoma with a panel of CLU antibodies

CLU is up-regulated in CIN and down regulated in invasive disease with all three antibodies

Cervical cell line	Cytospin	localisation	Upstate	Santa Cruz
NHFK	Formalin fixed	Nuclear	1+/-	1+
HeLa	Formalin fixed	Cytoplasmic	2+	2+
CaSKI	Formalin fixed	Cytoplasmic	1+/2+	2+
SiHa	Formalin fixed	Cytoplasmic	2+	2+
C33a	Formalin fixed	Cytoplasmic	1+	1+
W12E	Formalin fixed		-	-
W12E2	Formalin fixed		-	-
W12p11	Formalin fixed	Cytoplasmic	1+/-	1+/-
W12p12	Acetone fixed		-	-
W12p13	Acetone fixed		-	1+
W12p14	Acetone fixed	Cytoplasmic	-	1+/-
W12p15	Acetone fixed		-	-
W12p16	Acetone fixed		-	-
W12p17	Formalin fixed	Cytoplasmic	1+/-	1+/-
W12p18	Acetone fixed		-	-
W12p15	Formalin fixed	Cytoplasmic	1+	1+
W12p56	Formalin fixed	Cytoplasmic	1+/-	1+

Table 3.5: Immunohistochemical staining of cytospins taken from cervical cell lines and in serial passage of the W12 cell line.

There is an up-regulation of CLU at the protein level in HeLa, CaSki and SiHa compared with NHFK. With serial passage of the W12 cell line there was no apparent change in CLU expression across all W12 passages examined.

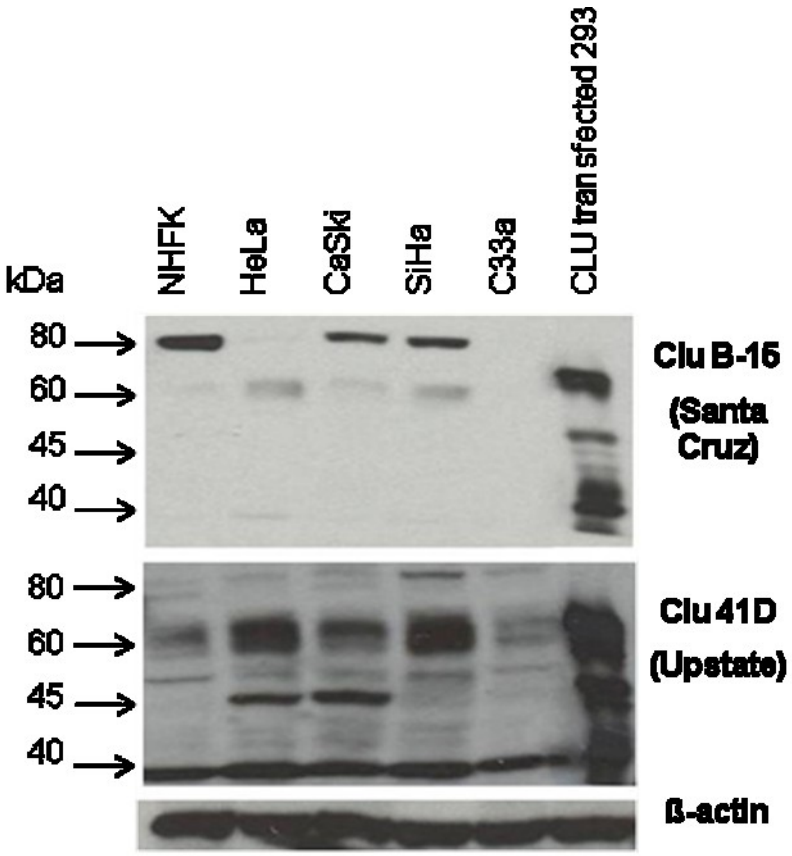


Figure 3.18: CLU protein expression in cervical cell lines compared with normal human foreskin keratinocytes.

Expression of CLU in cervical cancer cell lines compared with that in normal human foreskin keratinocytes (NHFK). CLU transfected HEK 293 cells were used as a positive control. CLU is overexpressed in HeLa, CaSki and siHa compared with NHFK

3.8.3. Evaluation of the methylation status of the CLU promoter

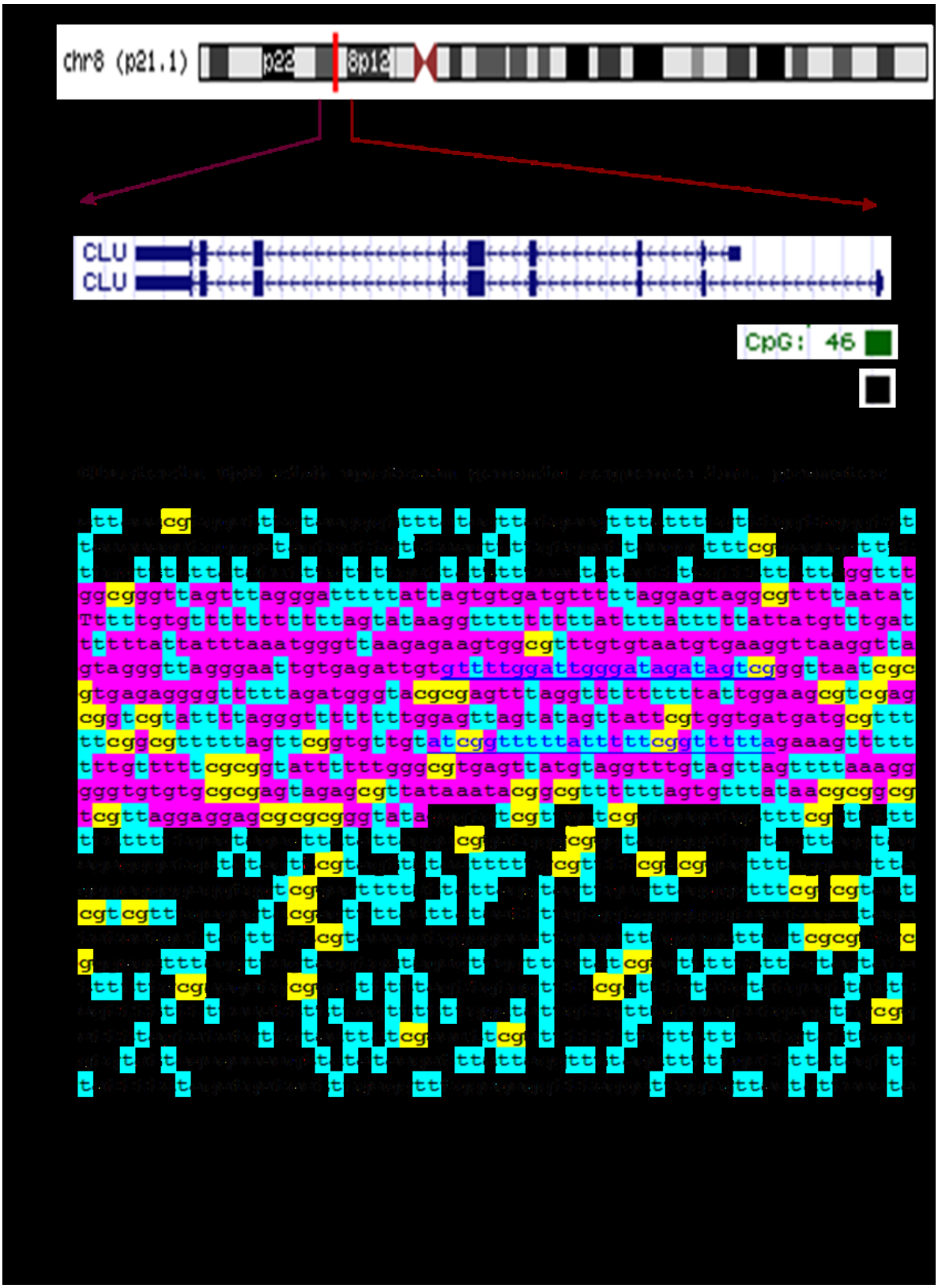
MSP is limited as it can only provide information on the CpG residues in the primers and not across the whole promoter and CpG island, so other techniques are required for a more detailed analysis of promoter methylation, such as bisulphite genomic sequencing. Figure 3.19A shows the two CLU transcripts and the predicted CpG island within the promoter region. Figure 3.19B illustrates the location of the MSP primers (blue underlined text) in the CLU promoter (pink), with the abundance of CpG dinucleotides highlighted in yellow.

3.8.3.1. Methylation of CLU in HeLa and control DNA using bisulphite genomic sequencing

Bisulphite genomic sequencing (BGS) was used to determine the pattern of promoter methylation across the entire CpG island in the promoter region of CLU in DNA extracted from HeLa. This confirmed the presence of the same methylated CpGs as detected by MSP analysis. However, as Figure 3.20 shows, less than 5% of HeLa cells show CLU methylation by BGS. This emphasises the bias and sensitivity of using MSP to detect methylation, which had previously given the false impression that HeLa cells were heavily methylated when in fact quantification by BGS has shown that only a small proportion of the CpGs are truly methylated. For comparison purposes, figure 3.21 shows an entirely unmethylated CLU promoter, with very low methylation of the adjacent CpG rich region in normal human cervical keratinocytes. Interestingly, BGS analysis shows that in positive methylated control DNA the CLU promoter is in fact unmethylated, but the adjacent CpG rich region is heavily methylated (figure 3.22). After these data were obtained the University of California Santa Cruz (UCSC) website changed its prediction of the CLU promoter,

and gave a new prediction that matched up with the heavily methylated CpG rich region of CLU, thus reinforcing this conclusion.

Methylation specific PCR is typically not very informative about methylation of CpG islands as it only provides methylation data for CpG di-nucleotides that are located where the primers lie. Figure 3.23 shows that the CpG di-nucleotides within the primers were heavily methylated, which explains my previous result which had shown a strong methylated band by MSP (section 3.3); whilst the CpGs in-between are predominantly unmethylated. This led to a premature conclusion that in HeLa cells the CLU promoter was heavily methylated. The heavy methylation of the few CpG dinucleotides identified appears to have some regulation on CLU transcription, given that subsequent treatment of HeLa with 5-Aza-2'-deoxycitidine was sufficient to cause an increase in CLU expression. However, it is possible that this is an result of the acetylation by Trichostatin A and not a result of demethylation as no change in methylation status could be detected by MSP post treatment with 5-Aza-2'-deoxycitidine. Alternatively, this could be a non-specific effect on CLU expression by the treatment itself.


chr8 (p21.1)
p22
8p12
CLU
CLU
CpG: 46

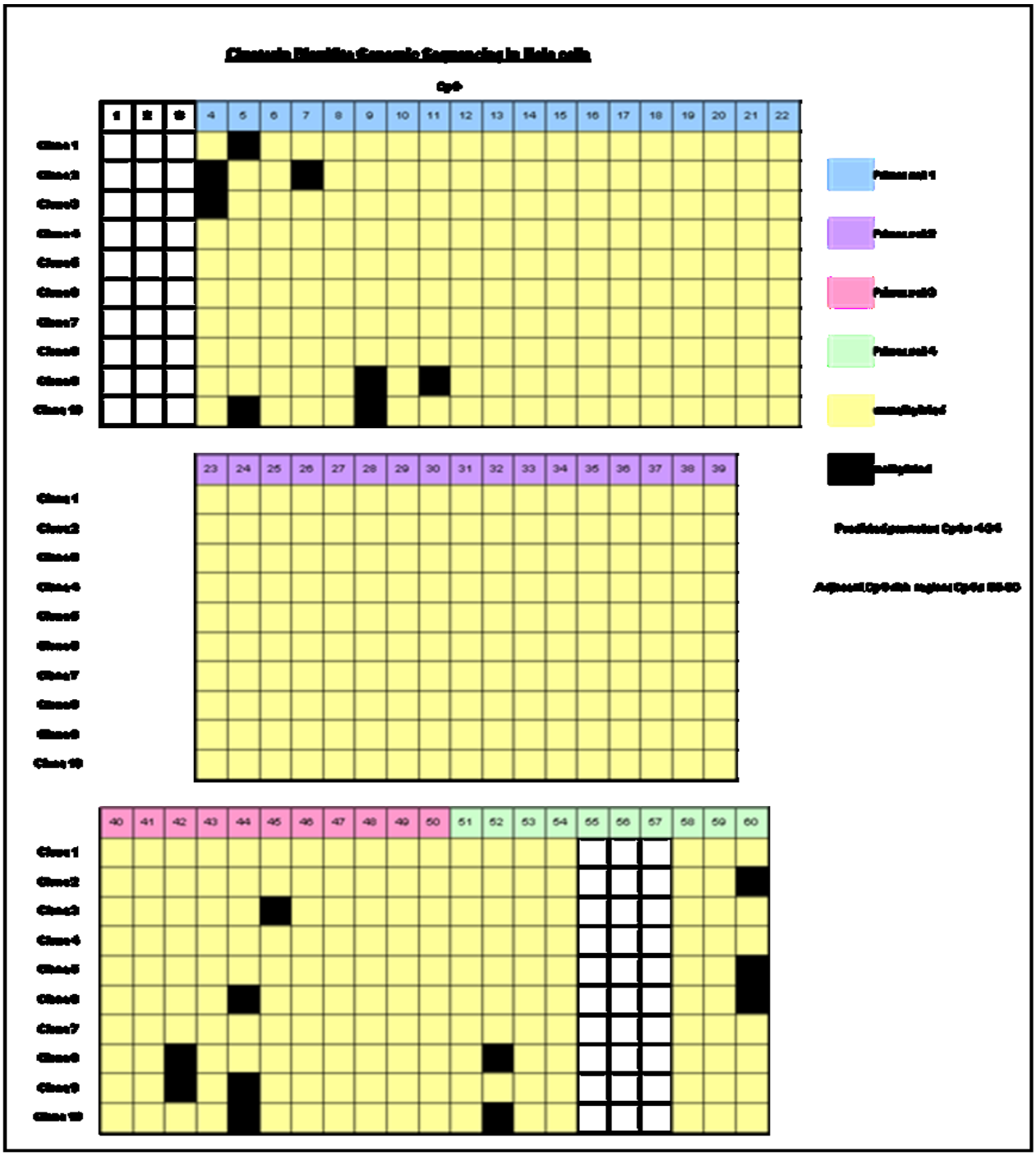


Figure 3.20: Bisulfite genomic sequencing of the Clu promoter and adjacent CpG rich region in Hela cells

Numbers across the top represent CpG dinucleotides and along the side clones. Yellow squares represent an unmethylated CpG dinucleotide and black a methylated CpG. The majority of CpG dinucleotides are frequently unmethylated.

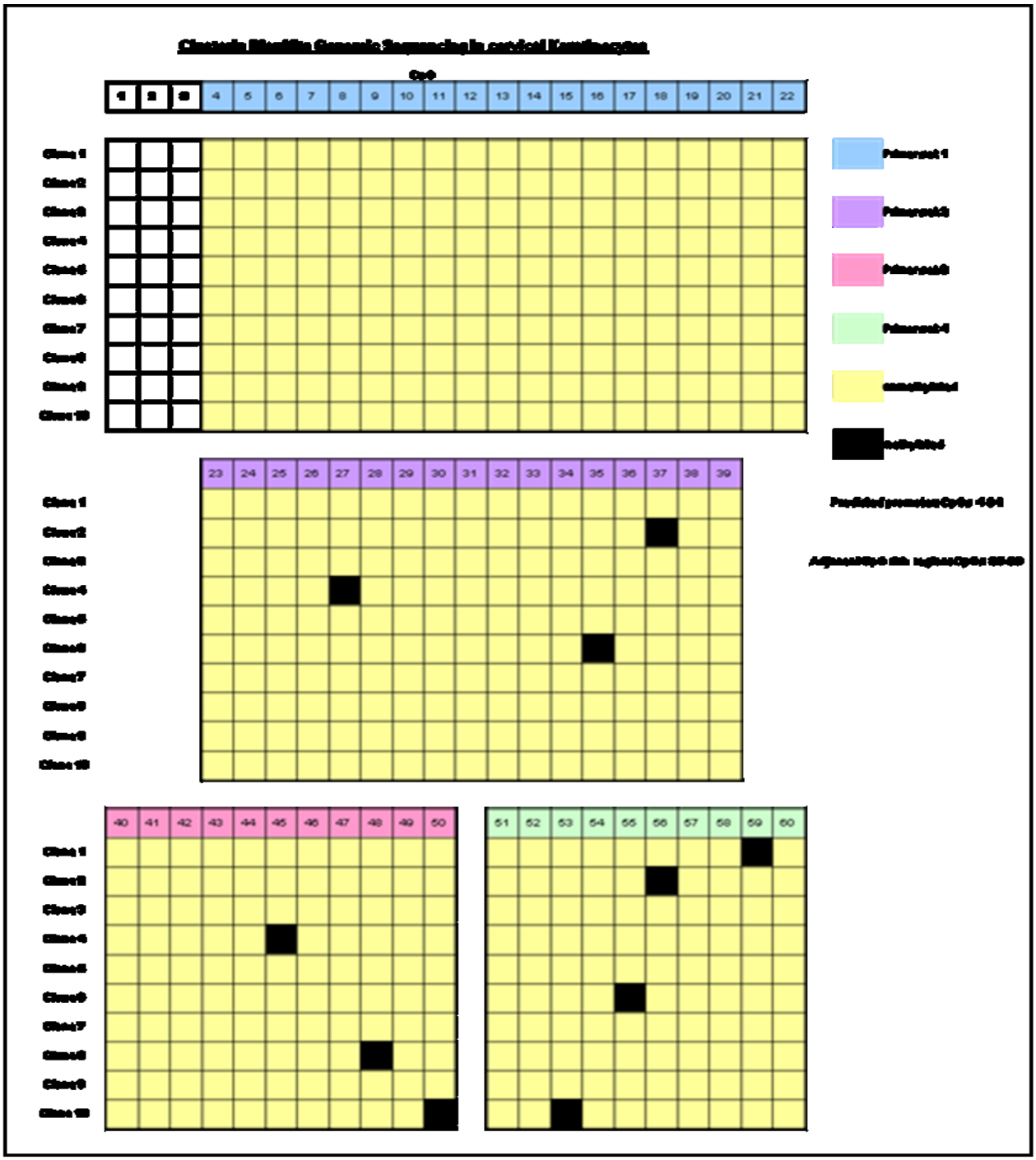


Figure 3.21: Bisulfite genomic sequencing of the CLU promoter and adjacent CpG rich region in cervical keratinocytes

Numbers across the top represent CpG dinucleotides and along the side clones. Yellow squares represent an unmethylated CpG dinucleotide and black a methylated CpG. All CpG di-nucleotides are predominantly unmethylated.

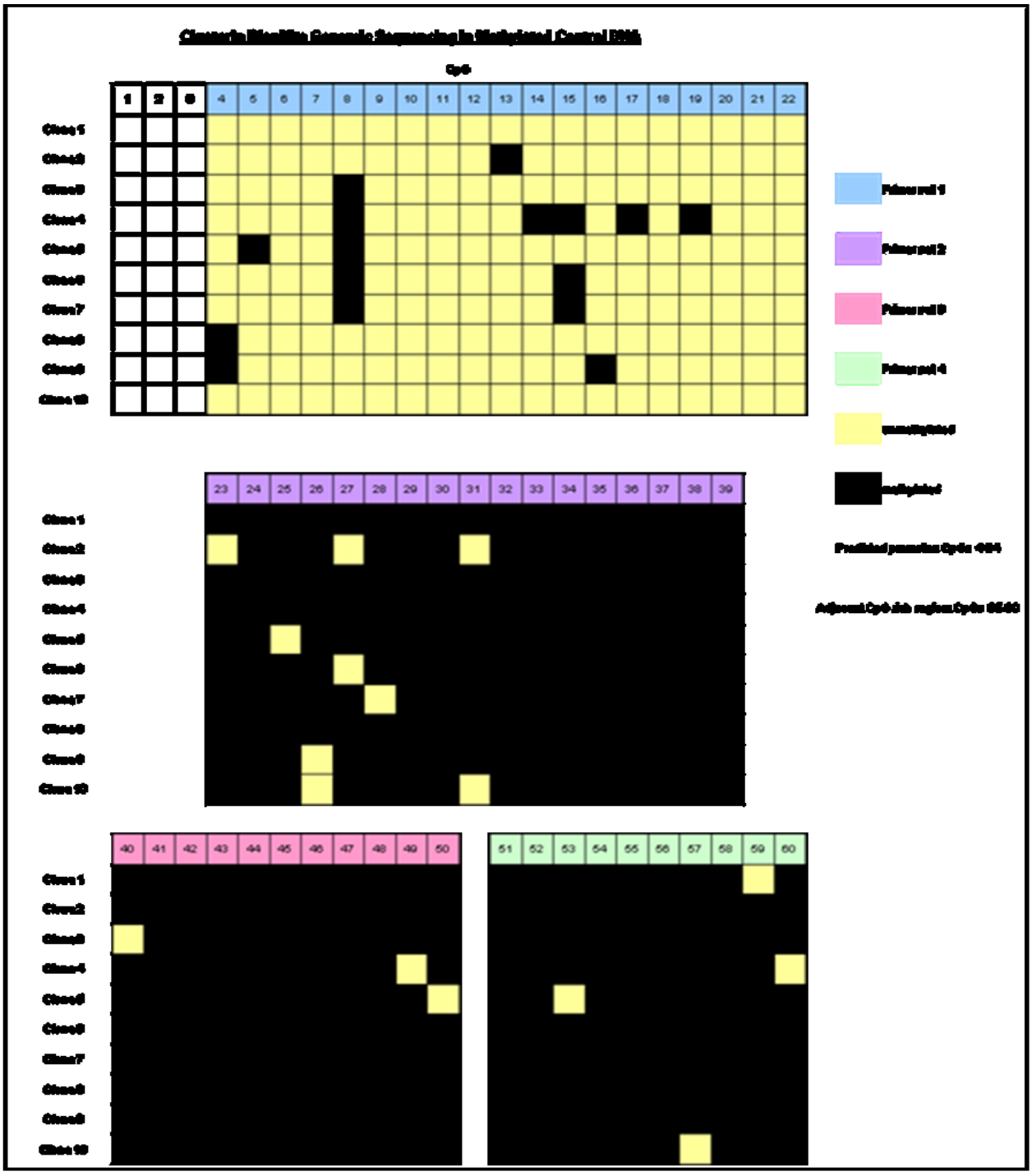


Figure 3.22: Bisulfite genomic sequencing of the CLU promoter and adjacent CpG rich region in methylated control DNA

Numbers across the top represent CpG dinucleotides and along the side clones. Yellow squares represent an unmethylated CpG dinucleotide and black a methylated CpG. The CpG rich region of CLU is heavily methylated.

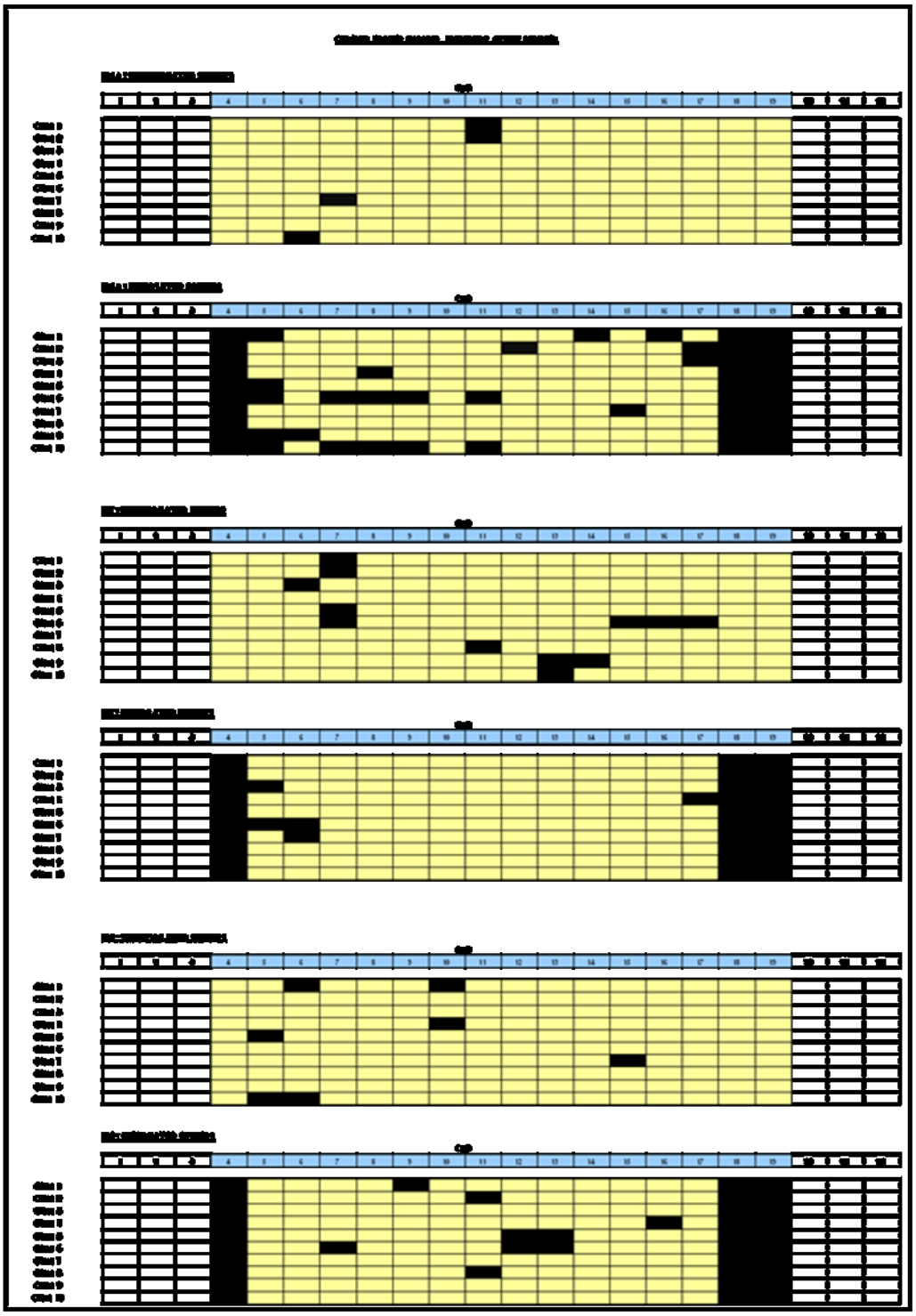


Figure 3.23: Bisulfite genomic sequencing of CLU MSP products in HeLa and methylated control DNA

Numbers across the top represent CpG dinucleotides and along the side clones. Yellow squares represent an unmethylated CpG dinucleotide and black a methylated CpG. Only CpGs that lie within the MSP primers are frequently methylated

3.8.3.2. Methylation of CLU in cervical cancer cell lines using pyrosequencing

Pyrosequencing was used to confirm the pattern of promoter methylation of CLU in DNA extracted from cervical cancer cell lines and in the W12 cell line model. 4 sets of pyrosequencing primers were designed to span the entire CLU promoter and were tested initially on both the commercially available unmethylated and methylated controls. The set of primers that gave the most reproducible data with successive runs was taken forward for analysis in all DNA samples. Table 3.6 and figure 3.24 show methylation in 5 CpG dinucleotides of the CLU promoter and their means. These 5 CpGs were selected as they were shown to be preferentially methylated by bisulphite genomic sequencing analysis. In HeLa cells the mean methylation was 12%, 62% in CaSki, 13% in SiHa and 64% in C33a, with unmethylated control DNA giving 5% methylation and methylated control DNA 91%. When HeLa cell were treated with 5-Aza-2'-deoxycitine there was no significant reduction in mean methylation from 15% to 14%, although there are slightly larger differences in the first two CpG dinucleotides showing a decrease from 16 % to 13% and 22% to 17% respectively. This provided further confirmation that in HeLa cells the CLU promoter is not regulated by DNA methylation. The high level of methylation in CaSki and C33a suggests that other cervical cell lines still may be epigenetically regulated by DNA methylation.

	CpG methylation (%)					
	1	2	3	4	5	Mean
HeLa	13	14	12	10	9	12
CaSki	77	81	67	49	36	62
SiHa	9	18	12	18	10	13
C33a	66	78	58	66	47	64
HeLa control	16	22	8	18	11	15
HeLa + 5 Aza	13	17	9	17	12	14
NHFK	12	14	8	10	5	10
NHCK + HPV18	34	36	25	33	27	31
W12 p9	9	9	6	10	5	8
W12 p11	22	19	12	18	10	16
W12 p12	3	4	2	5	2	3
W12 p21	8	9	3	8	3	6
W12 p22	13	11	7	11	8	10
W12 p32	16	11	6	10	6	10
W12 p44	14	10	5	11	6	9
W12 p58	15	16	9	18	11	14
UC	4	4	2	9	5	5
MC	96	91	83	95	88	91

Table 3.6: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and their means

Percentage CLU methylation for each of 5 CpG dinucleotides of the CLU promoter are confirmed in cervical cancer cell lines . High levels of methylation are seen in CaSki and C33a with increases and decreases in methylation of the CLU promoter with serial passage of the W12 cell line.

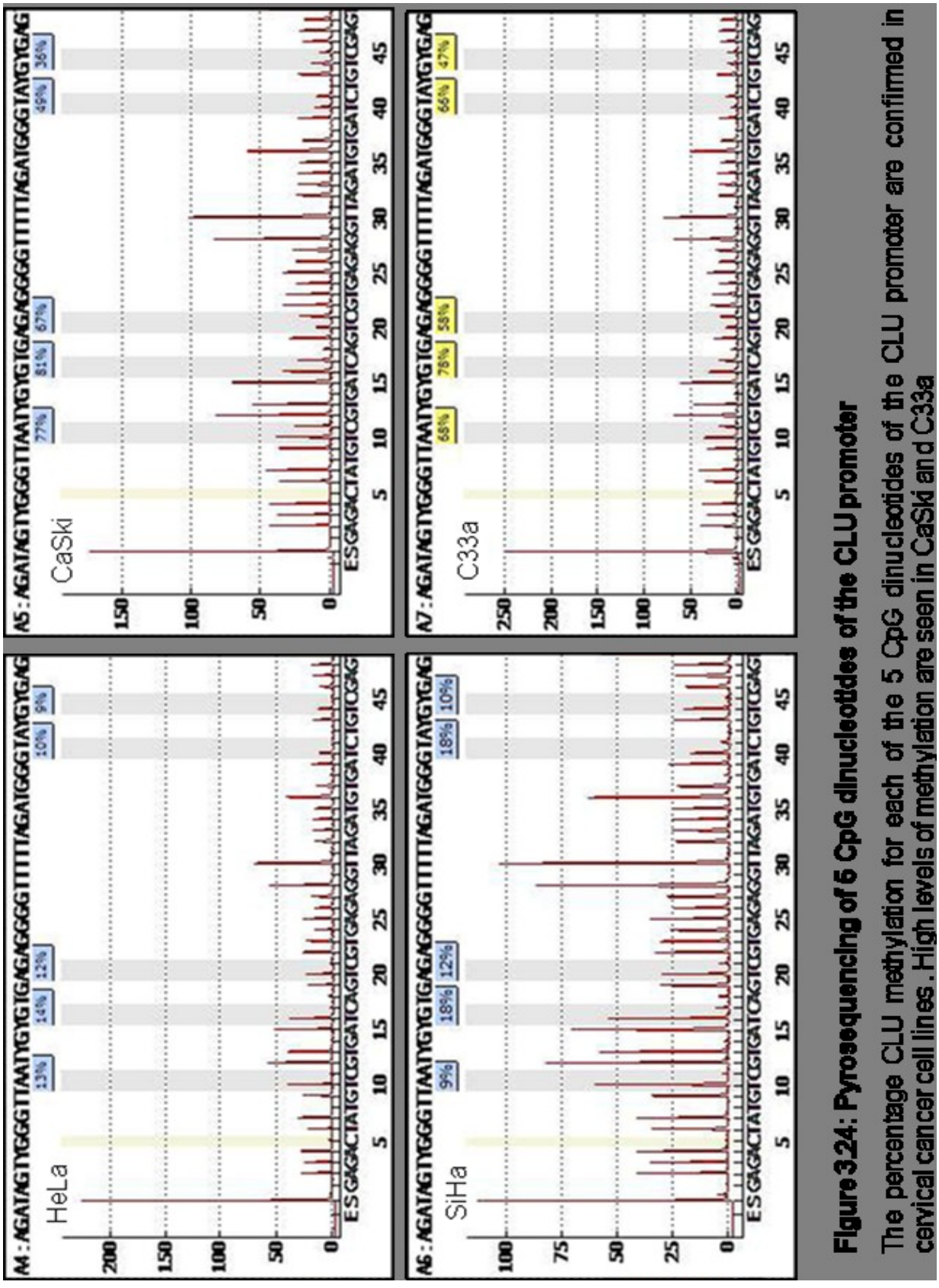


Figure 3.24: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter

The percentage CLU methylation for each of the 5 CpG dinucleotides of the CLU promoter are confirmed in cervical cancer cell lines. High levels of methylation are seen in CaSki and C33a

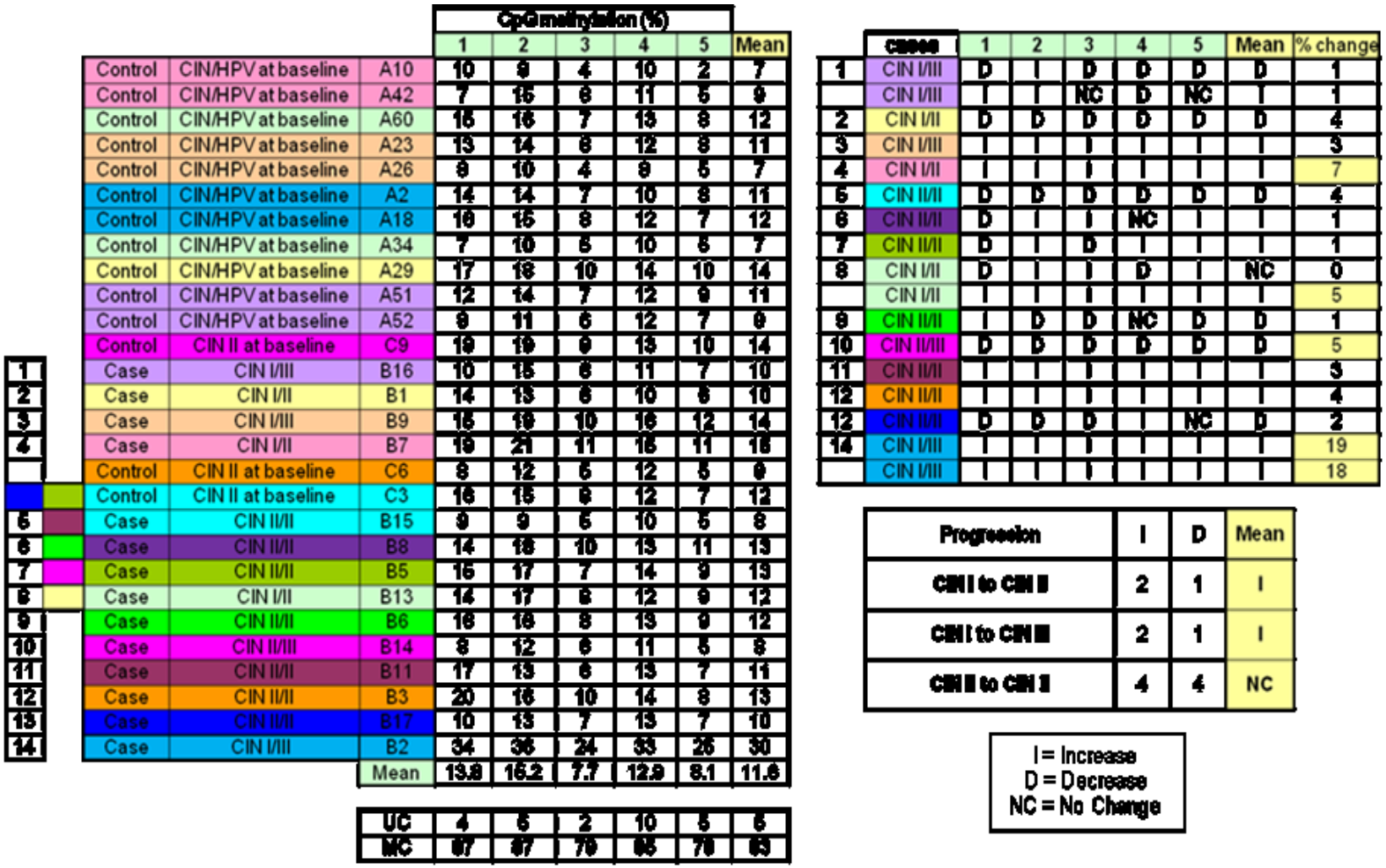
3.8.3.3. Methylation of CLU in primary human cervical keratinocytes and W12 using pyrosequencing

NHFK showed a similar methylation to that of HeLa, a result consistent with that obtained by BGS. When NHCK were transfected with HPV18 there was a much higher level of methylation, with a mean CLU promoter methylation of 31%. W12 passages showed variation in methylation status, with methylation increasing from 8% to 16% from passage 9 to 11, decreasing to 3% in passage 12 and then increasing from passage 21, returning to a mean methylation of 14% in passage 58. These slight changes in methylation may be responsible for the variation seen in RNA expression in these passages (section 3.3). It is possible that the changes in methylation status between passages 9 and 11 may be related to the loss of episomes and HPV integration.

3.8.3.4. Analysis of methylation of the CLU promoter in primary tissue by pyrosequencing

As we have seen by immunohistochemical staining, CLU expression is increased in CIN and then down-regulated in invasive disease. In order to validate the relevance of any correlating epigenetic changes *in vivo*, a natural history cohort was used that included women who were referred for investigation of cytological abnormality, as described earlier. However, these women were not treated immediately, as usually happens, but instead they were kept on follow up to see if their disease spontaneously regressed. Women who progressed to high grade CIN were treated immediately. Therefore, we can compare the patterns of epigenetic change in those women who progressed and in those who did not. The risk of high grade CIN can potentially be related to the presence of epigenetic changes in baseline material taken at the time of study entry.

Table 3.7 shows the percentage methylation of 5 CpG dinucleotides within the CLU promoter in DNA extracted from cervical smears of 14 women with CIN. These cases are matched against 1, 2 or 3 controls that showed either progression to higher grade abnormality or no change in CIN. These show both increases and decreases in the methylation status of the CLU promoter. Changes in methylation were small with both increases and decreases in methylation status associated with disease progression and no change in disease. However, overall an increase in methylation status correlated with disease progression from CIN I to CIN II and CIN III. If this is compared with expression of CLU at the protein level, there was a transient increase in CLU expression between normal cervical epithelium and high grade CIN and a down-regulation of CLU or loss of CLU in invasive disease. This down-regulation in invasive cancer preceded the overall increase in methylation of the CLU promoter and could be a consequence of CLU methylation. However, the fact that there were both increases and decreases in methylation of CLU means that no conclusion can be drawn from this subset and analysis is required in a wider cohort of patient samples.



				CpG methylation (%)					
				1	2	3	4	5	Mean
	Control	CIN/HPV at baseline	A10	10	9	4	10	2	7
	Control	CIN/HPV at baseline	A42	7	15	6	11	5	9
	Control	CIN/HPV at baseline	A60	15	15	7	13	8	12
	Control	CIN/HPV at baseline	A23	13	14	6	12	8	11
	Control	CIN/HPV at baseline	A26	9	10	4	9	5	7
	Control	CIN/HPV at baseline	A2	14	14	7	10	8	11
	Control	CIN/HPV at baseline	A18	16	15	6	12	7	12
	Control	CIN/HPV at baseline	A34	7	10	5	10	5	7
	Control	CIN/HPV at baseline	A29	17	18	10	14	10	14
	Control	CIN/HPV at baseline	A51	12	14	7	12	9	11
	Control	CIN/HPV at baseline	A52	9	11	6	12	7	9
	Control	CIN II at baseline	C9	19	19	9	13	10	14
1	Case	CIN I/III	B16	10	15	6	11	7	10
2	Case	CIN I/II	B1	14	13	6	10	6	10
3	Case	CIN I/III	B9	15	19	10	16	12	14
4	Case	CIN I/II	B7	19	21	11	16	11	16
	Control	CIN II at baseline	C6	8	12	5	12	5	9
	Control	CIN II at baseline	C3	16	15	6	12	7	12
5	Case	CIN II/II	B15	9	9	5	10	5	8
6	Case	CIN II/II	B8	14	18	10	13	11	13
7	Case	CIN II/II	B5	15	17	7	14	9	13
8	Case	CIN I/II	B13	14	17	6	12	9	12
9	Case	CIN II/II	B6	16	16	6	13	9	12
10	Case	CIN II/III	B14	8	12	6	11	5	8
11	Case	CIN II/II	B11	17	13	6	13	7	11
12	Case	CIN II/II	B3	20	16	10	14	8	13
13	Case	CIN II/II	B17	10	13	7	13	7	10
14	Case	CIN I/III	B2	34	36	24	33	26	30
			Mean	13.8	16.2	7.7	12.9	8.1	11.6
	UC			4	5	2	10	5	5
	MC			87	87	76	85	76	83

	Cases	1	2	3	4	5	Mean	% change
1	CIN I/III	D	I	D	D	D	D	1
	CIN I/III	I	I	NC	D	NC	I	1
2	CIN I/II	D	D	D	D	D	D	4
3	CIN I/III	I	I	I	I	I	I	3
4	CIN I/II	I	I	I	I	I	I	7
5	CIN II/II	D	D	D	D	D	D	4
6	CIN II/II	D	I	I	NC	I	I	1
7	CIN II/II	D	I	D	I	I	I	1
8	CIN I/II	D	I	I	D	I	NC	0
	CIN I/II	I	I	I	I	I	I	5
9	CIN II/II	I	D	D	NC	D	D	1
10	CIN II/III	D	D	D	D	D	D	5
11	CIN II/II	I	I	I	I	I	I	3
12	CIN II/II	I	I	I	I	I	I	4
12	CIN II/II	D	D	D	I	NC	D	2
14	CIN I/III	I	I	I	I	I	I	19
	CIN I/III	I	I	I	I	I	I	18

Progression	I	D	Mean
CIN I to CIN II	2	1	I
CIN I to CIN III	2	1	I
CIN II to CIN II	4	4	NC

I = Increase
D = Decrease
NC = No Change

Table 3.7. Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and the means in a number of matched cases of CIN

Percentage methylation of 5 CpG dinucleotides within the CLU promoter in DNA extracted from cervical smears of 14 women with CIN. These cases are matched against 1, 2 or 3 controls that showed no change in CIN. These show both increases and decreases in the methylation status of the CLU promoter, with no meaningful pattern.

3.9. Summary of results

Following a promising set of microarray results and a thorough literature review, I have investigated seven genes which I believed to be candidate tumour suppressor genes in cervical neoplasia. Although KLF4 and RNASET2 were up-regulated post-demethylation of the HeLa cell line, oddly methylation could not be detected in cervical cancer cell lines and so these genes were not pursued as candidates. AKAP12 showed variable methylation is cervical cancer cell lines, but no change in expression in CIN compared with normal cervical epithelium. Having found it to be hemi-methylated in HPV16 positive CaSki, the absence of any methylation in pre and post integration passages of W12 was unexpected. I might have expected to see methylation of AKAP12 in late but not early passages of W12, with methylation being associated with viral integration. This is because in CaSki cells methylation is accompanied by more than 600 integrated HPV-16 copies present; however this was not the case in my results. DKK3, TIMP1, CLU and CADM1 were methylated in cervical cell lines and showed progressive increase in methylation with serial passage of the W12 cell line. Unfortunately, no convincing down-regulation of these genes was seen with serial passage of W12; although it is possible that the methylation may precede transcriptional changes which will only become apparent in passages of the W12 beyond those tested. These four genes showed little or no expression in RNA extracted from cervical cancer patients, however these expression patterns were not substantiated at the protein level in the staining of cervical tissue, which showed no difference in expression between CIN and normal cervical samples.

The starting point of pursuing CLU as a candidate tumour suppressor gene in the pathogenesis of cervical neoplasia was the finding that CLU was up-regulated in the initial demethylation experiment performed in my laboratory. CLU was found to be methylated by MSP in cervical cancer cell lines and this was confirmed by pyrosequencing, with 50% of the cervical cell lines showing high levels of methylation of the CLU promoter. However, although 20 fold up-regulation of CLU was seen post-treatment of the HeLa cell line with 5-Aza-2'-deoxycitidine and TSA, the cell line showed low levels of methylation between 5 and 15% by BGS and pyrosequencing. Additionally, when pyrosequencing was carried out on the demethylated HeLa, no significant change in methylation status was detected; this would suggest that the substantial up-regulation post-treatment of HeLa is more likely to be due to acetylation by the Trichostatin A. In the W12 cell line model, there was variation in the methylation status of CLU with a distinct up-regulation of CLU between passages 9 and 11 proceeded by a significant decrease in methylation in passage 12; these changes in the methylation status of the CLU promoter may be related to the loss of episomes and viral integration.

Methylation of CLU in cervical cancer was initially identified by Methylation Specific PCR, which is although is a very sensitive technique, it is biased towards the detection of even low levels of methylation, which could potential give false positive results. For example, in the HeLa cell line the CLU promoter was thought to be heavily methylated based on methylation detection by MSP, yet quantitative analysis by bisulphite genomic sequencing and pyrosequencing revealed only a small promotion of cells are methylated. Also, when this cell line were

demethylated in vitro it was also treated with the acetylating agent trichostatin A and so expression changes observed may not solely be due methylation, as discussed in chapter 6.16.1.

When cohorts of women with CIN were analysed at the protein level, there was an up-regulation of CLU in high grade CIN compared with normal cervical epithelium, with a down-regulation or loss of CLU expression in squamous carcinoma and adenocarcinoma of the cervix. Q RT-PCR data is not entirely convincing given the inherent problems of housekeeping gene normalisation and is discussed in Chapter 6. Protein expression data is more reliable as it is not subject to the same sensitivity issues when comparing gene expression to housekeeping gene expression.

In DNA extracted from cervical smears from women with CIN the majority showing an increase in methylation status correlating with disease progression from CIN I to CIN II and CIN III. All of the methylation frequencies seen in cervical smears were much lower than those in the cervical cell lines, with the exception of the HeLa cell line, suggesting that cell lines do not accurately represent the gene methylation frequencies observed *in vivo*, which is further discussed in chapter 6.16.4. However, the methylation status of the CLU promoter was only examined in CIN and not in invasive disease, and had we examined invasive tumour samples the methylation frequencies could have been much higher, especially given the expression of CLU was very low level or lost in squamous and adeno-carcinoma samples examined. Alternatively, it is possible that high levels of methylation could be a feature of a small subset of cervical tumours.

Moreover, the story may not simply be confined to the cervix. For example, when Dr John Arrand used SNP analysis in the NPC cell line C666-1, this highlighted a possible deletion encompassing the clusterin locus on chromosome eight. Victor Lopes, a head and neck surgeon, has illustrated a possible deletion at this locus in oral cancer tissue samples. Gene expression profiling has also demonstrated that compared with disease free controls, a down-regulation of CLU is observed in the vast majority of oral cancers taken from patients in the UK and Sri Lanka. Microarray analysis has also shown that compared with disease free controls, a down-regulation of CLU in 18 of 25 NPC samples. There is therefore a compelling case for considering clusterin as a putative tumour suppressor gene in squamous cancer.

Chapter 4: Results 2

Investigation of the role of clusterin in oral and nasopharyngeal carcinoma

Overview

To further investigate the role of CLU in squamous cancer, I next set out to investigate the expression of CLU, and its regulation in oral and nasopharyngeal cancer. I first outline the reasons which led me to explore a possible tumour suppressor role for CLU in the pathogenesis of these cancers. Then, for each cancer in turn, I describe using cell lines and whole tissue how the expression of CLU varies at the RNA and protein level with disease state. I next report how methylation of the CLU promoter varies across these samples. In the section on nasopharyngeal cancer, I examine the possibility that CLU expression in the NPC cell line Ad-AH might be regulated by EBV or its latent genes. Finally, I focus in some detail on the NPC cell line C666-1 which is a potential model for exploration of the consequences of restoring CLU expression, and its possible mechanism of action.

4.1. Reasons for investigating the expression of CLU, and its regulation in oral cancer

The decision to investigate the expression and regulation of CLU in oral cancer was prompted by the results of a microarray experiment performed by Dr Victor Lopes, a PhD student in the Institute for Cancer Studies. The tissue samples included in this array were collected from oral cancer patients in the United Kingdom and in Sri Lanka (Kandy). Samples from each region were analysed separately in order to avoid potential confounding factors caused by different geographic localities, such as ethnicity, environment and diet, which can give rise to distinct origins in certain diseases. The analysis revealed the down-regulation of CLU in 21 out of 25 UK oral cancers and all 27 Kandy tumours when compared with tissue samples from 5 cancer-free controls (Figure 4.1). Down-regulation of CLU in 11 of 12 of these oral cancers was confirmed using Q RT-PCR by Ms P Gan, a BMedSci student attached to the Institute (Figure 4.2).

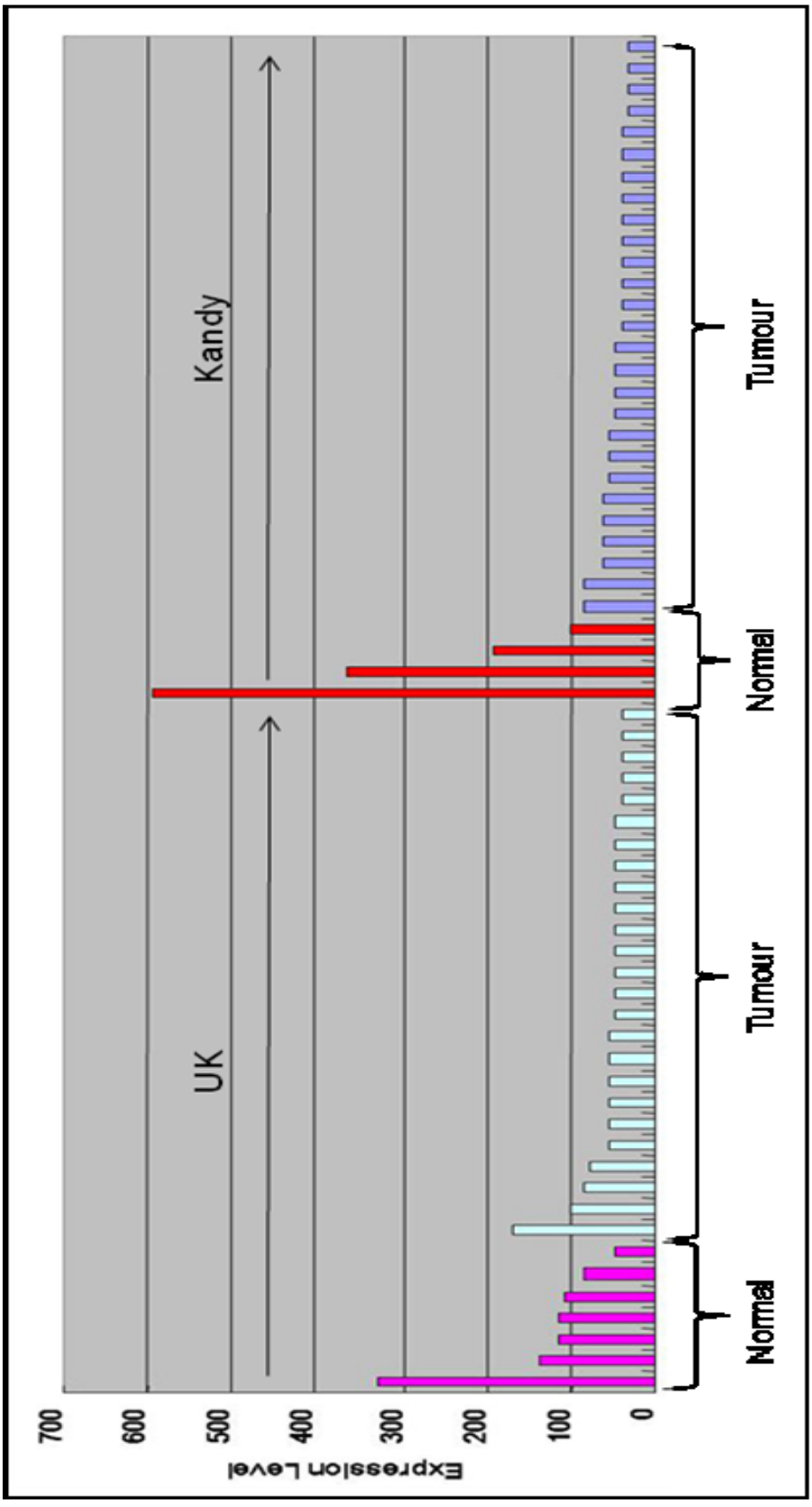


Figure 4.1: Gene expression profiling of CLU in normal tissues and oral cancer samples in both UK and Kandy groups.
Gene expression profiling showed that compared with disease free controls there is a down regulation of CLU in the majority of all oral cancer samples tested

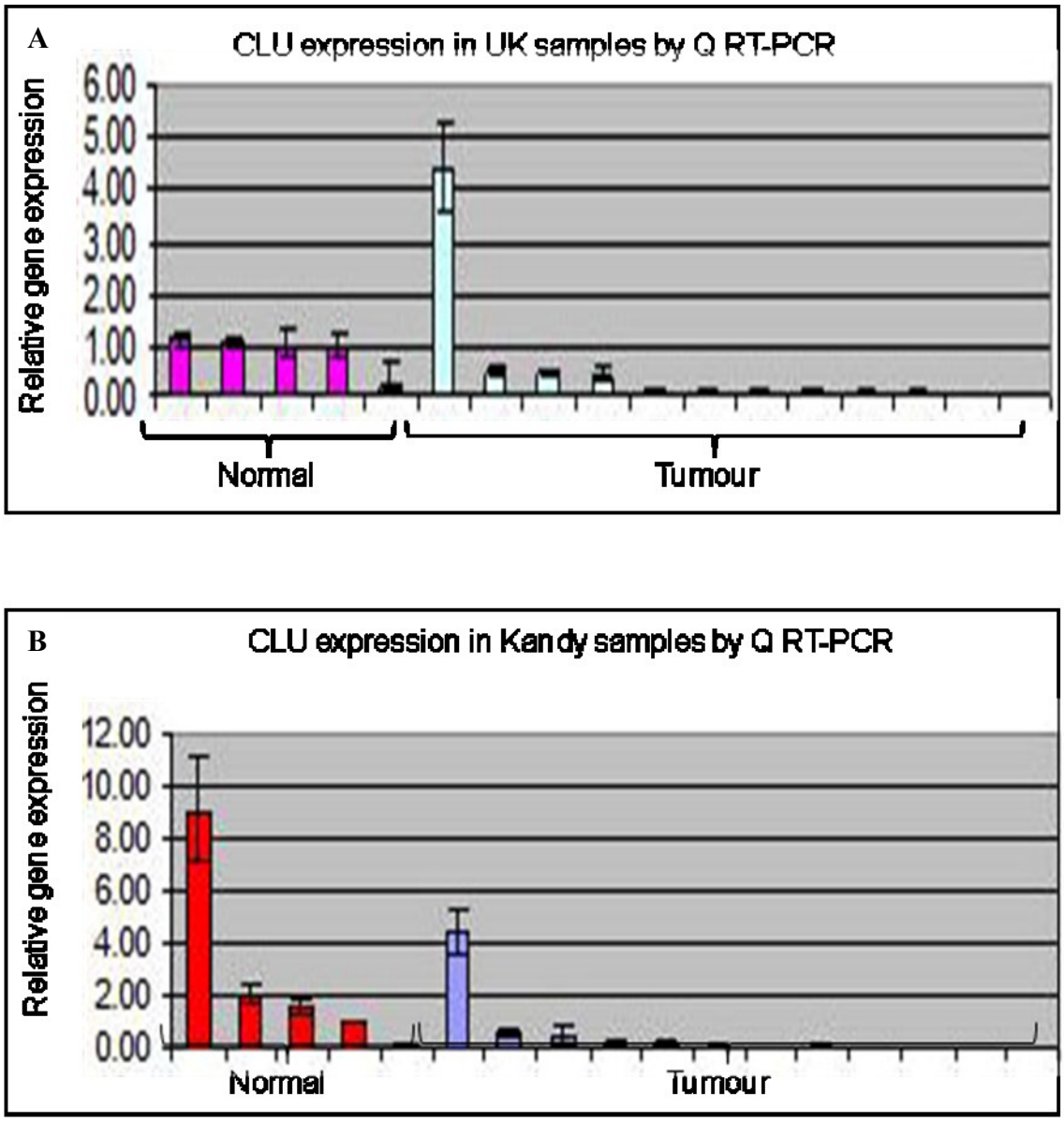
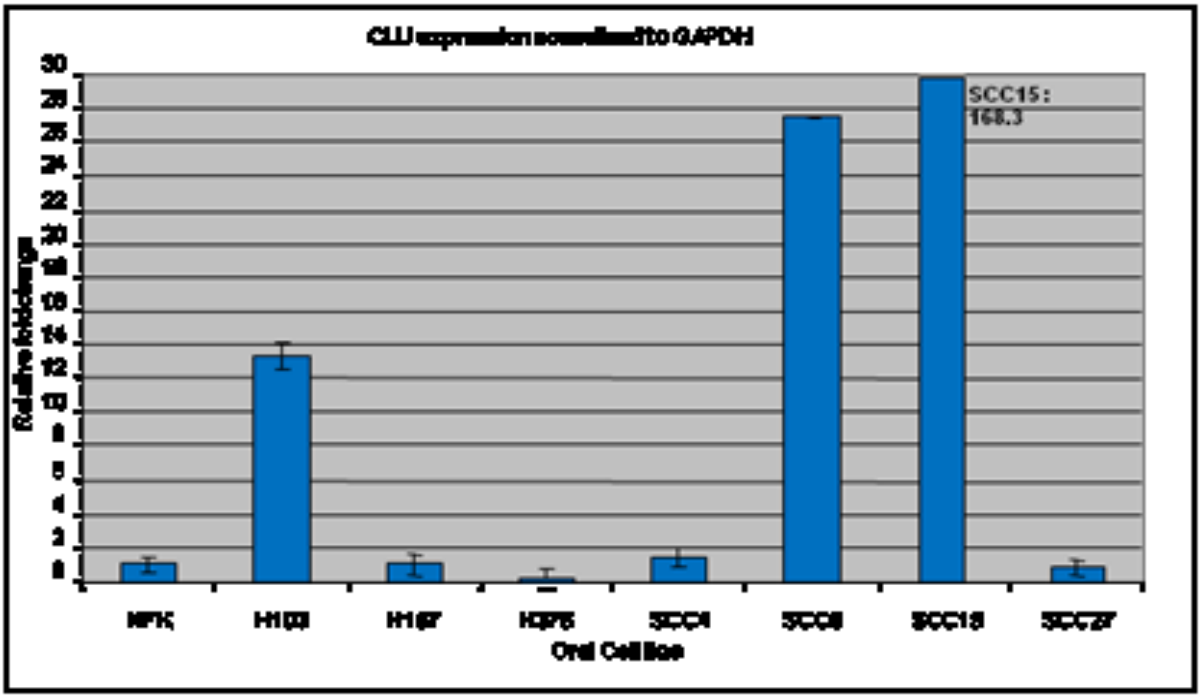


Figure 4.2A and B: Q RT-PCR validation of CLU expression in normal tissues and oral cancer samples from the UK and Sri Lanka

Expression of CLU in the 12 oral cancers compared with 5 normal controls for both (**A**) UK and (**B**) Kandy samples, confirms a down-regulation of CLU in 11 out of 12 tumours.

4.1.1. Expression of CLU at the RNA level in oral cancer cell lines

The expression of CLU at the RNA and protein level was investigated in seven oral cancer cell lines (H103, H157, H376, SCC4, SCC9, SCC15, SCC27) provided by Dr Chris Dawson, and in neonatal foreskin keratinocytes provided by Dr Sally Roberts. Figure 4.3 shows the mRNA expression of CLU in seven oral cancer cell lines compared with that found in normal human foreskin keratinocytes by Q RT-PCR using two different housekeeping genes, GAPDH and B2M. Although B2M had been shown to be a more consistent house keeping gene in cervical cancer cell lines, the scenario might be different in other cancer cell lines and so 2 housekeeping genes were used for comparison in oral cancer cell lines. When expression was normalised against GAPDH, CLU was found to be down regulated compared with NHFK in one oral cancer cell line (H376), and up regulated in three (H103, SCC9 and SCC15). When expression was normalised against B2M, a somewhat different pattern emerged: CLU was found to be down regulated in H103, and up-regulated in SCC4. Because of this uncertainty I examined expression levels using semi-quantitative RT-PCR, which confirmed the results obtained using B2M as a housekeeping gene.



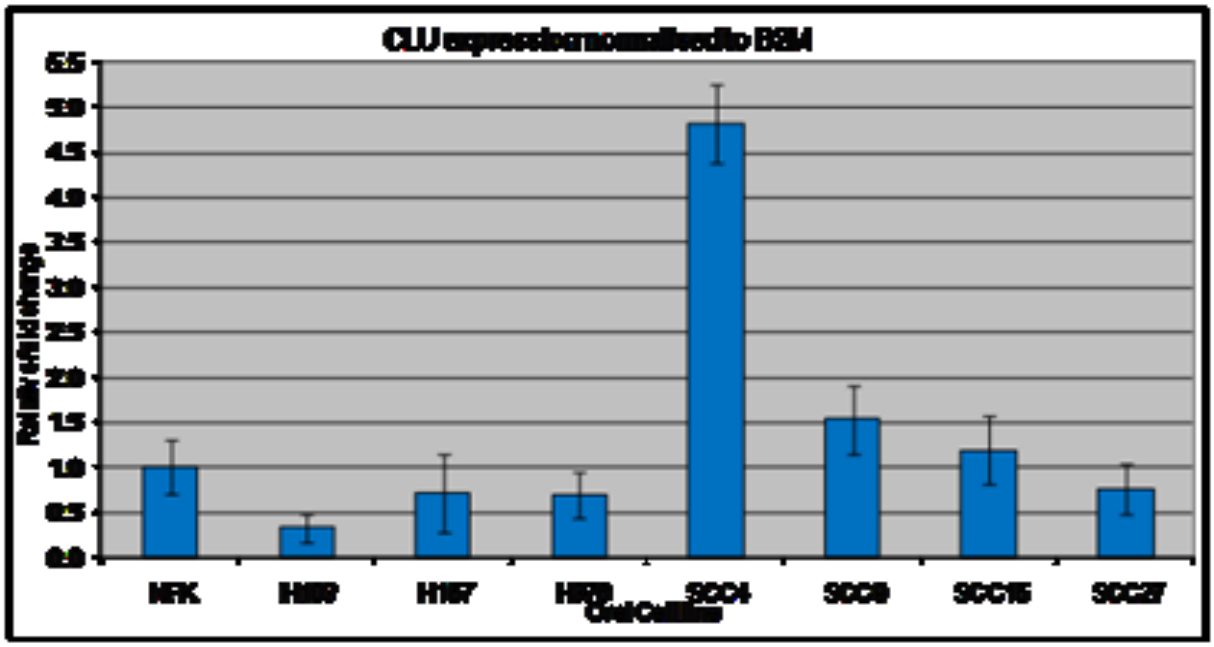


Figure 4.3: Q RT-PCR analysis of CLU expression in oral cancer cell lines

Expression of CLU in the seven oral cancer cell lines compared with expression levels in normal foreskin keratinocytes, using both GAPDH and B2M housekeeping genes for comparison, showed an up-regulation in some cell lines whilst a down-regulation was observed in others.

4.1.2. Expression of CLU at the protein level in oral cancer cell lines using Western blotting

I investigated the expression of CLU using immunohistochemistry and Western blotting of the same 7 oral cancer cell lines. Figure 4.4 shows the expression of CLU using Western blotting and two commercially available antibodies. The 60kDa and 40kDa CLU proteins are present in all cell lines. CLU appears to be over-expressed at the protein level in SCC4 compared with normal keratinocytes and with the other oral cancer cell lines (H103, H157, H376, SCC9, SCC15, SCC27), with no evidence of down-regulation of CLU at the protein level in these cell lines. These results mirror those obtained at the RNA level when normalised to B2M, and are contrary expression levels in oral cancers by microarray and Q RT-PCR. This suggests that the NHFK control available at the time of this study may not have been a representative control for these samples or that these cell lines are not representative of tumour samples from patients.

4.1.3. Expression of CLU at the protein level in oral cancer cell lines using immunohistochemistry

I next used immunohistochemistry with two antibodies to examine the expression of CLU in cytospins prepared from the seven oral cancer cell lines (Figure 4.5 and 4.6). Compared with the other oral cancer cell lines, the staining appears to be more intense in SCC4 cells, and less intense in SCC27, H157 and H376 cell lines. Gradation of this staining shows that H103, SCC4, SCC9 and SCC15 display very strong (3+) cytoplasmic staining; H157, H376 and SCC27 show moderate (2+) to low (1+) cytoplasmic staining. These observations are consistent with those seen by western blot and Q RT-PCR results obtained following normalisation of expression

against B2M, but not with those obtained following normalisation against GAPDH, suggesting that B2M is a more representative and suitable house keeping gene for these samples (section 4.2.1). Both antibodies resulted in the same trend in staining pattern, however, staining intensity in SCC4, SCC9 and SCC15 was much greater using the Santa Cruz antibody than the Upstate antibody. This was also the case when staining a number of other tissues with the antibody, such as in cervix, suggesting it may have a greater binding affinity for its epitope.

4.1.4. Expression of CLU at the protein level in oral cancers using immunohistochemistry

I next used immunohistochemistry with two CLU antibodies to investigate the expression of CLU in 40 oral cancers (Figure 4.7 and Table 4.1). Only 2 out of 40 cancers showed expression of CLU, which was very weak (1+), whereas the remaining cancers did not express CLU. Where CLU expression was detected, it was the necrotic cells that stained positive for CLU, which is not surprising given the role of CLU in the regulation of apoptosis.

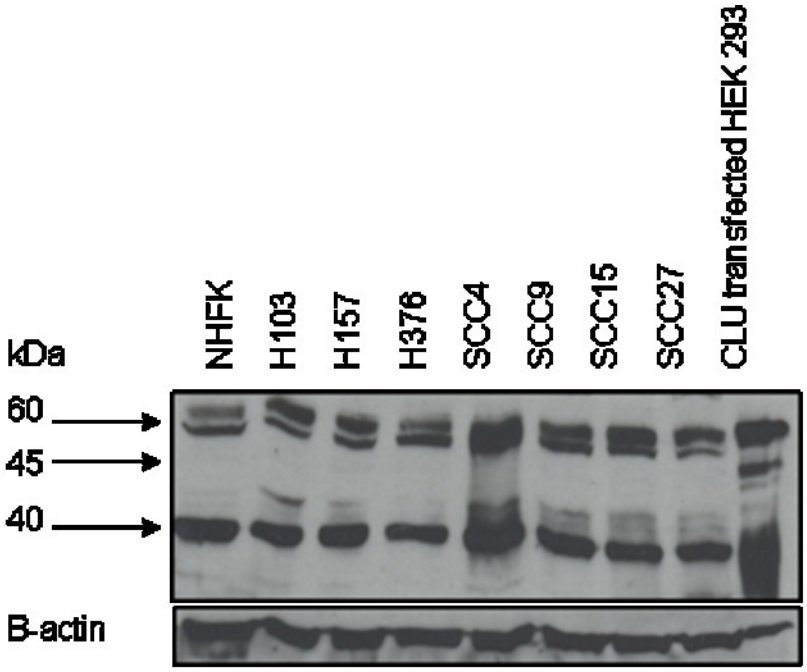


Figure 4.4: CLU expression in a panel of oral cancer cell lines compared with normal human foreskin keratinocytes
55kDa and 40kDa proteins are present in all cell lines and over expression in SCC4 compared with normal keratinocytes and the other oral cancer cell lines.

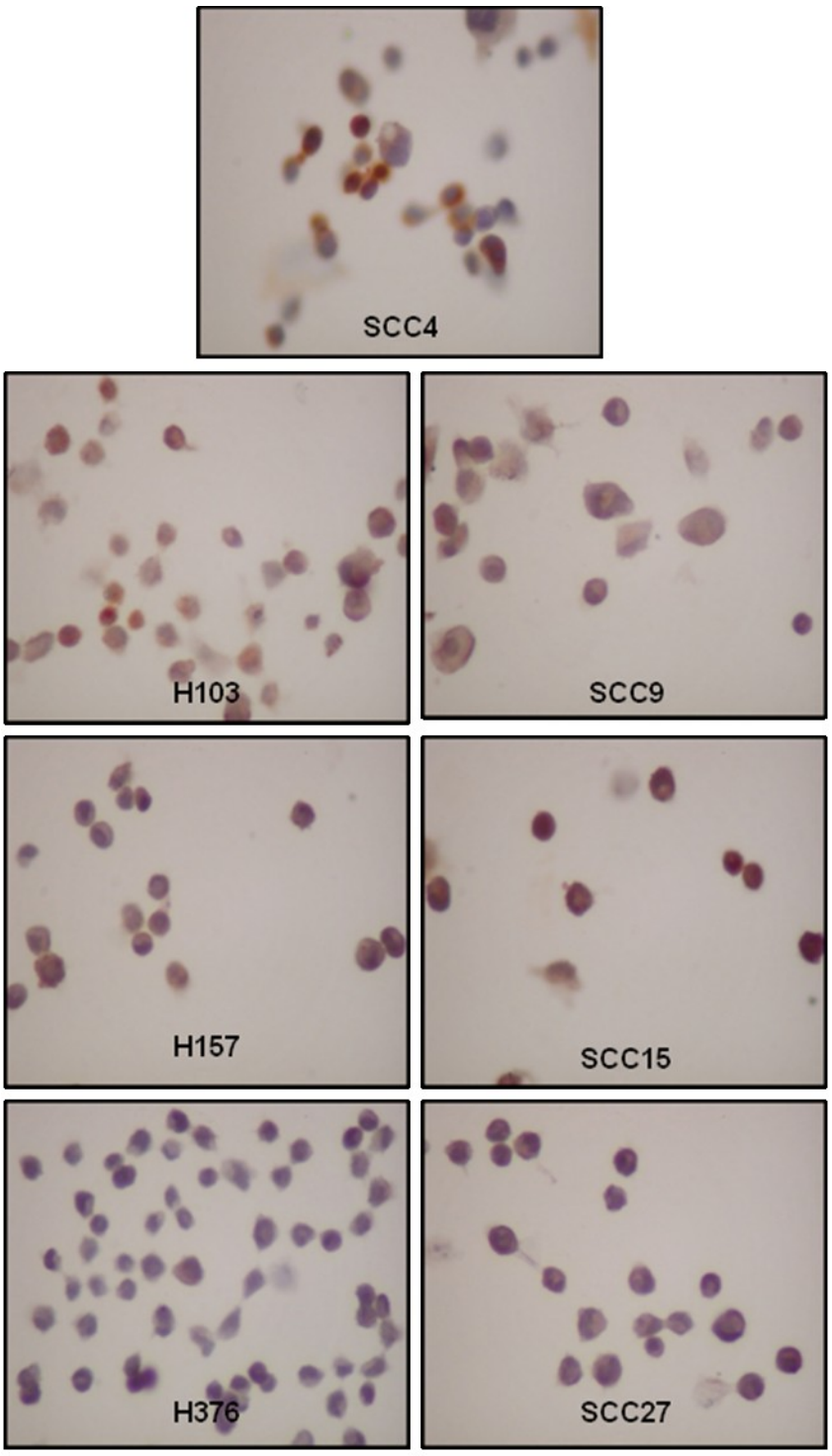


Figure 4.5: Immunohistochemical staining of cytospins from oral cancer cell lines using CLU 41D antibody (Upstate)

This figure shows over expression of CLU at the protein level in SCC4 compared with the other oral cancer cell lines and decreased expression in SCC27, H157 and H376 cell lines.

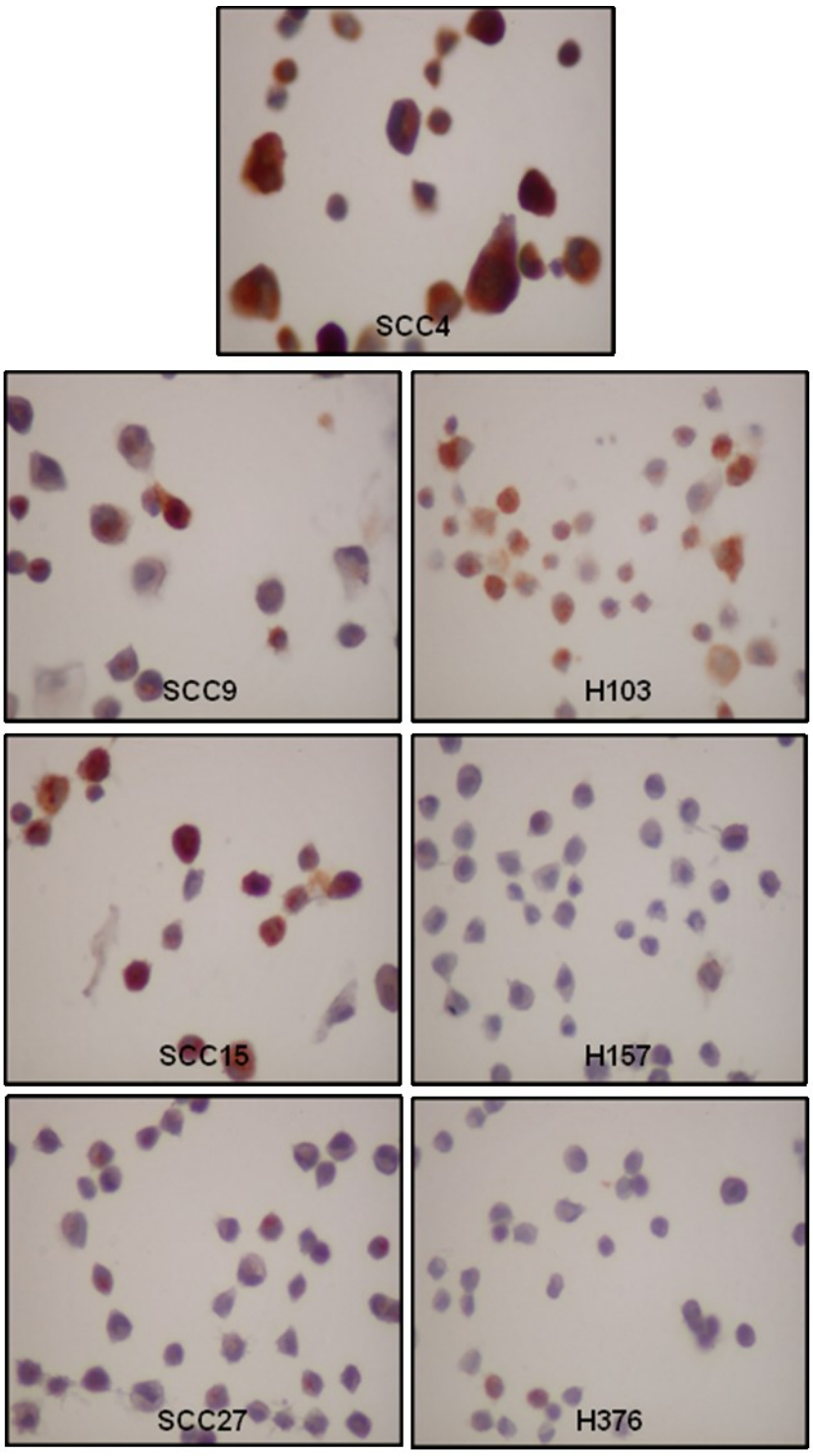


Figure 4.6: Immunohistochemical staining of cytospins from oral cancer cell lines using CLU B-5 antibody (Santa Cruz)

Immunohistochemistry shows over expression of CLU at the protein level in SCC4 compared with the other oral cancer cell lines and decreased expression in SCC27, H157 and H376

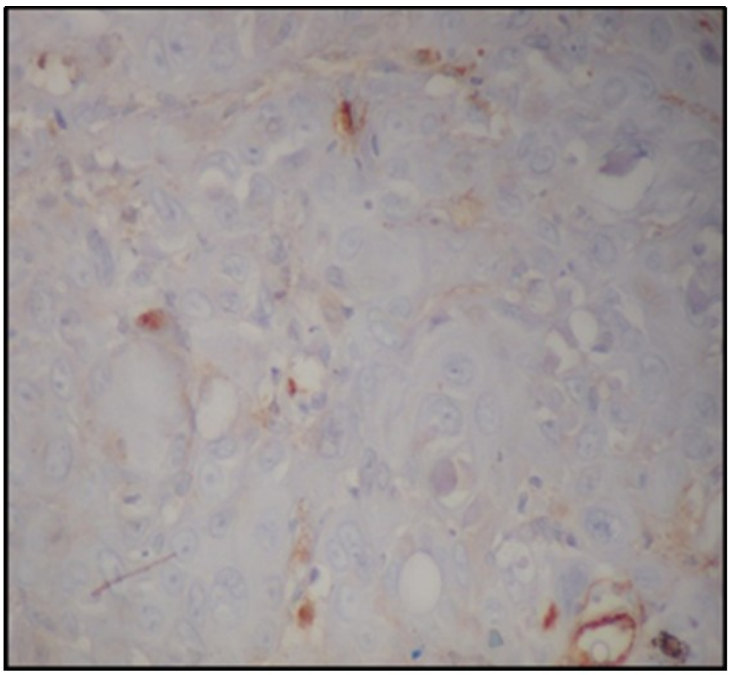


Figure 4.7: Immunohistochemical staining of oral cancer

Immunohistochemistry shows weak 1+ staining for CLU expression in oral cancers. However, the absence of CLU staining of normal oral mucosa means that we cannot determine whether this weak staining is reflective of a down-regulation of CLU in oral cancer.

Oral cell lines	Upstate	Santa Cruz
H103	3+	3+
H157	2+	1+/2+
H376	2+	1+
SCC4	3+	3+
SCC9	2+	3+
SCC15	3+	3+
SCC27	2+	2+

Oral Tumour	Upstate	Santa Cruz
940494	-	-
930726	-	-
942838	-	-
942852	-	-
930801	-	1+
932100	1+	2+
951285	-	-
951112		-
932128	-	-
931847	-	-
930498	-	-
932850	-	-
950080	-	-
952410	-	-
952366	-	-
9511886	-	-
951738	-	-
932223	-	-
9510400	-	-
930891	-	
942933	-	-
932053	-	-
931396	-	-
931829	-	2+
932059	-	
950622	-	-
941828/1	-	-
941862	-	-
941207	-	-
943120/B	-	-
95284/11	-	-
94/1230		1+
931068/2	-	-
942751	-	-
942335	-	-
942311	-	-

Table 4.1: Immunohistochemical staining of oral cancer cell lines and oral tumours.

A low level of CLU expression is evident in almost all oral tumours, however without normal oral epithelium this cannot be said to be down-regulated.

4.2. Allelic loss and epigenetic regulation of CLU in oral cancer

A contemporaneous in-house investigation using SNP analysis performed on whole tissue had revealed a possible deletion encompassing the CLU locus (8p21) in a number of oral cancers. This analysis was performed by Dr. J Arrand in the Institute for Cancer Studies, who subsequently confirmed using genomic PCR, loss of one CLU allele in oral cancers (Figure 4.8). Although this region includes 11 genes which are reported to have a tumour suppressive function (BNIP3L, CLU, NKX3-1, PPP2R2A, RHOBTB2, TNFRSF10A and TNFRSF10B), only CLU was found to be down-regulated in the gene expression array.

4.2.1. Epigenetic regulation of CLU expression in oral cancers and cell lines

As previously described (section 3.7), promoter methylation may have a strong influence on gene silencing. To evaluate whether this phenomenon governed CLU expression at other sites of cancer, the possible contribution of CpG island methylation to the loss of CLU expression in oral cancer cell lines and tissue was investigated using MSP and pyrosequencing.

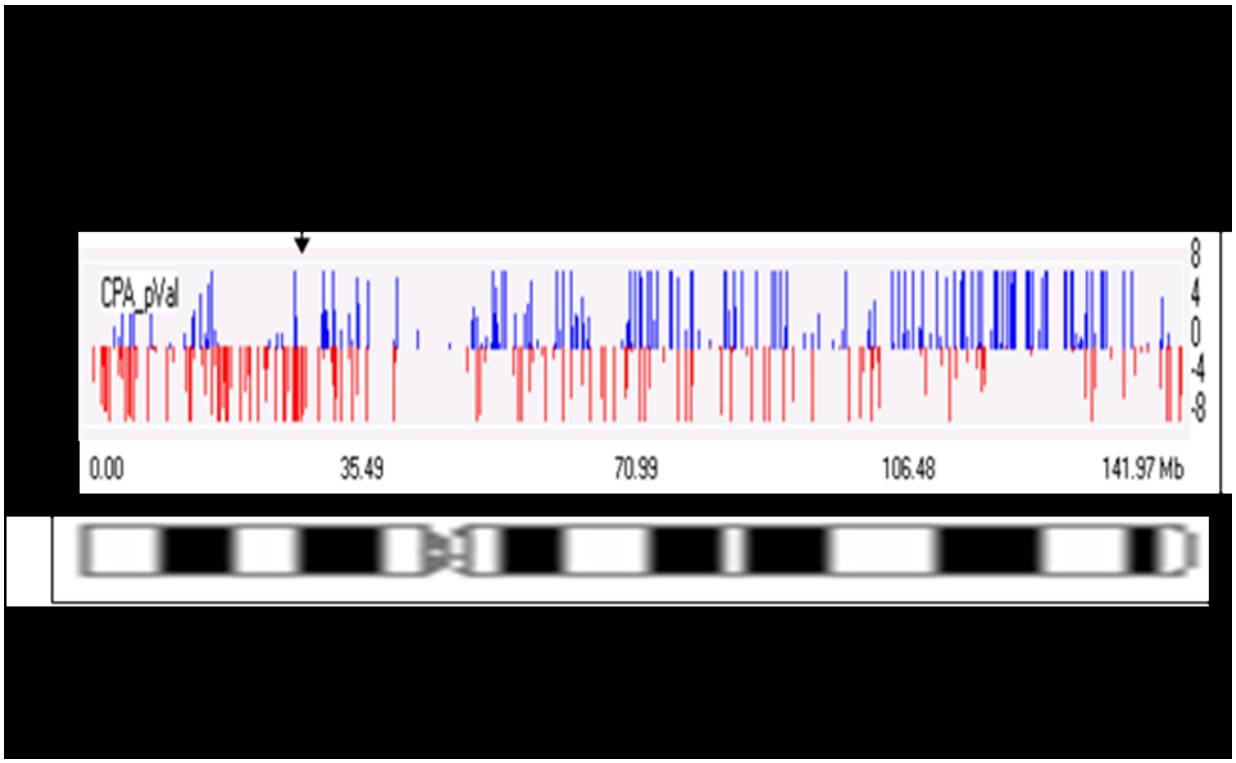
4.2.1.1. Frequency of methylation of the CLU promoter in oral cancer cell lines as determined by MSP

MSP was used to determine the presence of methylated and unmethylated forms of CLU in six oral cancer cell lines, H103, H157, H376, SCC4, SCC9, SCC15 (Figure 4.9). Single discrete bands of the expected size of amplified PCR products were detected for all samples and controls

behaved as expected throughout. Where evidence of methylation was detected, results were repeated alongside unmethylated results to confirm that the results were reproducible. Methylated forms were detected in all cell lines, with four of the five also showing weak unmethylated bands (H157, SCC4, SCC9, SCC15).

4.2.1.2. Frequency of CLU promoter methylation in oral cancer cell lines as determined by pyrosequencing

Pyrosequencing was used to confirm the pattern of promoter methylation of CLU in DNA extracted from oral cancer cell lines. As in section 3.7.3.4, 4 sets of pyrosequencing primers were optimised on commercially available controls, the most reproducible set taken forward for analysis in all DNA samples. Table 4.2 shows methylation in 5 CpG dinucleotides of the CLU promoter and the mean of these. In H103 the mean methylation was 28%, 24% in H157, 22% in H376, 16% in SCC4, 17% in SCC9, 20% in SCC15 and 25% in SCC27. The controls behaved as expected throughout with unmethylated control DNA showing a mean methylation of 5% and methylated control DNA 83%. Interestingly, SCC4 which appeared to have the highest level of CLU expression at the RNA and protein level of all the cell lines (section 4.2) also had the lowest level of methylation of the 7 cell lines.


CPA_pVal
8
4
0
-4
-8
0.00
35.49
70.99
106.48
141.97 Mb

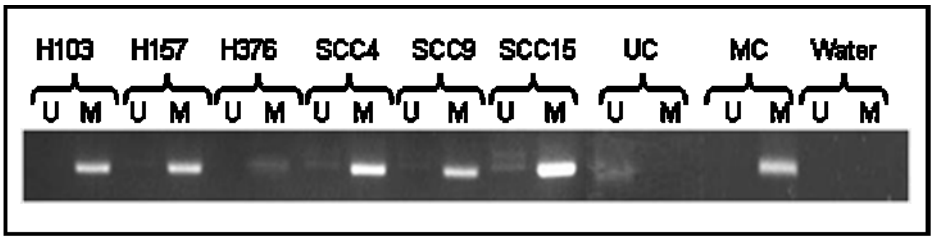


Figure 4.9: MSP analysis CLU promoter methylation in oral cancer cell lines.

Methylation of CpGs within the CLU promoter was detected in 5 of 6 cell lines (H103, H157, SCC4, SCC9, SCC15), with very weak detection of an additional unmethylated product in 4 of these (H157, SCC4, SCC9, SCC15)

	CpG methylation (%)					
	1	2	3	4	5	Mean
H103	28	30	23	28	29	28
H157	17	30	13	28	29	24
H376	14	27	14	30	36	22
SCC4	16	16	9	18	21	16
SCC9	12	17	8	25	20	17
SCC15	13	26	12	27	22	20
SCC27	22	30	15	30	27	25
Mean	17.4	25.1	13.4	26.6	26.3	21.7
UCA	4	5	2	10	5	5
MC	87	87	79	85	78	83

Table 4.2: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and their means for oral cancer cell lines

Percentage CLU methylation for each of 5 CpG dinucleotides of the CLU promoter are confirmed in oral cancer cell lines, showing a range of methylation between 8% and 30% across all CpGs

4.2.1.3. Frequency of CLU promoter methylation in oral cancers as determined by MSP

Again using MSP, I determined the presence of methylated and unmethylated forms of CLU in 4 oral cancers and in oral tissue taken from a disease-free patient (Figure 4.10). Methylated and unmethylated forms were detected in all cancers and in the control patient, with no apparent difference between the disease-free control and the cancers. The presence of both unmethylated and methylated products, hemi-methylation, could be due to a mixed cell population with some cells being methylated while others are not, or mixture of methylated and unmethylated alleles within these tumours reflecting that the population as a whole is heterogeneous. It is also possible that in the tumour samples that adjacent normal cells may be contributing to the level of methylation detected and micro-dissected tumours would have provided a more accurate comparison.

4.2.1.4. Frequency of methylation of the CLU promoter in oral cancers as determined by pyrosequencing

10 randomly selected oral tumours from a cohort of 200 oral cancers were used to evaluate CLU promoter methylation frequency. Samples were randomly selected blinded to any clinical information about the tumour so as not to bias any subsequent data interpretation. Unfortunately, there was no remaining DNA from normal oral epithelium after MSP analysis and so this was unable to be validated by pyrosequencing. Table 4.3 shows methylation in 5 CpG dinucleotides of the CLU promoter and the mean of these. The mean methylation across all 5 CpG dinucleotides for all 10 tumours is 13%, with a range from 7 to 20%. CpG dinucleotides 1, 2 and

4 have consistently higher levels of methylation than CpGs 3 and 5 in all 10 of the oral cancers, and may be preferentially methylated in this subset of tumours. The controls behaved as expected throughout with unmethylated control DNA showing a mean methylation of 5% and methylated control DNA 83%.

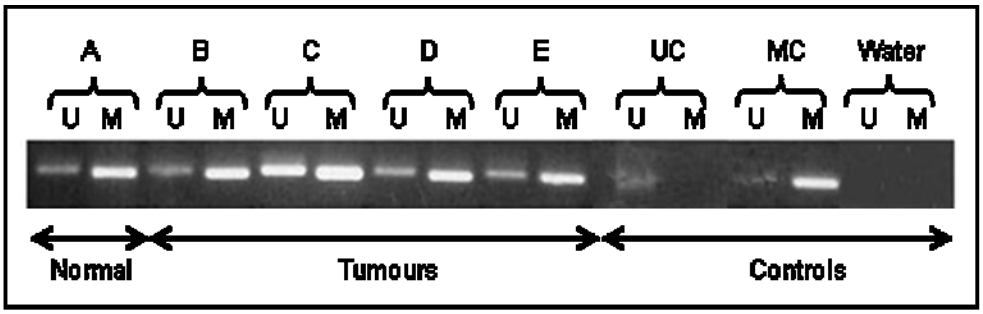


Figure 4.10: MSP analysis CLU promoter methylation in oral tissue.
Using MSP, both methylated and unmethylated forms can be detected in all tumours and in the disease free control.

		CpG methylation (%)					
		1	2	3	4	5	Mean
1	932225a	15	19	10	17	11	14
2	932440a	14	17	8	15	9	13
3	932667a	12	15	7	13	8	11
4	940705a	14	15	7	13	8	12
5	941556a	15	16	8	14	8	12
6	941857a	14	16	8	14	9	12
7	942023a	15	17	9	14	11	13
8	950415a	17	20	11	17	12	15
9	950106a	15	17	9	15	9	13
10	951473a	14	18	8	16	9	13
	Mean	14.5	17	8.5	14.8	9.4	12.8
	UC	4	5	2	10	5	5
	MC	87	87	79	85	78	83

Table 4.3: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and their means for oral tumours

Percentage CLU methylation for each of 5 CpG dinucleotides of the CLU promoter are confirmed in oral cancers. Methylation levels are lower in the tumours than in the oral cancer cell lines.

4.3. Reasons for investigating the expression of CLU, and its regulation in nasopharyngeal cancer

My interest in nasopharyngeal cancer (NPC) was prompted by the results of a microarray experiment which had been analysed in the Institute for Cancer Studies as part of an on-going collaboration with the Department of Oral and Dental Science, University of Bristol. The samples included in this array were collected from Malaysian patients by Dr. Lee-Fah Yap who was then a PhD student in Bristol. The analysis revealed the down-regulation of CLU in 19 (76%) of 25 nasopharyngeal cancers when compared with tissue samples from 3 cancer-free controls (Figure 4.11). A review of the literature also revealed down-regulation of CLU in two published arrays of primary NPC, one of which included whole tissue samples, the other, micro-dissected epithelium (Shi 2006, Sriuranpong 2004).

4.3.1. Expression of CLU in nasopharyngeal cancer cell lines

The expression of CLU at the RNA and protein level was investigated in the NPC cell lines C666-1, Ad-AH, CNE1, SUNE1, NPC TWO 1, NPC TWO 4, HONE 1. It was also measured in normal foreskin keratinocytes; in NP69, an SV40 immortalised normal nasopharyngeal line; and in HEK 293 cells, a human embryonic kidney cell line.

4.3.1.1. Expression of CLU at the RNA level in nasopharyngeal cancer cell lines

Figure 4.12A and 4.12B show the mRNA expression of CLU in the seven NPC cell lines compared with that found in normal foreskin keratinocytes using Q RT-PCR. When expression was normalised against GAPDH, CLU was found to be down regulated in two NPC cancer cell lines (C666-1 and NPC TWO 4) compared with normal human foreskin keratinocytes, and up regulated in three (Ad-AH, SUNE 1 and NPC TWO 1). When expression was normalised against B2M, a somewhat different pattern emerged: CLU was found to be down regulated in only one cell line (C666-1), and up regulated in four others (CNE 1, SUNE 1, NPC TWO 1 and HONE 1). Because of this uncertainty I examined expression levels using semi-quantitative RT-PCR, which verified the results normalised to B2M.

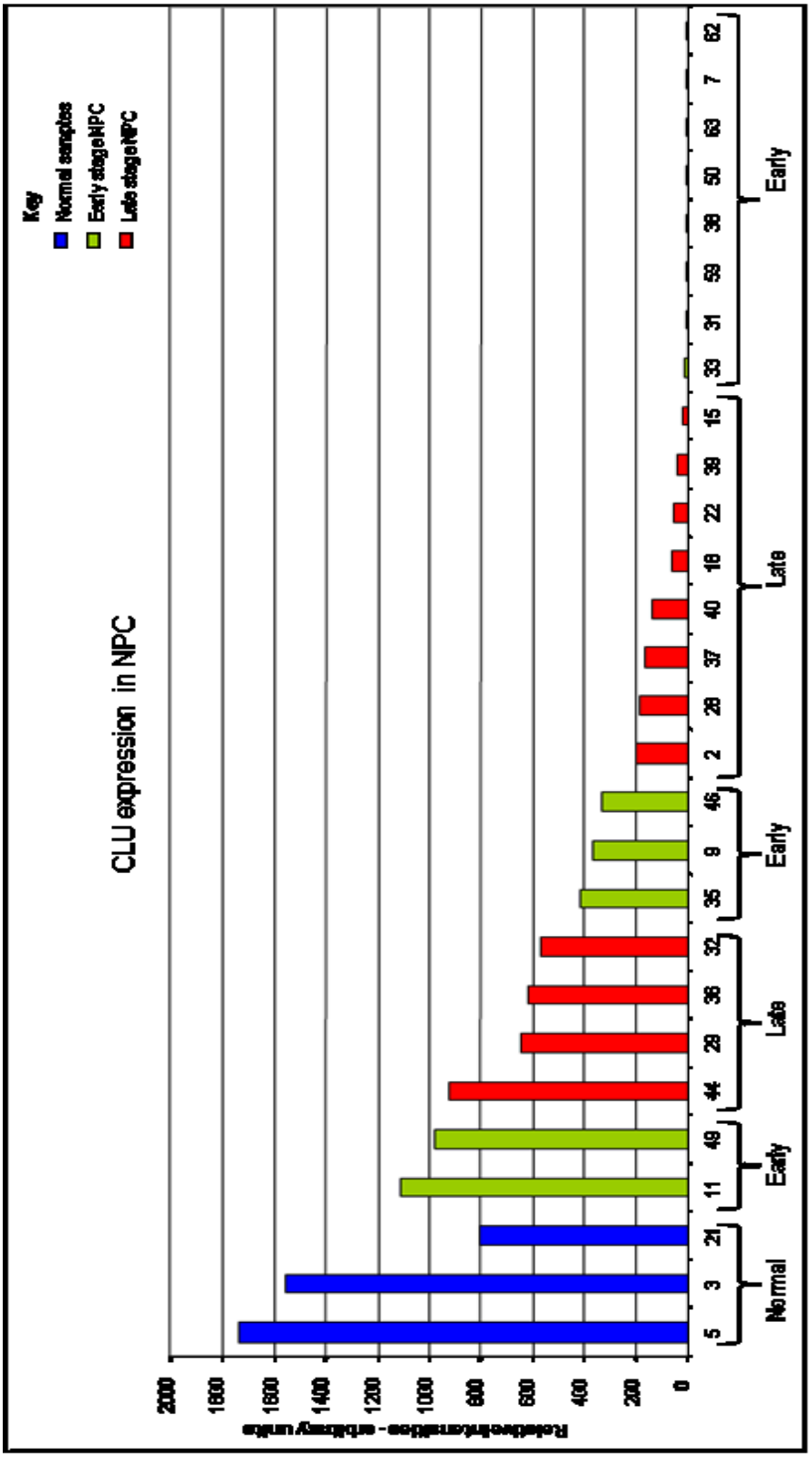
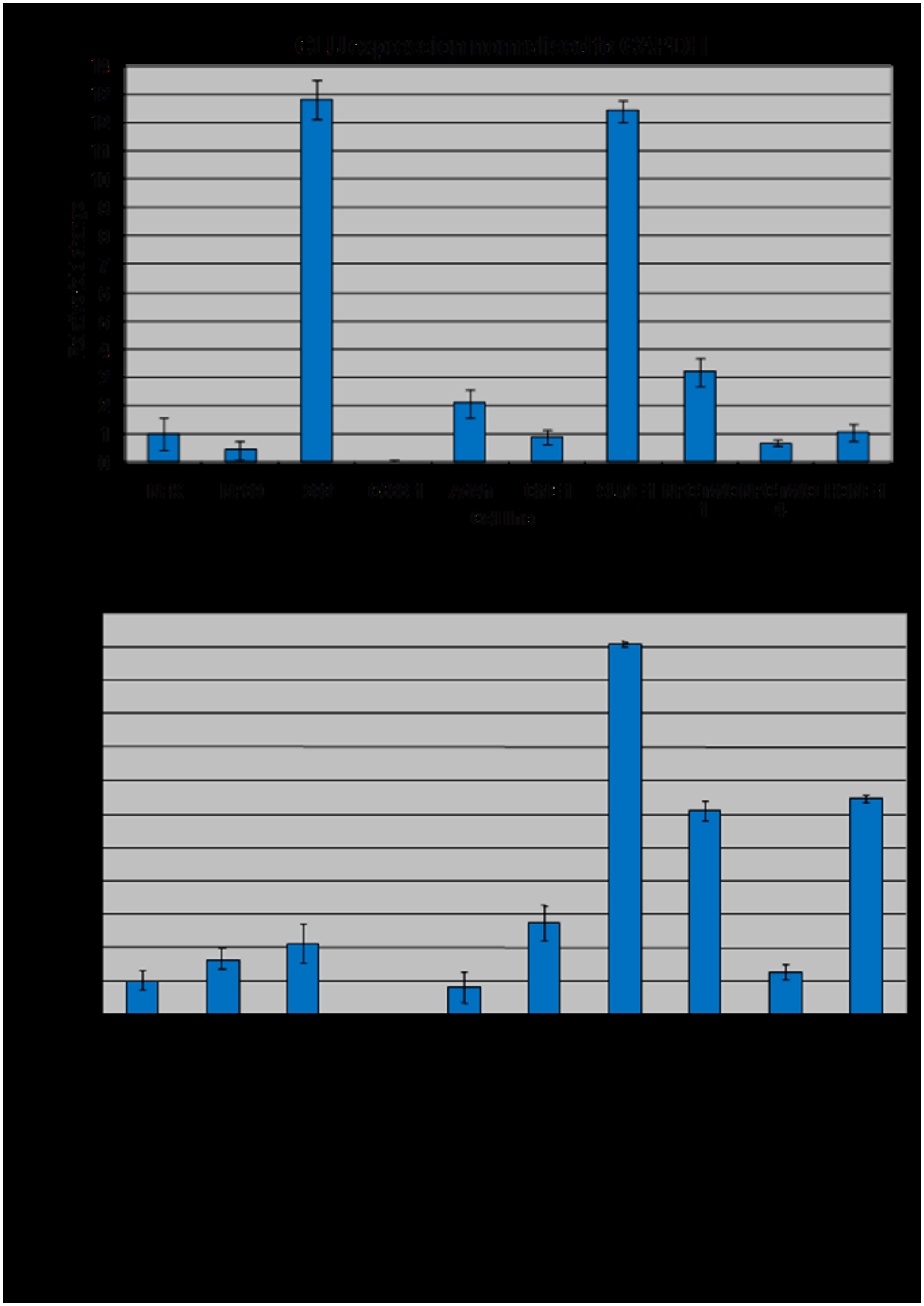


Figure 4.11: Microarray analysis showing the relative intensities of CLU in each of the early and late stage NPC samples as compared to 3 normal nasopharyngeal samples.

Microarray analysis revealed the down-regulation of CLU in 19 (76%) of 25 nasopharyngeal cancers when compared with tissue samples from 3 cancer-free controls



4.3.1.2. Expression of CLU at the protein level in nasopharyngeal cell lines using western blotting and immunohistochemistry

I used Western blotting with two CLU antibodies to evaluate the expression of CLU in lysates prepared from NHFK, NP69, Ad-AH, C666-1, HONE1, CNE2, AGS and following infection of Ad-AH, HONE 1, CNE 2 and AGS with EBV (Figure 4.13). C666-1 does not express any of the CLU proteins and mirrors findings at the RNA level that CLU is not expressed in this cell line. The 60kDa and 40kDa CLU proteins are present in other all cell lines. Ad-AH, HONE 1 and CNE 2 show higher levels of CLU expression than NHFK and NP69, and post-infection of these cell lines with EBV there is a significant over-expression of the 60kDa CLU protein. HONE 1 and CNE 2 additionally highly express a 45kDa CLU protein, as does Ad-AH, however this is at a much a lower level. This molecular weight corresponds to the size of nuclear CLU, but the antibodies used are not able to discriminate between CLU isoforms and so it is also possible that this is a precursor protein for cytoplasmic CLU. The gastric cancer cell line AGS displayed a similar profile of expression of the CLU protein as the NPC cell lines. The expression of CLU at the protein level was also examined by immunohistochemistry which confirmed that C666-1 did not express CLU. Ad-AH cells showed moderate (2+) to high (3+) levels of CLU which was predominantly nuclear in localisation using both Santa Cruz and Upstate antibodies (Figure 4.18 and Table 4.5).

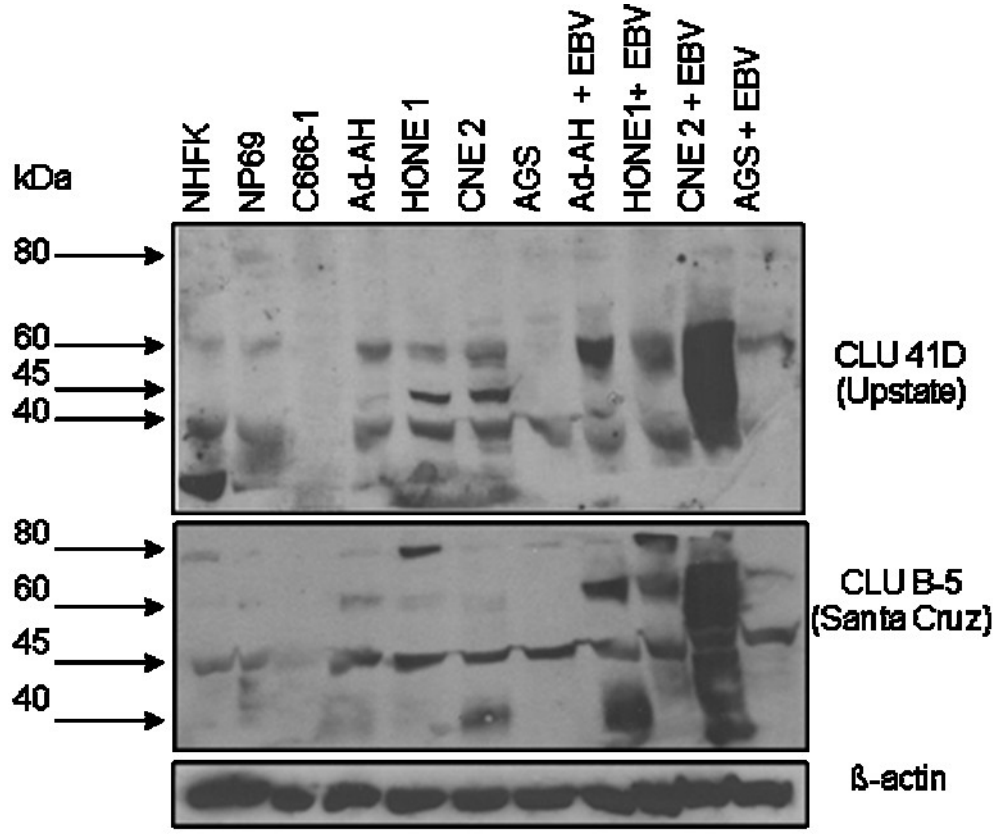


Figure 4.13: Expression of CLU at the protein level in nasopharyngeal cell lines using western blotting

C666-1 does not express any of the CLU proteins and the 60kDa and 40kDa CLU proteins are present in other all cell lines, with 45kDa CLU in Ad-AH, HONE 1 and CNE 2. The gastric cancer cell line AGS displays the same profile of expression of the CLU proteins. 60kDa CLU protein is over expressed in these cell lines post infection with EBV.

4.3.1.3. Expression of CLU at the protein level in nasopharyngeal cancers using immunohistochemistry

Figure 4.14 shows using immunohistochemistry the expression of CLU in normal tonsillar epithelium, normal nasopharyngeal epithelium, and in malignant NPC cells. This pattern of expression was confirmed using two antibodies. CLU is strongly expressed in the follicular dendritic cells (FDCs) within the tonsil; there is weak staining in normal epithelium adjacent to tumour tissue and complete absence of staining in malignant cells. Similar down regulation of CLU was seen in 10/12 tumours as summarised in table 4.4.

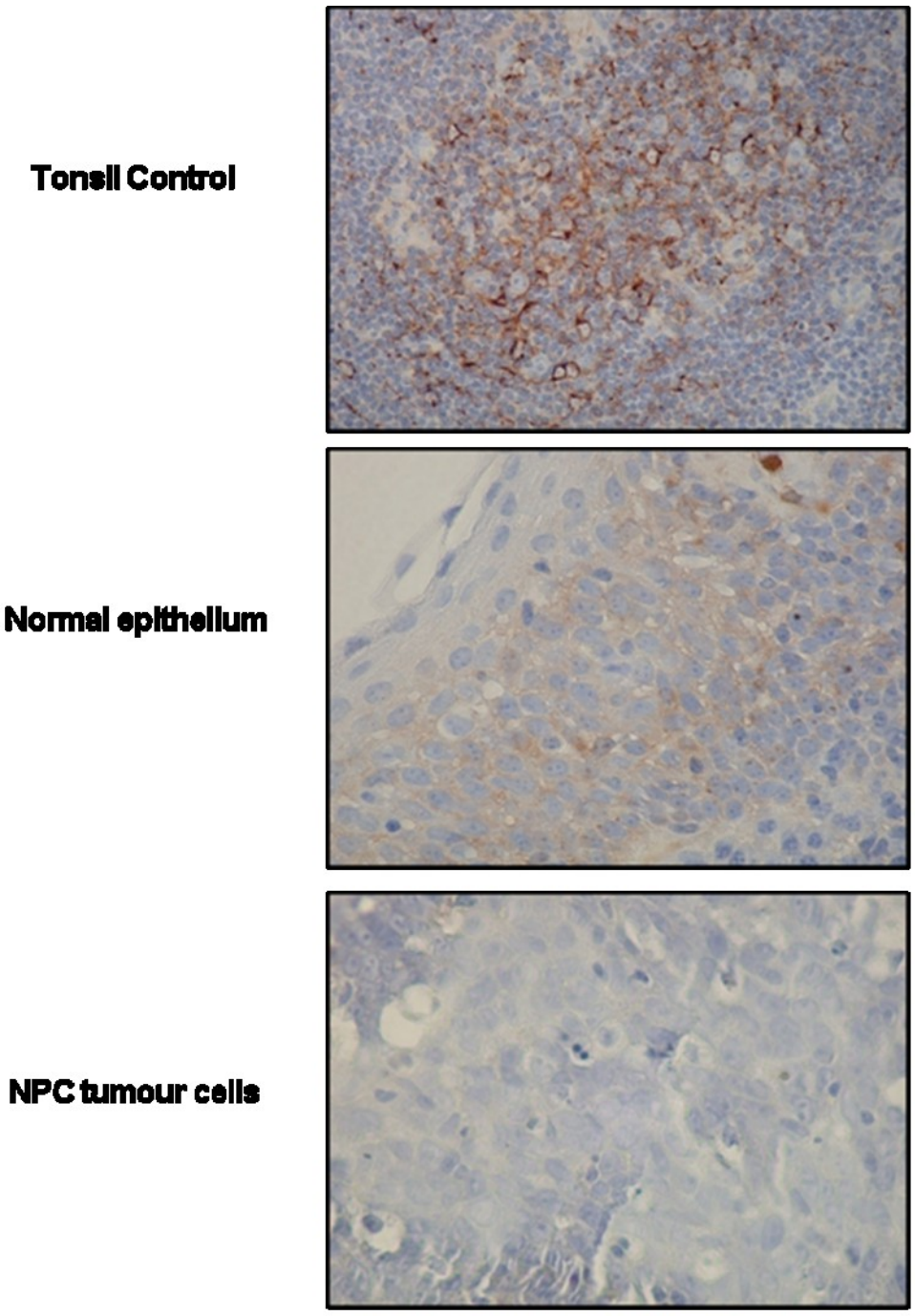


Figure 4.14: Immunohistochemistry in nasopharyngeal tissues

Absence of CLU can be seen in NPC biopsy, weak staining in normal epithelium and an intense stain in the follicular dendritic cells (FDC) within tonsil, which acted as a positive control.

NPC Tumours	localisation	Upstate	Santa Cruz
988163	Cytoplasmic	1+	-
199686		-	-
96767		-	-
988674		-	-
988615		-	-
987802	Cytoplasmic	1+/-	-
988510		-	-
987970		-	-
3585		-	-
95177B2		-	-
3578	Cytoplasmic	-	3+
179389		-	-

Table 4.4: Immunohistochemical staining of nasopharyngeal tumours

Immunohistochemical staining in a panel of NPC tumours compared with normal nasopharyngeal epithelium showed a down-regulation of CLU in 10 out of 12 NPC tumours.

4.4. Impact of EBV and its latent genes on the expression of CLU in the Ad-AH cell line

I next investigated the impact on CLU expression of the infection of the NPC cell line, Ad-AH, with EBV and its latent genes, EBNA1, LMP2A, LMP2B and LMP1. Ad-AH was also infected with a recombinant EBV in which the LMP2A gene had been deleted (ΔLMP2A EBV, Stewart et al. PNAS). Infections were performed by Dr John O'Neil, a member of the NPC group in the Institute for Cancer Studies.

4.4.1. Impact of EBV and its latent genes on the expression of CLU at the RNA level in Ad-AH cell line

Figure 4.15 shows the RNA expression of CLU as determined by Q RT-PCR in the parental Ad-AH line and following its infection with EBV and transfection with plasmids encoding individual latent genes or control plasmid containing a neomycin-resistance gene. CLU was substantially and significantly up-regulated following infection with EBV and ΔLMP2A EBV; 6.6 fold and 3.3 fold respectively when compared with the parental cell line. In the remaining comparisons, expression following infection with an EBV latent gene had been normalised against that observed following transfection with a vector containing a neomycin resistance gene under the same experimental conditions. Expression of the latent genes EBNA1, LMP2A, LMP2B and LMP1 in Ad-AH did not show any change in the expression of CLU at the RNA level compared with the parental cell line.

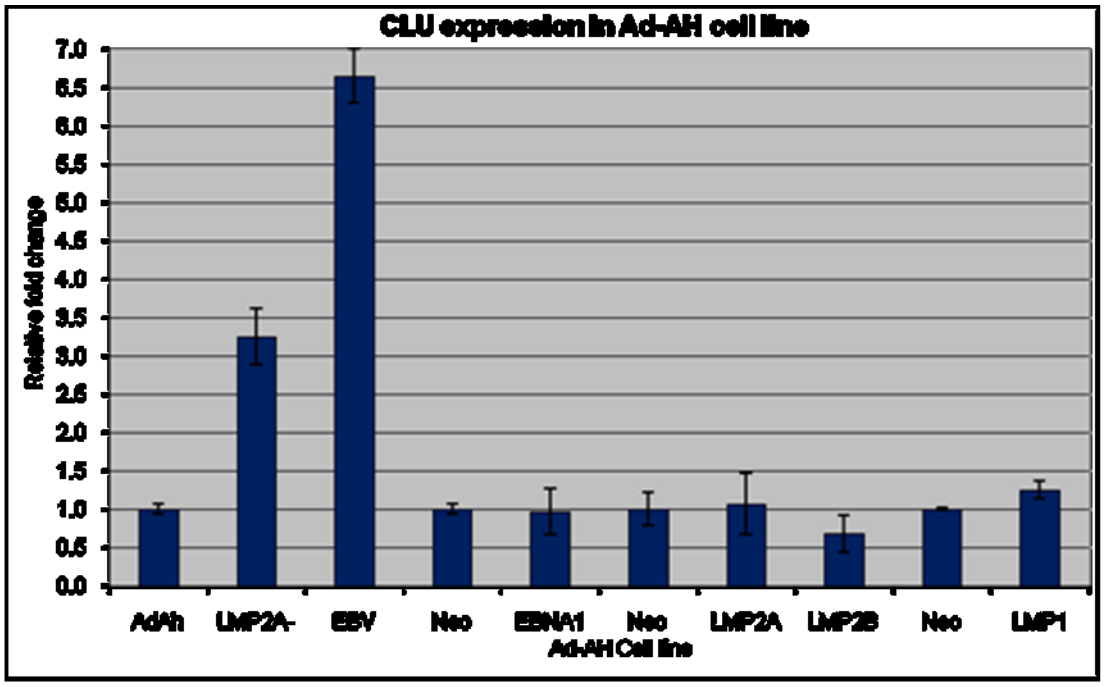


Figure 4.15: RNA expression of CLU as determined by Q RT-PCR in the parental Ad-AH line and following its infection with EBV, EBV latent genes and a neomycin-resistance cassette.

Compared with the parental cell line, CLU mRNA expression was found to be up- regulated following infection of Ad-AH with EBV and Ad-AH EBV with LMP2A knock-out virus.

4.4.2 Impact of EBV and its latent genes on the expression of CLU at the protein level in Ad-AH cell line

To evaluate whether changes in RNA expression were reflected in protein expression, I next investigated the impact of EBV infection and expression of its latent genes on the expression of CLU at the protein level in Ad-AH cells using a variety of techniques.

4.4.2.1 Western blotting

Figure 4.16 shows using Western blotting, the expression of CLU in the parental Ad-AH cell line, and following its infection with EBV, and transfection with EBV latent genes and a vector containing a neomycin-resistance gene. Compared with the parental cell line, the 60kDa CLU protein is over-expressed following infection with EBV and ΔLMP2A EBV, with the 40kDa α and β subunits also over-expressed post infection with EBV; these results confirm those observed at the RNA level (4.4.1). CLU is additionally up-regulated at the protein level post-transfection with LMP1 in Ad-AH cells compared to parental cells and its neo-counterpart. The 60kDa CLU protein appears to be down-regulated following transfection of Ad-AH cells with LMP2A and LMP2B, which was not seen at the RNA level. The 45kDa CLU protein seems to be very weakly expressed in these two cell lines, but not in the parental cell line or post-transfection any of the others. This is interesting given that the 60kDa protein seems to be down-regulated post transfection of Ad-AH with LMP2A or LMP2B and in these we also see the expression of a 45kDa protein, corresponding to the molecular weight of nuclear CLU.

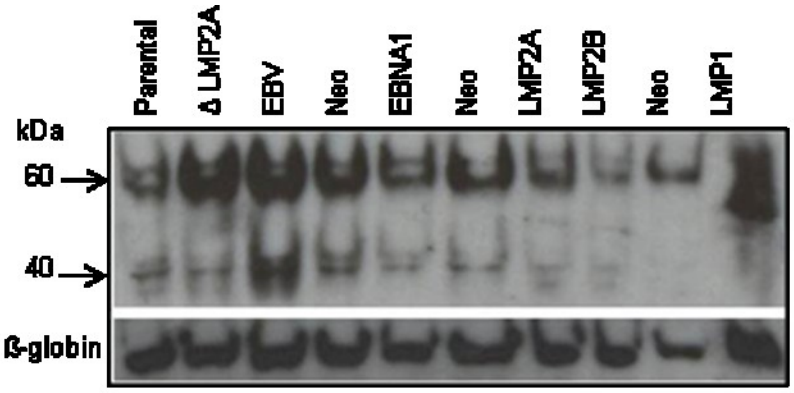


Figure 4.16: The expression of CLU as determined by Western blot in the parental Ad-AH line, and following its infection with EBV, EBV latent genes and a vector containing a neomycin-resistance gene.

Down regulation of CLU following transfection of Ad-AH with LMP2A and LMP2B and up-regulation of CLU following infection of Ad-AH with EBV wt and ΔLMP2A.

4.4.2.2 Immunofluorescence

To explore the possible down-regulation of CLU in Ad-AH cells transfected with LMP2A or LMP2B I used immunofluorescence and three antibodies to stain methanol-fixed cells which were prepared from the Ad-AH cell line after stable transfection with LMP2A or neomycin control gene (Figure 4.17). Both nuclear and cytoplasmic staining are observed; and using the Vector antibody, staining of the Golgi apparatus can also be seen. However, there is no compelling evidence that following transfection with LMP2A alters either the intensity of staining or the cellular localisation of CLU in this cell line. Interestingly in both the cell lines nuclear CLU expression was observed in cells that appeared to be undergoing apoptosis.

4.4.2.3 Immunohistochemistry

The expression of CLU at the protein level was further examined by immunohistochemistry in the C666-1 cell line and in the Ad-AH parental cell line and following its infection with EBV, and transfection with EBV latent genes and a vector containing a neomycin-resistance gene. Figure 4.18 shows the expression of CLU in whole cell lysates prepared from parental Ad-AH and Ad-AH infected with EBV and shows up-regulation of CLU at the protein level post infection of this cell line with EBV. Immunohistochemical analysis also confirmed that C666-1 did not express CLU, with Ad-AH cell lysates showing moderate (2+) to high (3+) levels of CLU, which was nuclear in localisation (Table 4.5).

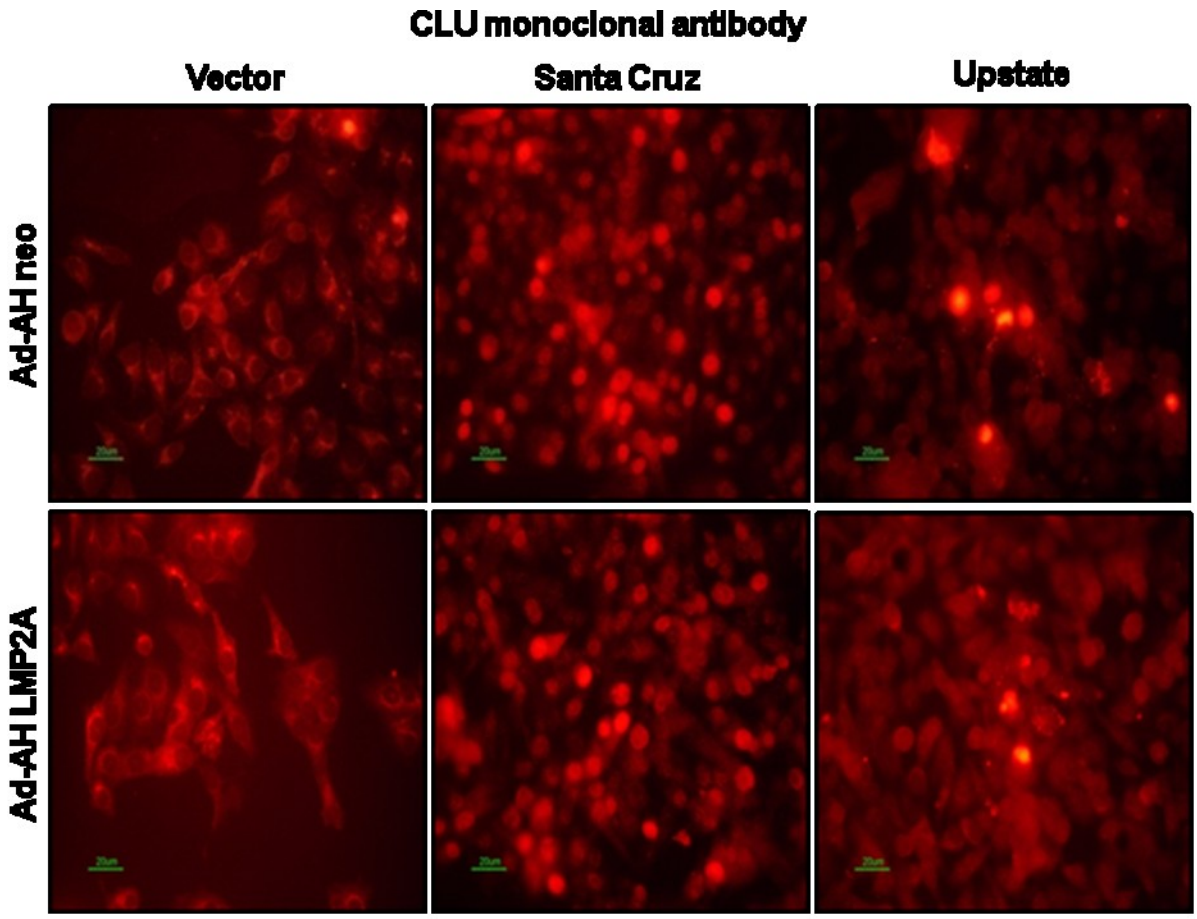


Figure 4.17: Immunofluorescence in Ad-AH and Ad-AH transfected with LMP2A

Immunofluorescence using three antibodies, showing the expression of CLU in methanol-fixed cells which were prepared from the Ad-AH cell line after stable transfection with LMP2A or neomycin control.

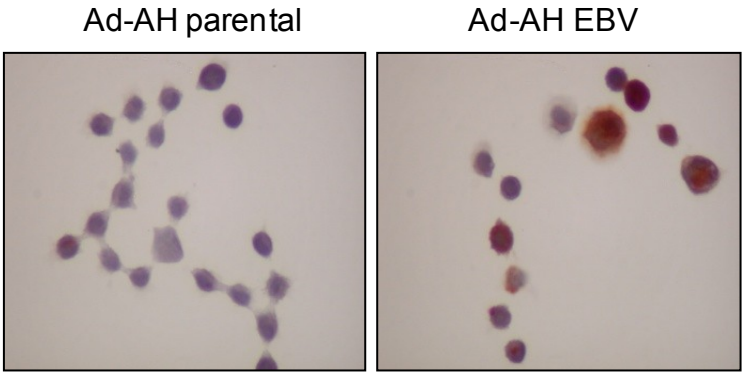


Figure 4.18: Immunohistochemical staining for CLU expression in the Ad-AH cell line.

Expression of CLU in cytospins prepared from the Ad-AH parental and infected cell line. Compared with the parental cell line, the staining pattern appears to be more intense in the cytospins prepared from Ad-AH transfected with EBV.

Cytospin	localisation	Upstate	Santa Cruz
Parental	Nuclear	2+	2+
LMP2A k/o	Nuclear	3+	2+
EBV	Nuclear	2+	3+
neo	Nuclear	2+	2+
EBNA1	Nuclear	2+	2+
neo	Nuclear	2+	2+
LMP2A	Nuclear	2+	2+
LMP2B	Nuclear	3+	2+
neo	Nuclear	2+	2+
LMP1	Nuclear	2+	2+
C666-1		-	-

Table 4.5: Immunohistochemistry in Ad-AH cell line and in C666-1 cell line

Immunohistochemical staining in the Ad-AH cell line and following its infection with EBV and transfection with its latent genes showed a strong nuclear stain in all cell lines.

4.5. Allelic loss and epigenetic regulation of CLU in the Ad-AH and C666-1 cell lines

I decided to focus in more detail on the C666-1 nasopharyngeal cancer cell line because a contemporaneous in-house investigation using SNP analysis had revealed in this cell line, and in the Ad-AH cell line, a possible deletion encompassing the CLU locus (8p21). This analysis was performed by Dr. J Arrand in the Institute for Cancer Studies, who subsequently confirmed using genomic PCR, a 7.6mb deletion with loss of one CLU allele in C666-1 (Figure 4.19A and 4.19B). Allelic loss having been demonstrated, I have investigated the methylation status of the CLU promoter in the remaining allele using bisulphite genomic sequencing and pyrosequencing. I have also examined using RT-PCR, changes in CLU expression following demethylation of this cell line with 5-aza, 2'-deoxycitidine.

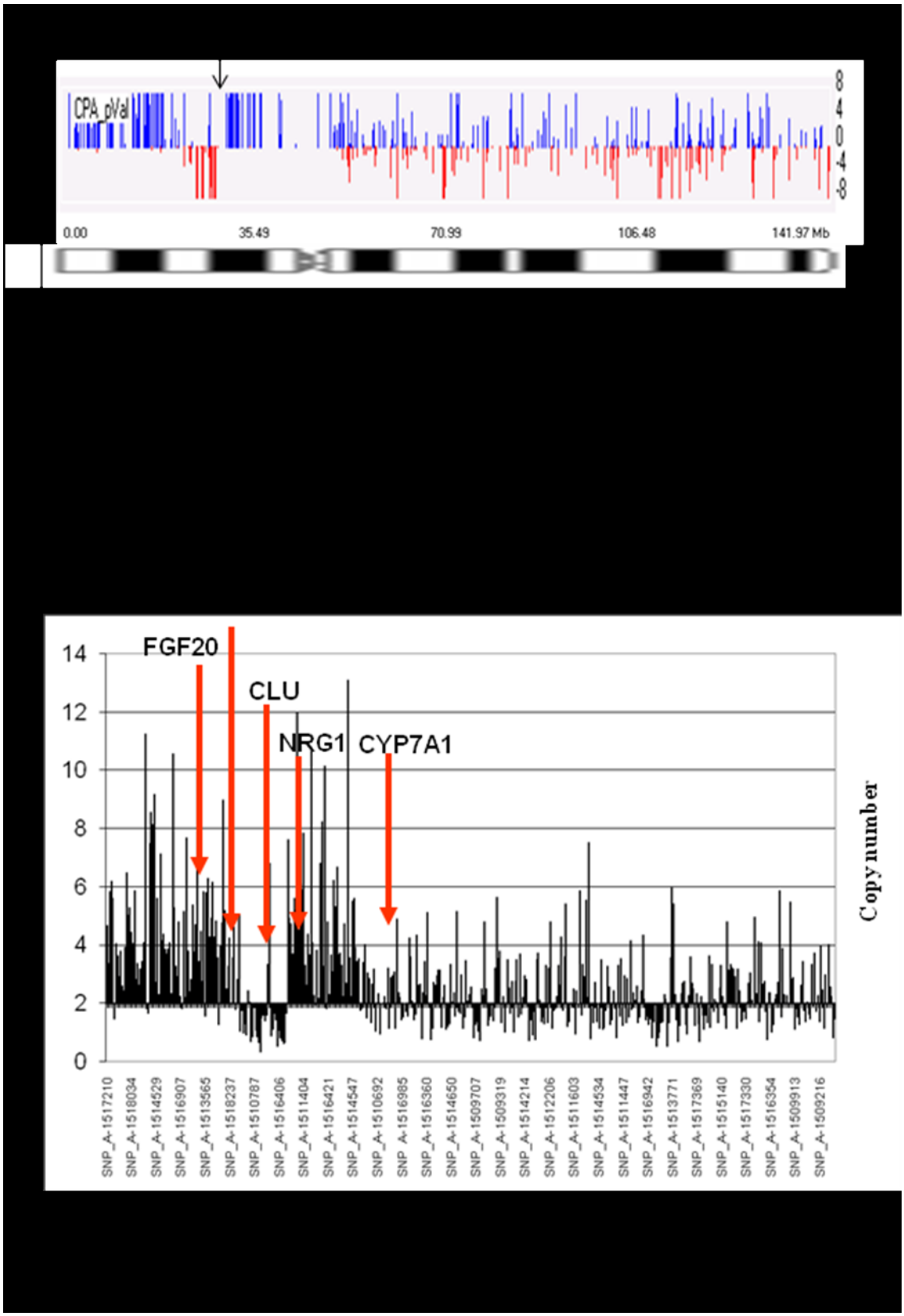
4.5.1. Methylation status of the CLU promoter in Ad-AH and C666-1 as determined by MSP

MSP was used to determine the presence of methylated and unmethylated forms of CLU in the Ad-AH and C666-1 cell lines (figure 4.20). Single discrete bands of the expected size of amplified PCR products were detected for all samples and controls behaved as expected throughout. Where evidence of methylation was detected, results were repeated alongside unmethylated results to confirm that the results were reproducible. Both methylated and unmethylated forms of CLU were detected in both cell lines, with the methylated band being predominant, especially in C666-1. The methylation status of the CLU promoter was also determined following infection of Ad-AH with EBV, EBV latent genes and a neomycin-

resistance gene (figure 4.21). There appears to be a reduced level in methylation following infection of the parental line with EBV and its latent genes, however it is difficult to determine by MSP as it is not quantitative and requires further investigation by pyrosequencing (section 4.9).

4.5.2. Transcriptional and epigenetic changes in CLU expression following demethylation of C666-1

I investigated whether demethylation of C666-1 would be followed by the up-regulation of CLU. Figure 4.22 shows using RT-PCR up-regulation of CLU mRNA following treatment with the demethylating agent 5-Aza-2'-deoxycidine. Examination of the intensity of promoter methylation before and after treatment with 5-Aza, using MSP shows that drug treatment results in significant demethylation of the CLU promoter (Figure 4.23). This change was confirmed on pyrosequencing; which showed a decrease in the methylation of the CLU promoter from 77% to 31% (section 4.5.4).


CPA_pVal
8
4
0
-4
-8
0.00
35.49
70.99
106.48
141.97 Mb
FGF20
CLU
NRG1
CYP7A1
14
12
10
8
6
4
2
0
Copy number
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SNP_A-1516907
SNP_A-1513565
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SNP_A-1516985
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SNP_A-1509707
SNP_A-1509319
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SNP_A-1512206
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SNP_A-1514534
SNP_A-1511447
SNP_A-1516942
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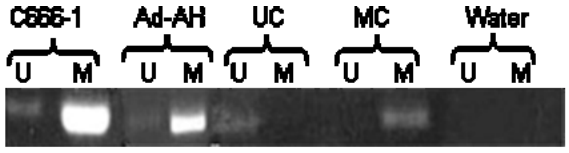


Figure 4.20: : MSP analysis of CLU promoter methylation in NPC cell lines.

Methylation of CpGs within the CLU promoter was detected in both Ad-AH and C666-1 cell lines, with very weak detection of an additional unmethylated product in these.

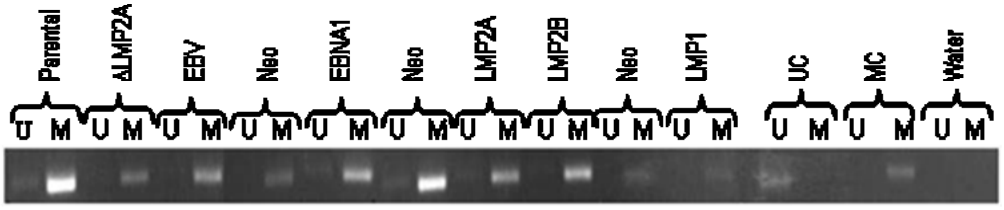


Figure 4.21: MSP analysis CLU promoter methylation in Ad-AH series

Methylated forms were detected in all Ad-AH cell lines by MSP, with the parental line and in Ad-AH transfected with EBNA1 also showing weak unmethylated bands were present.

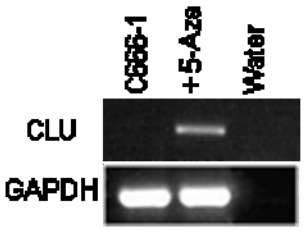


Figure 4.22: RT-PCR analysis of CLU expression in C666-1 pre and post treatment with 5-Aza-2'-deoxycitidine

RT PCR showing RNA expression pre- and post- demethylation of the NPC cell line C666-1. Expression levels of CLU were examined pre and post demethylation of C666-1 using 5'Aza2'deoxycytidine at 20uM.

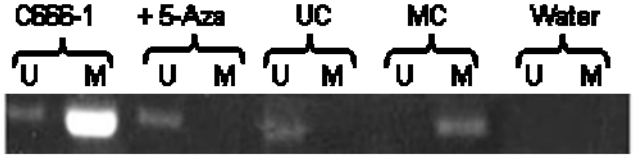


Figure 4.23: MSP analysis of CLU promoter methylation in C666-1 pre and post treatment with 5-Aza-2'-deoxycitidine.

Methylation Specific PCR shows the intensity of promoter methylation before and after treatment with 5-Aza. Drug treatment appears to have been followed by demethylation of the CLU promoter.

4.5.3. Analysis of the CLU promoter in C666-1 cells using bisulphite genomic sequencing

Bisulphite genomic sequencing (BGS) was used to confirm the pattern of promoter methylation across the entire CpG island in the promoter region of CLU in DNA extracted from C666-1 cells. Figure 4.24 shows the distribution of methylated CpGs within the CLU promoter and the adjacent CpG rich region. Approximately 85% of CpGs in the CLU promoter were found to be methylated by BGS in this cell line, and BGS confirming the methylation of CpG di-nucleotides covered by the MSP primers. Additionally, this analysis shows that the majority of CpG di-nucleotides are frequently methylated.

4.5.4. Frequency of methylation of the CLU promoter in NPC cell lines as determined by pyrosequencing

Pyrosequencing was used to confirm the pattern of promoter methylation of CLU in DNA extracted from nasopharyngeal cancer cell lines. As in section 3.7.3.4, 4 sets of pyrosequencing primers were optimised on commercially available controls, the most reproducible set taken forward for analysis in all DNA samples. Table 4.6 shows methylation in 5 CpG dinucleotides of the CLU promoter and their mean levels. In C666-1 cells the mean methylation was 84%, 17% in Ad-AH, CNE2 is 21%, 24% in SUNE1 and 12% in HONE1. The mean methylation in C666-1 not treated with drugs was 77% across all 5 CpGs analysed, and post treatment with 5-Aza-2'deoxycidine this was reduced to 51%. Of all 5 CpGs the most upstream one showed the highest levels of methylation (90%), and the level of methylation successively decreased across the adjacent CpGs. The controls behaved as expected throughout with unmethylated control DNA

showing a mean methylation of 5% and methylated control DNA 91%. All NPC cell lines were more methylated than the primary foreskin keratinocyte cell line, which showed a mean methylation of 10%.

Further analysis of the Ad-AH cell line showed that compared to the 17% methylation in the parental cell line, methylation increased to 39% in LMP2A knockout cells, and 62% following infection with wild type EBV. LMP2A and LMP2B transfected cells showed 37% and 29% methylation respectively compared with 30% methylation in their neo counterpart. EBNA1 transfected cells showed 29% methylation compared to its neo counterpart at 40%, and LMP1 19% compared with 5% in its neo counterpart. In these runs, the unmethylated control was 9% methylated and the methylated control had a mean methylation of 71%.

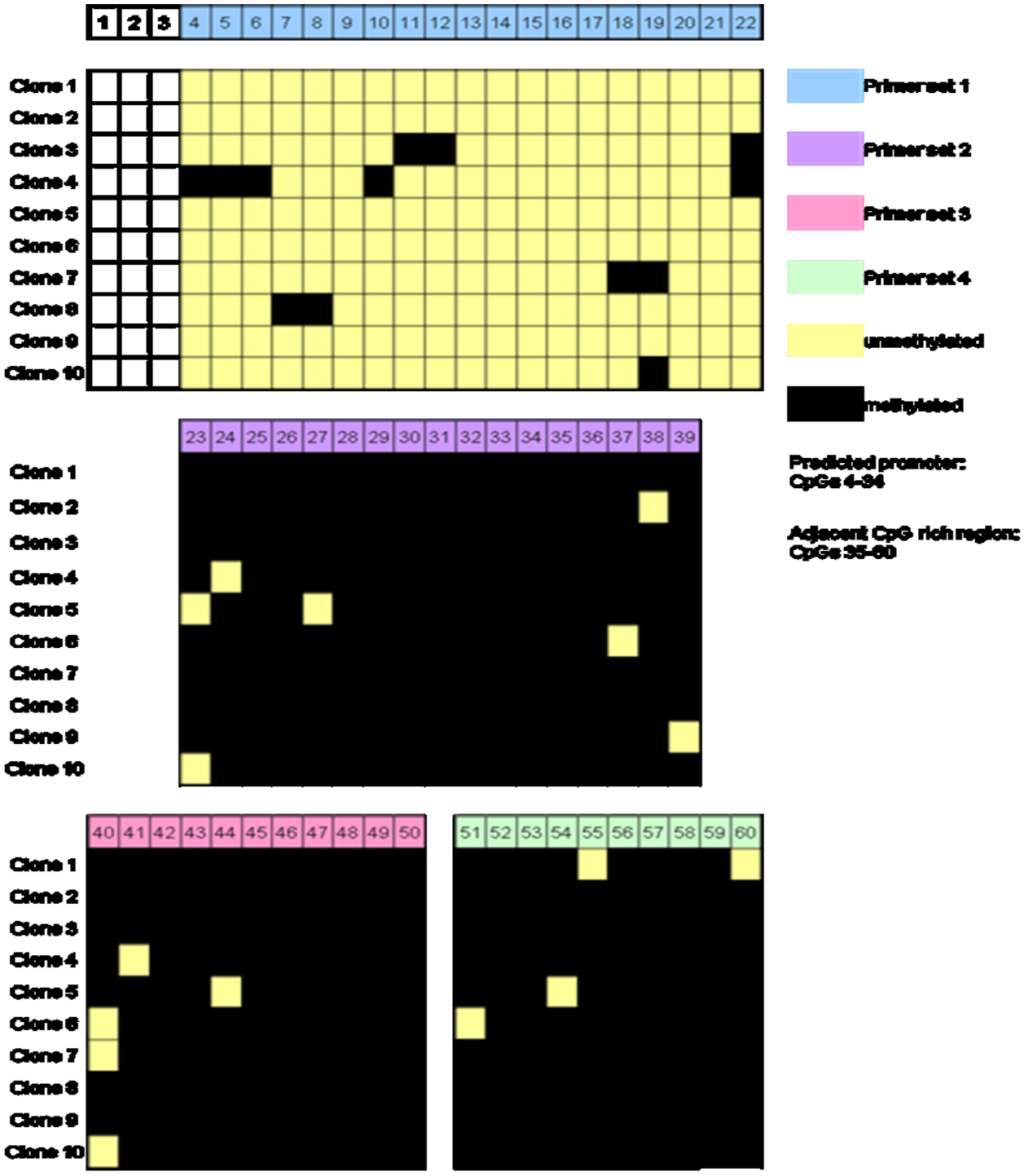


Figure 4.24: Bisulfite genomic sequencing of the CLU promoter in C666-1

Numbers across the top represent CpG dinucleotides and along the side clones. Yellow squares represent an unmethylated CpG dinucleotide and black a methylated CpG. Approximately 85% of CpGs in the CLU promoter were found to be methylated by BGS in this cell line.

	CpG methylation (%)					
	1	2	3	4	5	Mean
C666-1 control	90	83	75	75	60	77
C666-1 + 5 Aza	55	60	61	50	38	31
293	22	29	15	29	31	25
NFK	12	14	8	10	5	10
CNE2	18	27	17	23	17	21
SUNE1	26	38	19	26	12	24
HONE1	12	18	7	14	7	12
C666-1	94	82	73	94	80	84
Ad-AH parental	18	25	16	16	12	17
LMP2A K/O	41	52	36	36	29	39
EBV	63	76	59	59	53	62
Neo	32	40	27	31	22	30
LMP2A	39	47	33	37	26	37
LMP2B	35	43	23	26	19	29
Neo	40	48	40	40	32	40
EBNA1	29	39	29	26	20	29
Neo	5	8	3	7	4	5
LMP1	21	29	15	21	11	18
UC	4	4	2	9	5	5
MC	96	91	83	95	88	91

Table 4.6: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and their means for NPC cell lines.

In C666-1 the mean methylation was 84%, 17% in Ad-AH, in CNE2 21%, 24% in SUNE1 and 12% in HONE1. The mean methylation in C666-1 not treated with drugs was 77% across all 5 CpGs analysed, and post treatment with 5-Aza-2'deoxycidine this was reduced to 31%.

4.6. Allelic loss and epigenetic regulation of CLU expression in nasopharyngeal cancers

A SNP analysis performed by Dr John Arrand in the Institute for Cancer Studies suggested a possible deletion at the CLU locus on 8p21 in 2 of 14 nasopharyngeal cancers from which epithelium had been removed by micro-dissection. Although this region includes 7 genes which are reported to have a tumour suppressive function (BNIP3L, CLU, NKX3-1, PPP2R2A, RHOBTB2, TNFRSF10A and TNFRSF10B), only CLU was found to be down-regulated in the gene expression array (Hui 2006). Unfortunately, this material had been exhausted, and therefore I have explored the methylation status of CLU in whole tissue taken from a separate series of 10 nasopharyngeal tumours.

4.6.1. Frequency of methylation of the CLU promoter in nasopharyngeal cancer as determined by pyrosequencing

I next used 10 randomly selected NPC tumours from a larger cohort of nasopharyngeal cancers, for which we also have expression data from three probe sets on a microarray to investigate the mean methylation across all 5 CpG dinucleotides (Table 4.7). The results show a mean methylation for all 10 tumours of 23%, with a range from 10 to 49%. Three of the NPC tumours showed much higher levels of methylation than the other seven tumours (NPC 2, 39 and 62). However, this did not necessarily correlate with CLU expression since in some of the less methylated tumours expressed CLU at a lower level than others. The controls behaved as expected throughout with unmethylated control DNA showing a mean methylation of 5% and methylated control DNA 83%.

	CpG methylation (%)						Expression on microarray			
	1	2	3	4	5	Mean	209791_at	209792_s_at	222043_at	Mean
NPC2	30	30	29	37	29	31	196	49	13	85
NPC9	29	29	16	20	15	21	382	144	19	182
NPC15	25	27	17	21	15	21	69	29	11	30
NPC37	21	21	16	16	16	16	136	69	14	70
NPC39	16	20	11	16	13	15	39	16	10	21
NPC39	39	44	22	16	12	29	69	24	10	31
NPC40	16	22	11	20	14	17	104	49	11	59
NPC44	19	21	16	15	20	18	936	340	66	447
NPC59	20	21	11	21	13	17	39	16	10	21
NPC62	44	49	37	47	44	44	25	15	8	16
Mean	27	29	17	23	19	23	199	79	17	98

UCA	4	5	2	10	5	5
MC	67	67	70	65	70	69

Table 4.7: Pyrosequencing of 5 CpG dinucleotides of the CLU promoter and their means for NPC tumours.

The results show a mean methylation for all 10 tumours of 23%, with a range from 10 to 49%. Three of the NPC tumours showed much higher levels of methylation than the other seven tumours (NPC 2, 39 and 62). However, this did not necessarily correlate with their expression since some of the less methylated tumours were also expressed at a lower level than others.

4.7. Summary of expression and methylation changes in oral cancer and NPC

I have shown a high level of CLU expression in oral cancer cell lines at the RNA and protein level, however these cell lines were not representative of CLU expression in oral cancer. Immunohistochemistry of oral cancer confirmed what had been shown by microarray and Q RT-PCR, which was a down-regulation or loss of CLU in oral cancer. The Q RT-PCR data is not entirely convincing, given the inherent problems of housekeeping gene normalisation (discussed further in 6.16.3). Protein expression data is more reliable as it is not subject to the same sensitivity issues when comparing gene expression to housekeeping gene expression. In oral cancer cell lines I showed a level of methylation, which was much lower in oral cancer, suggesting that methylation is unlikely to be the sole regulator of reduced CLU expression in oral cancer. It is also possible that in the tumour samples that adjacent normal cells may be contributing to the level of methylation detected and micro-dissected tumours would have provided a more accurate comparison. As in cervical cancer, methylation results were consistent with previous evidence suggesting that cell lines can acquire specific DNA methylation defects as a result of immortalisation and continuous culture that are unrelated to the tumour origin (discussed further in 6.17.4.1). SNP analysis revealed a possible deletion encompassing the CLU locus on chromosome 8p21 which may play a role in the down-regulation of CLU in oral cancer.

Contemporaneous in-house microarray experiments undertaken by other members of the department had shown that CLU was down-regulated in NPC cell lines, and in 19 (76%) of 25 NPC biopsies compared with cancer free controls. CLU was also found to be down-regulated

when micro-dissected epithelium from patients with NPC was compared with tissue taken from the same cancer free controls. In NPC cell lines, I was only able to confirm the down regulation of CLU in one of the cell line at the RNA and protein level, which as in cervix and oral cancers, was not reflective of the expression in NPC tumours. As in oral cancer, the Q RT-PCR data is not entirely convincing and protein expression data is more reliable for evaluating the expression of CLU. The lack of expression of CLU in C666-1, and its presence in Ad-AH could be because Ad-AH is an adenocarcinoma cell line, not squamous carcinoma. Using immunohistochemistry, a down-regulation of CLU was confirmed in 10 out of 12 cancers. Infection of the Ad-AH cell line with EBV appeared to up-regulated CLU, however, this did not correlate with methylation of the CLU promoter as there was also an increase in promoter methylation. Methylation frequencies were much higher in NPC cell lines than in NPC tumours and this is discussed in chapter 6 (section 6.17.4.1). The impact of EBV and its latent genes on CLU expression was not entirely convincing because although EBV and ΔLMP2A up-regulated CLU, transfection of LMP2 and LMP1 in Ad-AH cells did not have any effect on CLU; we would have expected LMP2 to down-regulate CLU and LMP1 to up-regulate it.

The NPC cell line C666-1 not only showed down-regulation of CLU but using genomic PCR had shown loss of one CLU allele in this cell line. I have shown using MSP, pyrosequencing and bisulphite genomic sequencing methylation of the CLU promoter in the remaining allele in the C666-1 cell line. The C666-1 cell line may be a more useful indicator of gene methylation status *in vivo* as tumour samples used were not micro-dissected and so there will be undoubtedly be a contribution from the surrounding normal cells masking the level of methylation detected. To

summarise, in this cell line I have shown loss of one CLU allele and epigenetic silencing by DNA methylation of the other. This is a novel observation, which is included in a manuscript being prepared for submission.

Chapter 5: Results 3

Regulation of clusterin in nasopharyngeal carcinoma

Overview

In this chapter, I investigate the phenotypic changes which follow the transient transfection of nuclear and full-length CLU into the NPC cell line C666-1. I then investigate the regulation of NF-kB activity by CLU. Towards this end, I first measured NF-kB activity following knock-down of CLU in the HeLa cell line and then measured NF-kB activity following its expression in C666-1. Both transfection and knockdown conditions were first optimized in HEK 293 cells.

5. 1. Transient transfection of 293 with full length CLU and nCLU

In order to determine whether the expression vectors were able to express the CLU proteins, I first transiently transfected HEK 293 cells with either a pIres plasmid containing full length CLU or a pIres plasmid containing nCLU, which lacks the leader sequence. Both of these plasmids were provided by Prof. Bettuzzi and generated using the published sequence for transcript variant 2. pIres mock plasmid was used as a transfection control, and expression levels of transcripts monitored at 12, 24 and 48 hours post transfection.

5.1.1 RNA expression following transfection with full length CLU and nCLU

In order to verify that transfection was successful, I examined the expression of transcript variants 1 and 2 in HEK 293 cells transiently transfected with full length CLU, nuclear CLU and empty vector by RT-PCR, using GAPDH as a loading control (Figure 5.1). Levels of transcript variant 1 do not change following transfection with either full length or nCLU. This observation

is not surprising because the plasmids used were generated from transcript variant 2 sequences. On the other hand, transcript variant 2, which is absent in the HEK 293 cell line, is expressed at 12, 24 and 48 hours following transfection with full length and nuclear CLU but not following transfection with the empty vector. Expression decreased with falling concentrations of plasmid, but was still detectable at a concentration of 62.3ng/µl. Expression levels appeared to be particularly high at 24 hours post transfection of nCLU and so an earlier time point of 12 hours was investigated. Plasmid concentrations from 250ng/µl to 37.3ng/µl were monitored by semi-quantitative RT-PCR, resulting in a fall in expression, with expression still detectable using 37.3ng/µl plasmid.

5.1.2. Protein expression following transfection with full length CLU and nCLU

I next examined expression at the protein level in the HEK 293 cell line 12, 24 and 48 hours post transient transfected with nCLU and full length CLU, using western blot and immunofluorescence.

5.1.2.1 Western blotting

Using Western blotting and two antibodies a 60kDa protein, corresponding to the cytoplasmic precursor of secreted CLU, a 40kDa protein corresponding the α/βcleaved cytoplasmic protein and a 45kDa protein corresponding to nuclear CLU can be detected following transfection with full length CLU (Figure 5.2). The 45kDa protein but not the 60kDa protein is detectable

following transfection with nCLU. Of interest is the observation that the 45kDa protein can only be detected 24 hours post transfection with full length CLU, but is expressed earlier post-transfection with nCLU. This is consistent with the possibility that the full length plasmid is also able to generate nuclear CLU by translocating to the nucleus and hence it not being detectable until 24 hours post transfection.

5.1.2.2. Immunofluorescence

To further verify CLU expression following transfection I used immunofluorescence (Figure 5.3). 24 and 48 hours after transfection strong expression was observed with full length CLU but not empty vector, and it appears to be cytoplasmic in localisation.

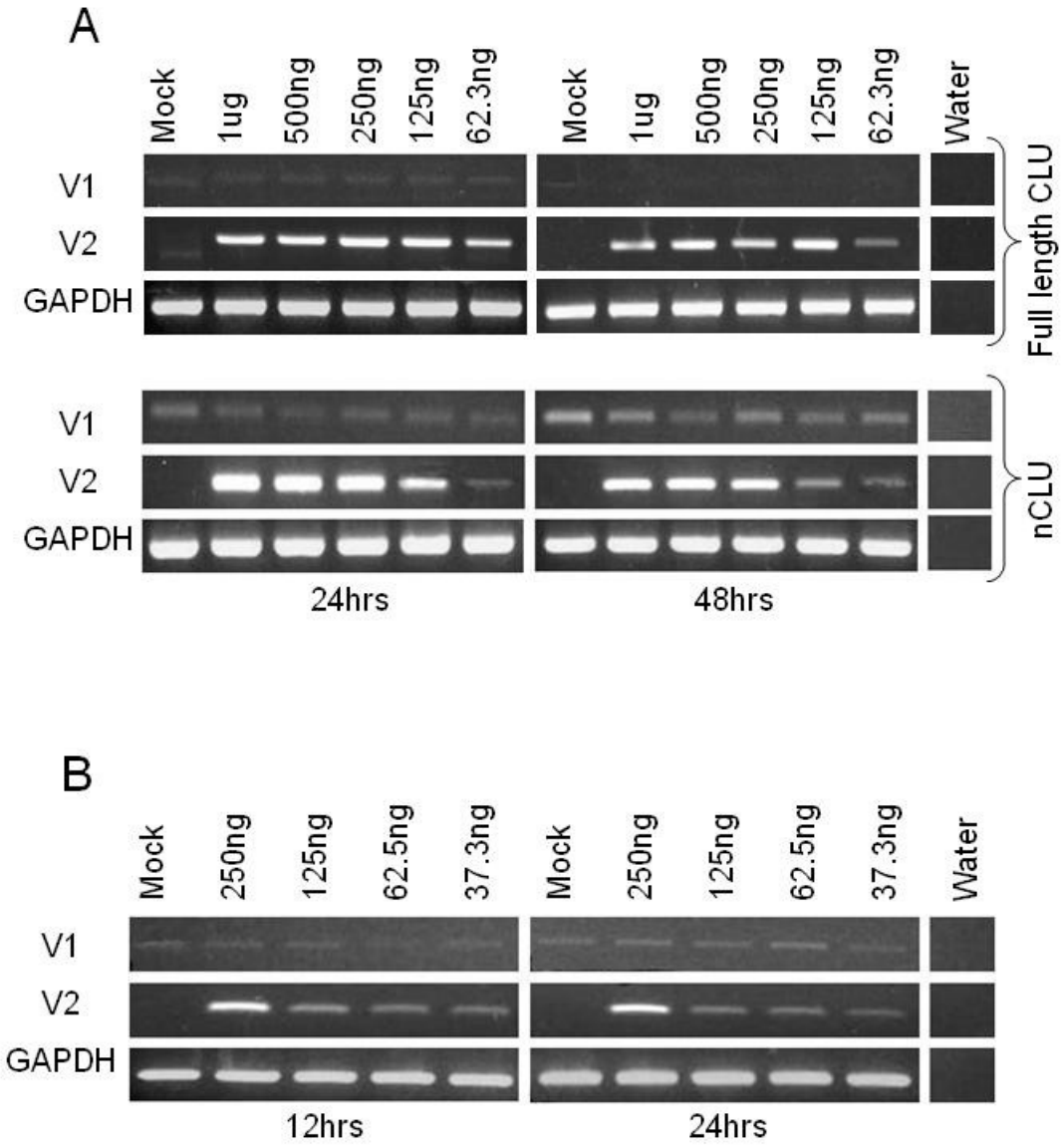


Figure 5.1 A and B: CLU expression in HEK 293 by RT-PCR

Expression of transcript variants 1 and 2 in HEK 293 cells transiently transfected with decreasing concentrations of vector encoding full length CLU or nCLU; GAPDH was used as a loading control.

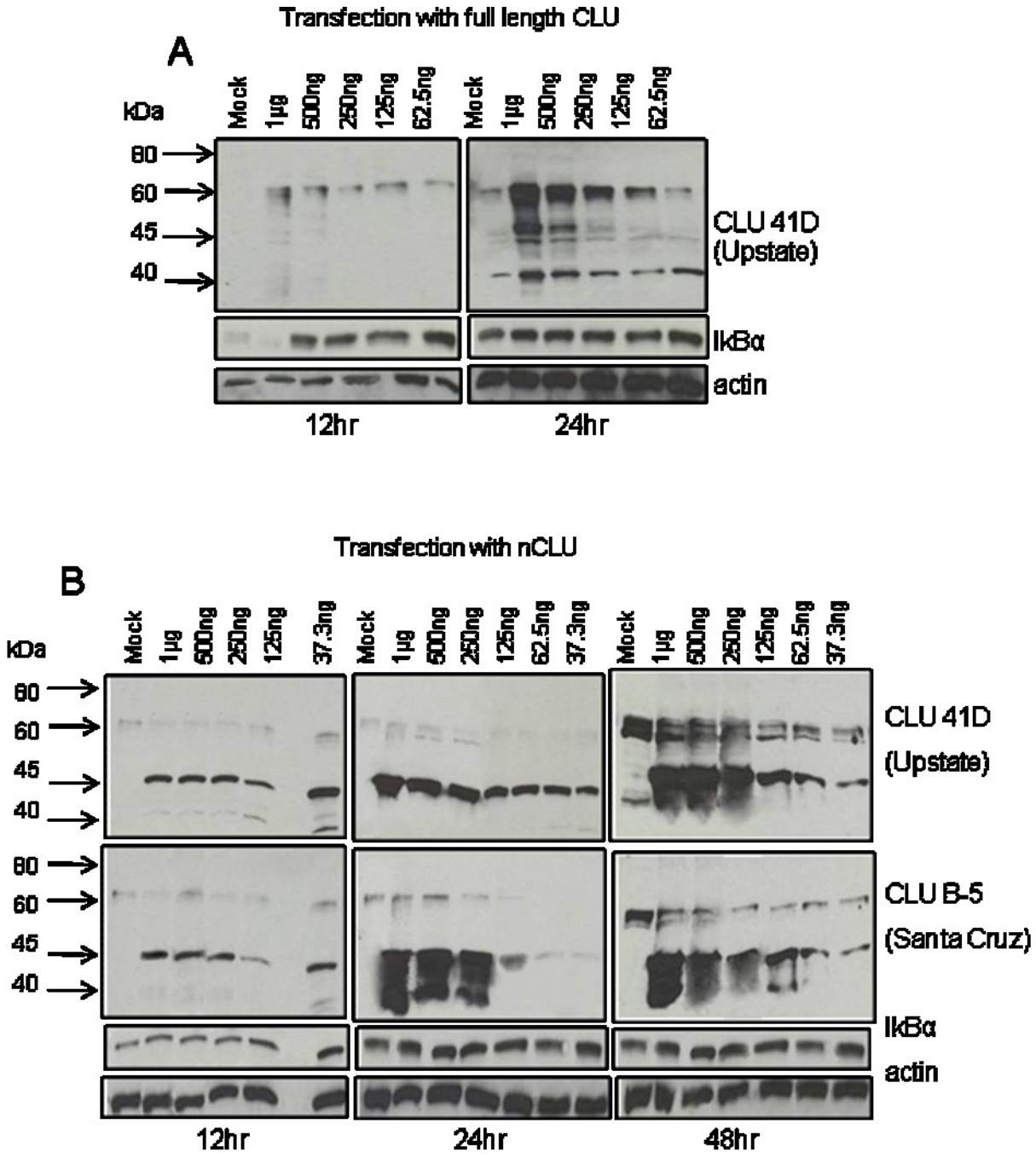


Figure 5.2A and B : CLU expression in HEK 293 by Western blotting

The expression of CLU in lysates prepared from 293 cells at 12, 24 and 48 hours post transfection with mock vector, nCLU and full length CLU showing over-expresion of 60kDa and 45kDa proteins.

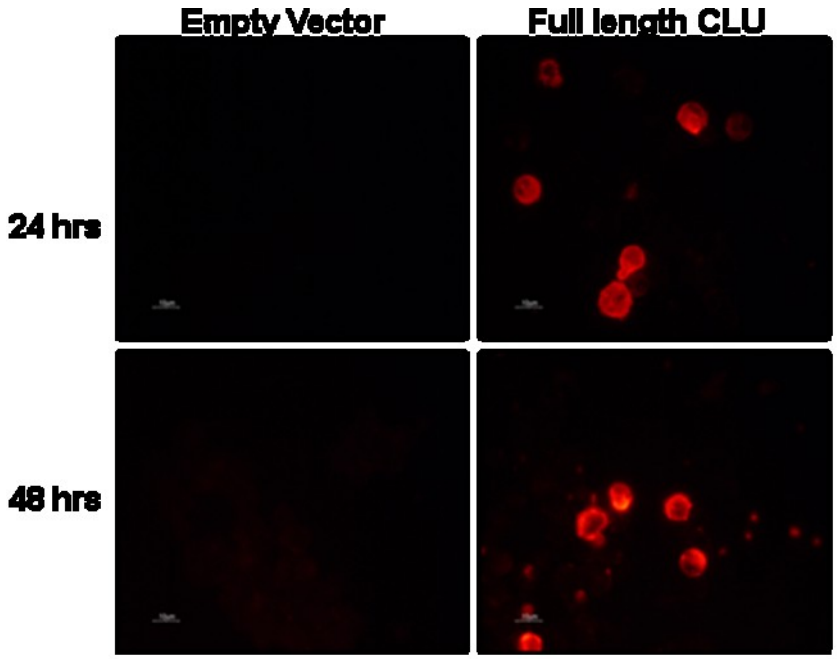


Figure 5.3: CLU expression in HEK 293 by immunofluorescence

The expression of CLU in HEK 293 cells transfected with empty vector and full length CLU, 24 and 48 hours post transfection. Strong expression is observed following transfection with full length CLU but not empty vector, and this appears to be cytoplasmic.

5.2. Transient transfection of C666-1 cells with nCLU and full length CLU

To evaluate the efficiency of transfection in C666-1 cells, I first transiently transfected C666-1 cells with a pIres GFP plasmid. By fluorescent microscopy over 70% of cells were GFP-positive (Figure 5.4). I next transiently transfected C666-1 cells with the same CLU plasmids. Expression levels were monitored at 6, 12 and 24 hours post transfection in order to determine initial expression of the CLU proteins in the C666-1 line.

5.2.1 RNA expression following transfection of C666-1 with full length and nCLU

Figure 5.5 shows, as before, that expression of transcript variant 1 was not induced following transfection with full length CLU or nCLU. Transcript variant 2 was induced from 6 hours post transfection with full length CLU and nCLU. However, transfection with full length CLU and nuclear CLU generated amplicons of different molecular weight – full length CLU generated an amplicon of 240bp whereas nCLU 181bp. This confirms that the nCLU plasmid is a truncated form of the full length CLU, but both of which arise from transcript variant 2.

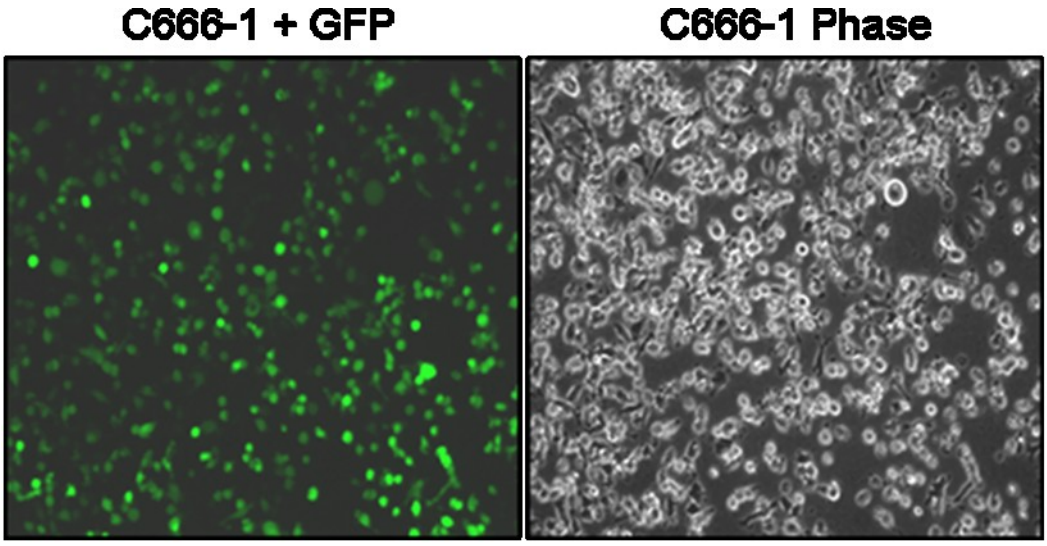


Figure 5.4 : Transient transfection of C666-1 with a GFP-plasmid

48 hours post transient transfection of C666-1 cells with a pIres GFP plasmid fluorescent microscopy show over 70% of cells are GFP-positive

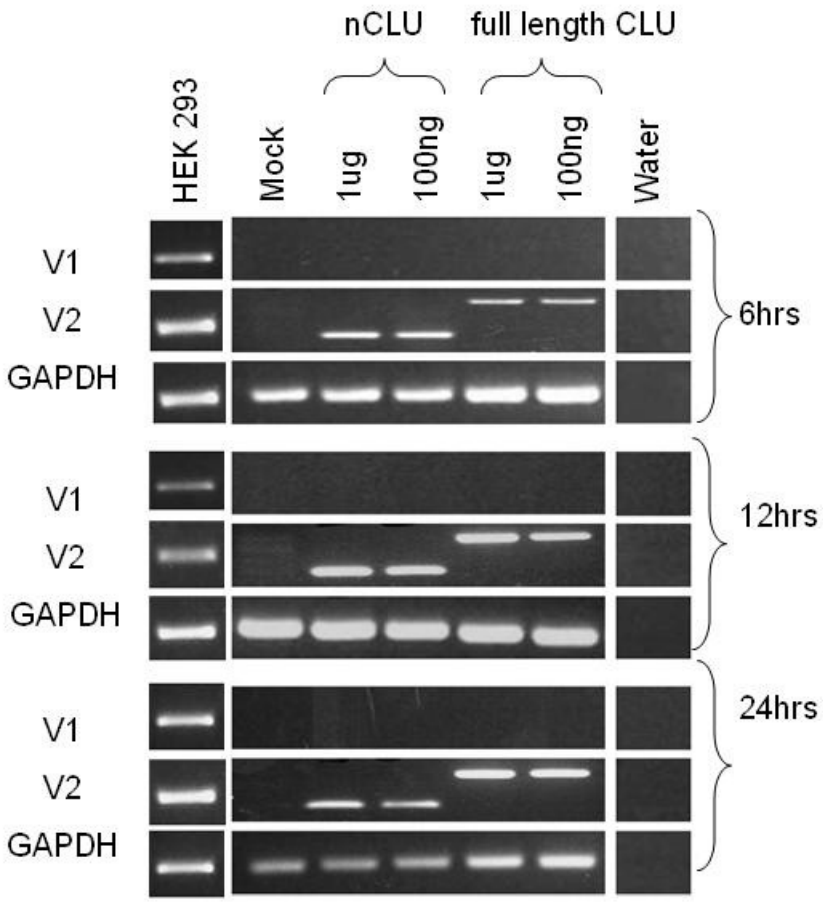


Figure 5.5: CLU expression C666-1 by RT-PCR

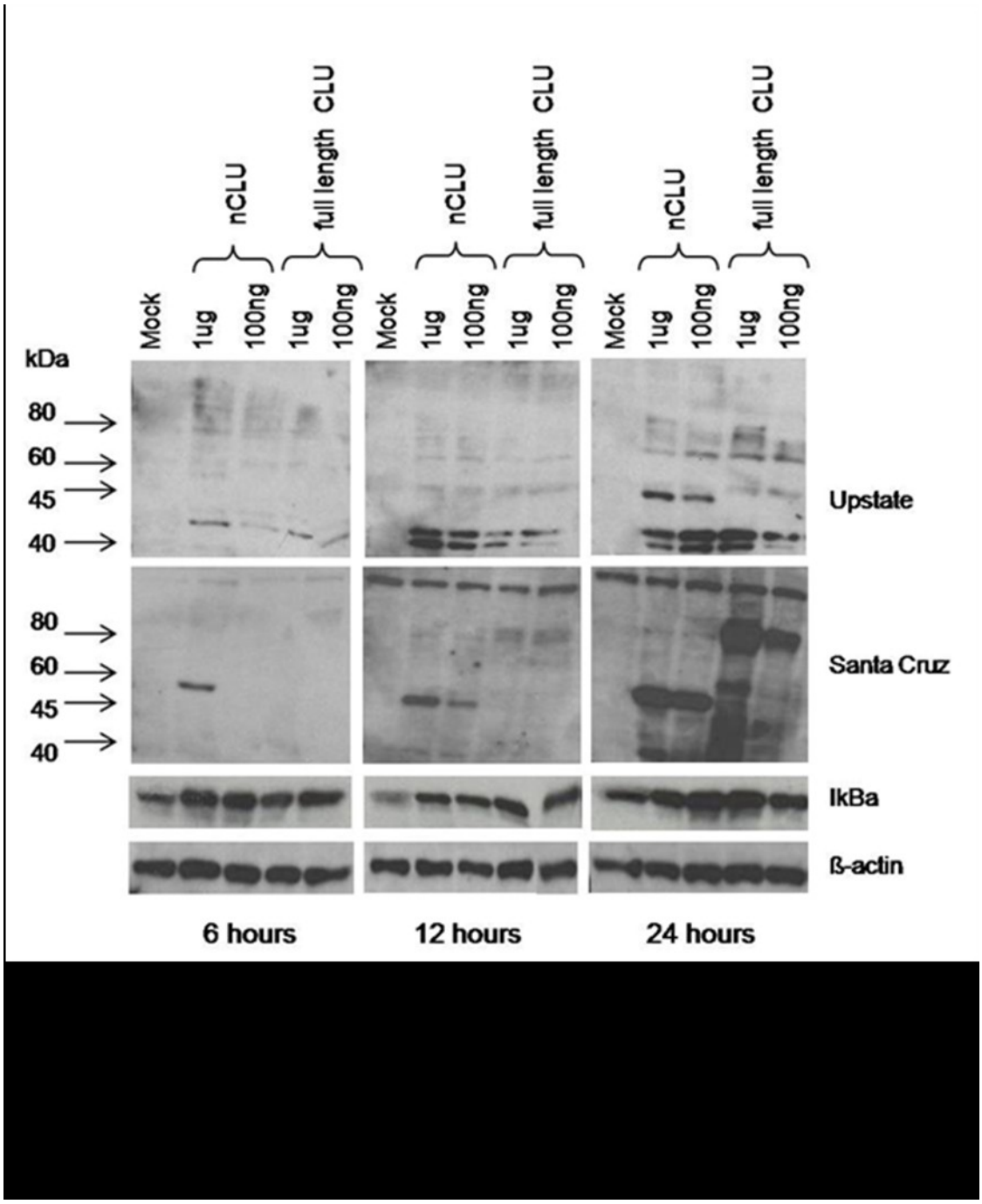
The expression of CLU transcript variants 1 and 2 in C666-1 cells transiently transfected with full length CLU, nCLU and empty vector; GAPDH was used as a loading control.

5.2.2 Protein expression following transfection of C666-1 with full length and nCLU

I next examined expression at the protein level in the C666-1 cell line transiently transfected with nCLU and full length CLU, using western blotting. Expression levels were monitored at 6, 12 and 24 hours post transfection.

5.2.2.1 Western blotting using whole cell lysates

I evaluated the expression of CLU in lysates prepared from C666-1 cells at 6, 12, 24 and 48 hours post transfection with mock vector, nCLU or full length CLU using Western blotting and 2 antibodies (Figure 5.6). A 60kDa protein, corresponding to the cytoplasmic precursor of secreted CLU, is detectable in C666-1 cells 24 hours post transfection with full length CLU but not with nCLU. In contrast to HEK 293 a 45kDa protein is detectable following transfection with nCLU but not full length CLU. A 50kDa protein band can also be seen using both antibodies at 24 hours post transfection with full length CLU; this is further discussed below. A weak 40kDa and an 80kDa protein band can be detected using one or both antibodies in all lysates. This is likely to be 80kDa secreted protein and the 40kDa doublet of bands representative of the alpha and beta chain of the mature protein.


nCLU
full length CLU
Mock
1ug
100ng
kDa
80
60
45
40
Upstate
Santa Cruz
IkBa
ß-actin
6 hours
12 hours
24 hours

5.2.2.2 Western blotting using nuclear and cytosolic extracts

In order to determine whether the 45kDa band obtained by Western blotting of whole cell lysates was indeed nuclear CLU, I carried out western blotting on nuclear and cytosolic extracts prepared from C666-1 cells at 12 and 24 hours post transfection with mock vector, nCLU or full length CLU. SP1 was used as a loading and purification control for nuclear extracts, and tubulin for cytosolic extracts (Figure 5.7). Transfections were performed under both high and low serum conditions to assess whether low serum conditions induced expression of differentially processed proteins. Low serum condition did not induce differential expression of CLU proteins.

The absence of SP1 from the cytosolic extract and the absence of tubulin from the nuclear extract suggest that these extracts have been successfully separated. Using both antibodies a 60kDa protein, corresponding to the cytoplasmic precursor of secreted CLU, can clearly be detected in the cytoplasmic extracts at 24 hours post transfection with full length CLU but not with nCLU or with empty vector. A 45kDa protein can clearly be detected using both antibodies at 12 and 24 hours post transfection with nCLU but not full length CLU in both cytosolic and nuclear extracts. It is therefore likely to be nuclear in localisation, with its presence in the cytosolic extract being the precursor for the nuclear protein that has not yet translocated to the nucleus. The 50kDa protein observed previously cannot be detected in nuclear and cytosolic extracts, and so is likely to be an artifact or break-down product.

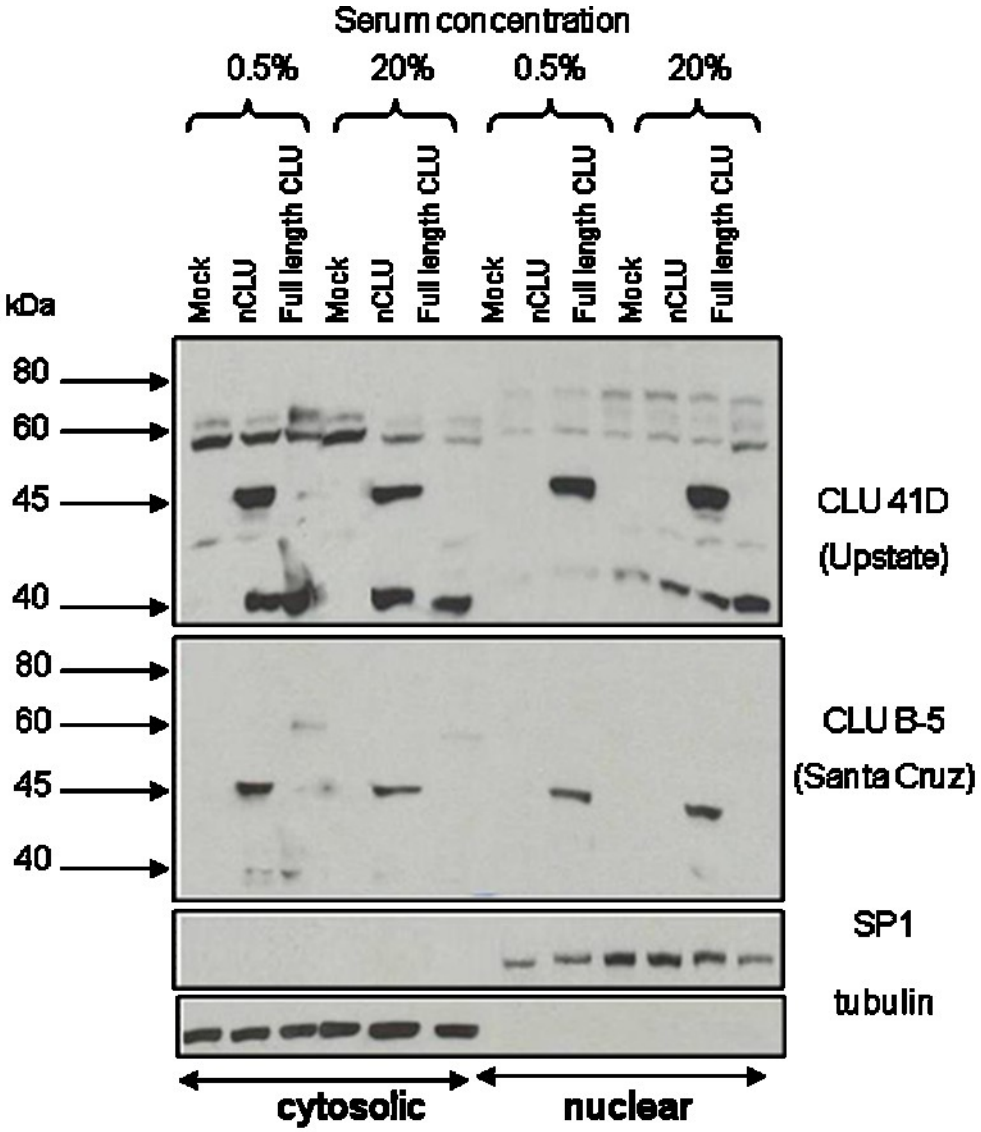


Figure 5.7: CLU expression in C666-1 nuclear and cytosolic extracts by Western blotting

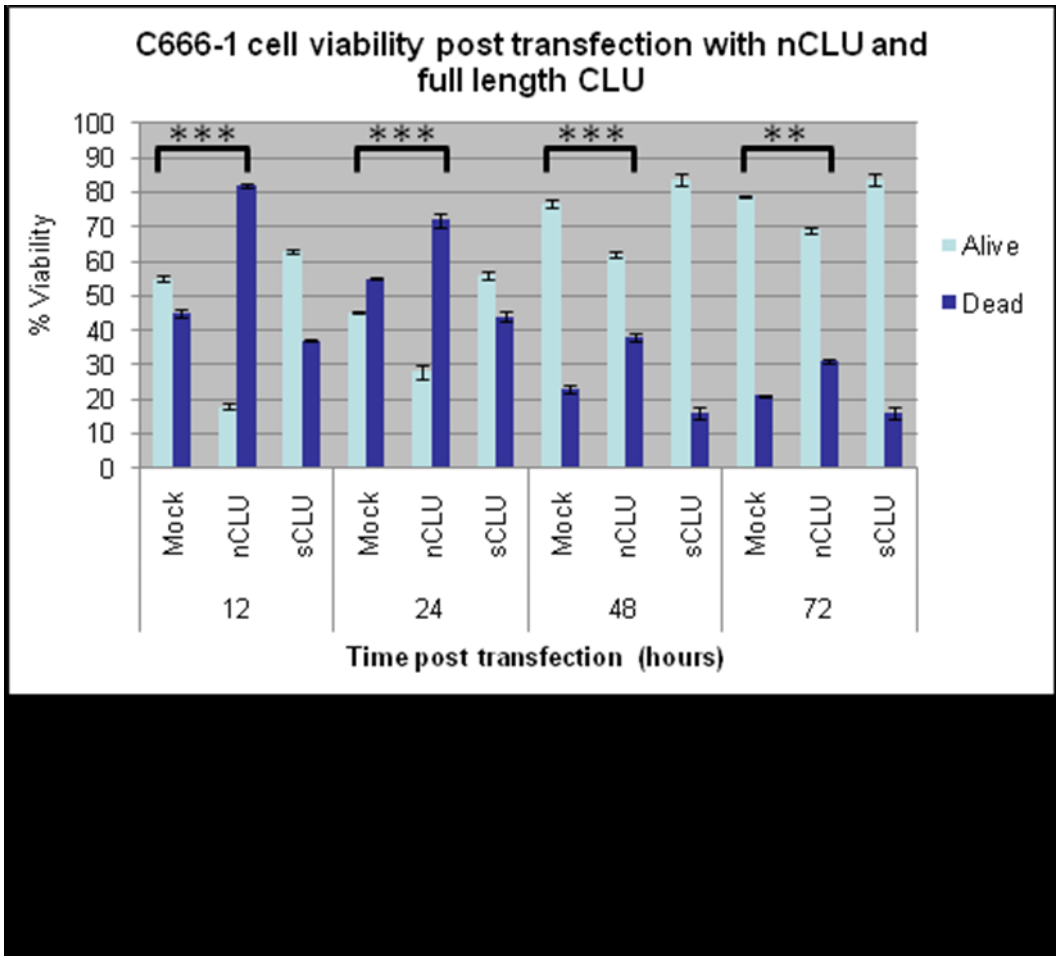
Western blotting shows the expression of CLU, SP1 and tubulin in nuclear and cytosolic extracts at 24 hours post transfection of C666-1 with full length and nCLU; the absence of SP1 from the cytosolic extract, and the absence of tubulin from the nuclear extract suggest that these extracts have been successfully separated.

5.2.3. Phenotypic changes following transfection with nCLU and full length CLU

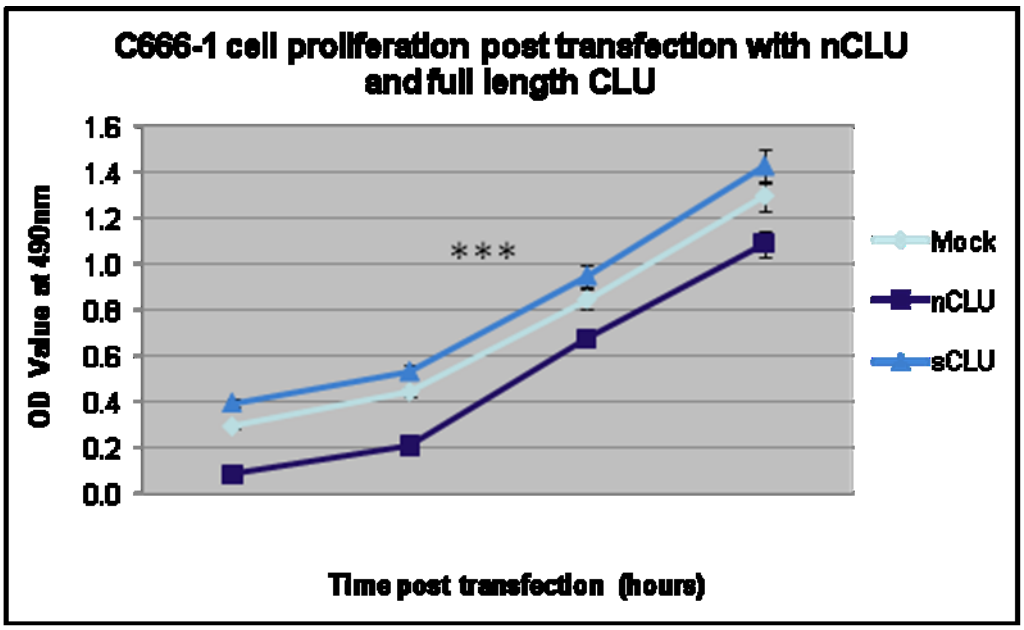
In order to investigate the phenotypic consequences of expression of nCLU and full length CLU in C666-1 cells I examined cell viability, proliferation and apoptosis. Figure 5.8 shows that compared to mock transfected cells at 12, 24 and 48 hours post transfection of C666-1 there is a decrease in the number of live viable cells. Decreased viability following transfection with nCLU was statistically significant at all time points, P-value <0.001 at 12, 24 and 48hrs and P-value <0.01 at 72hrs. There was a small increase in the number of viable cell transfected with length CLU, which is also able to generate nCLU by nuclear translocation. Figure 5.9 shows the number of proliferating cells as measured by MTS assay, where the number of proliferating cells in proportional to formazan production. Compared with mock transfected cells there is a 70% and 50% reduction in the number of proliferating cells at 12 and 24 hours respectively post transfection of C666-1 with nCLU. 12 hours post transfection of C666-1 with full length CLU results in a 30% increase in the number of proliferating cells, which starts to return to baseline from 24 hours. A one-way ANOVA shows that transfection with nCLU and sCLU statistically significantly reduces (nCLU)/increases (sCLU) cell proliferation of C666-1, with a P-value <0.001.

To determine the effect on apoptosis of transfection of CLU into C666-1 cells Annexin-V PI staining and subsequent analysis by flow cytometry was performed at 48 hours post transfection. Figure 5.10 shows results displayed as a bivariate distribution of Annexin V/FL1 and PI/FL3 fluorescent intensity. PI positive cells represent the dead cell population (upper left quadrant),

Annexin-V positive cells are early apoptotic cells (lower right quadrant), PI and Annexin-V positive cells are late apoptotic cells (upper right quadrant) and unstained cells are viable cells (lower left quadrant). Bold figures in the upper right and lower right quadrants show the percentages early and late apoptotic cells. Figure 5.11 Panel B and D show an increase in the number of early and late apoptotic cells following transfection with both nCLU and full length CLU, an effect which is ameliorated following TGFβ stimulation. TGFβ stimulates nuclear translocation of CLU (Reddy, Jin et al. 1996) so I would have expected this to increase the number of apoptotic cells. This result was unexpected – I would have expected to see an increase in the number of apoptotic cells following stimulation of nCLU transfected C666-1with TGFβ. This is possibly because apoptosis assays were carried out 48 hours post transfection when the effect of nCLU is masked by that of sCLU and so an earlier time point would have been more appropriate to study the levels of TGFβ induced apoptosis by nCLU. To some extent this can be seen in panel C, following stimulation of CLU transfected cells there is an increased number of dead cells compared with unstimulated cells.


C666-1 cell viability post transfection with nCLU and full length CLU
% Viability
100
90
80
70
60
50
40
30
20
10
0
***
***
***
**
Mock
nCLU
sCLU
12
24
48
72
Time post transfection (hours)
Alive
Dead

A.



B.

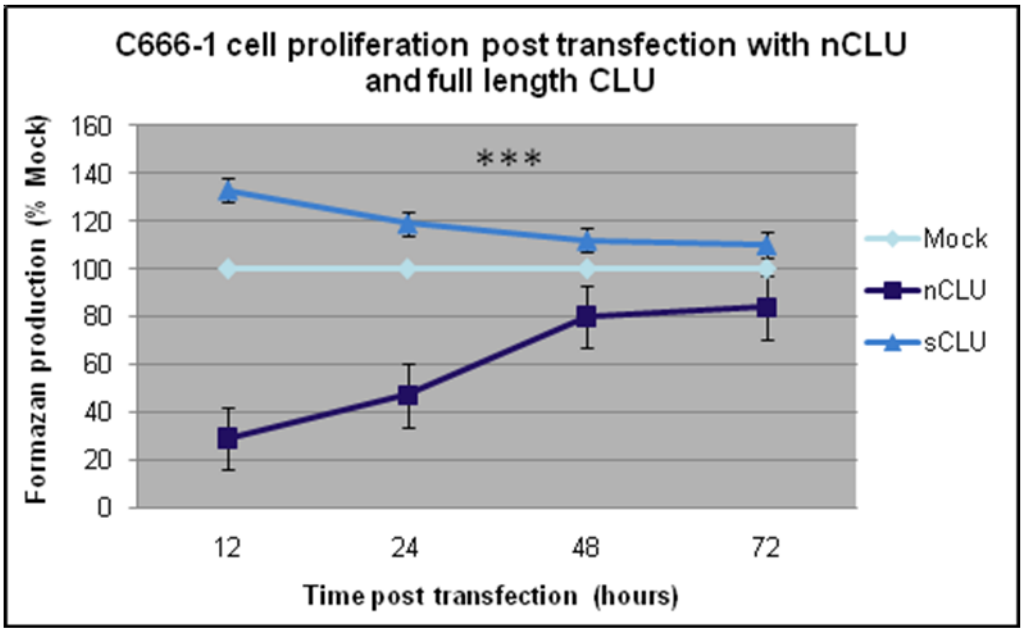
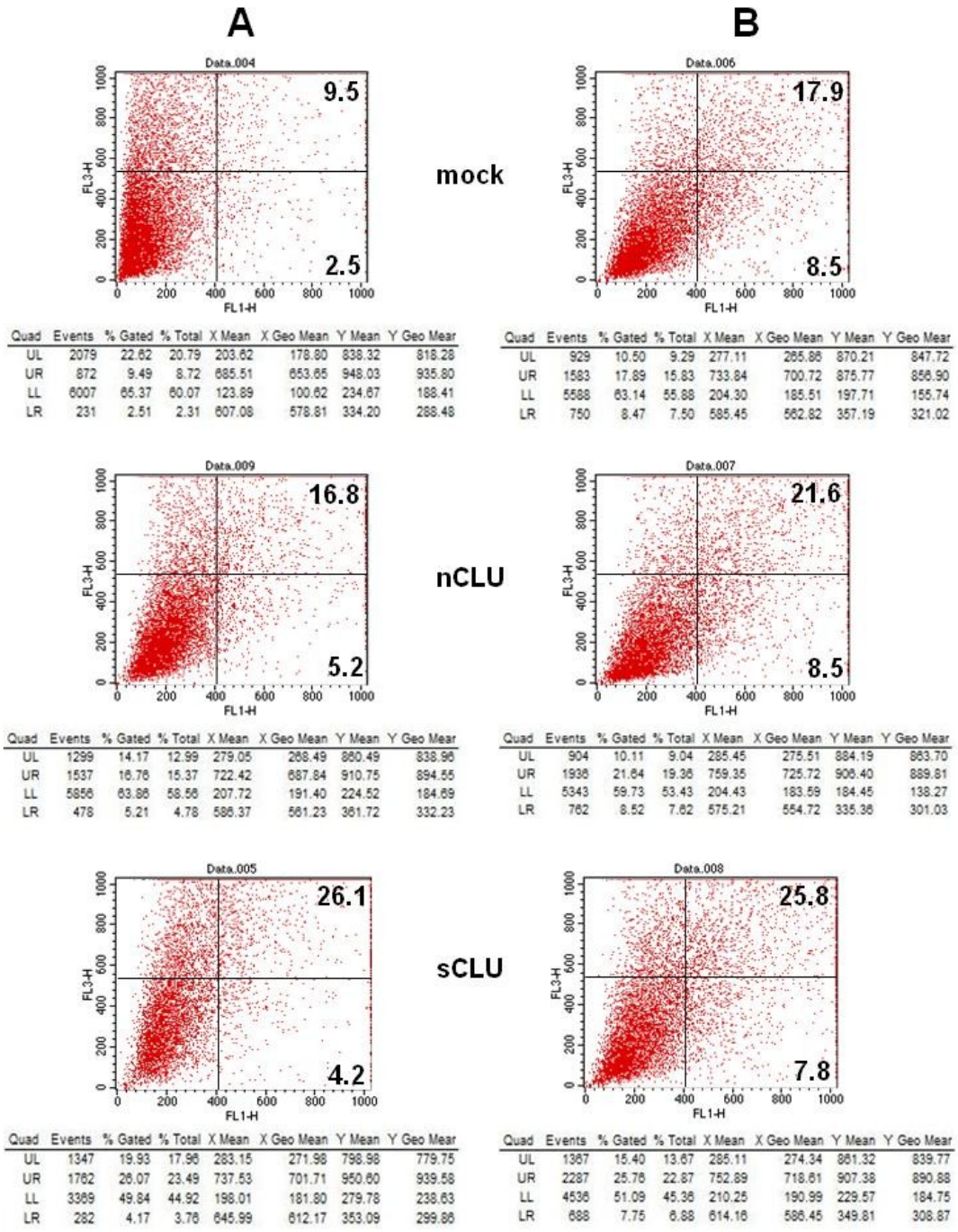


Figure 5.9A and B: Phenotypic changes observed following transfection of the C666-1 cell line with CLU- cell proliferation

MTS assay shows a reduction in cellular proliferation following transfection with nCLU in C666.1, and an increase in proliferation following transfection with full-length CLU, shown both as raw data (A) and expressed as a percentage of the control (B)
A one way ANOVA shows that transfection with nCLU and sCLU statistically significantly reduces (nCLU)/increases (sCLU) cell proliferation of C666.1, with a P value <0.001 (***).



Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	2079	22.62	20.79	203.62	178.80	838.32	818.28
UR	872	9.49	8.72	685.51	653.65	948.03	935.80
LL	6007	65.37	60.07	123.89	100.62	234.67	188.41
LR	231	2.51	2.31	607.08	578.81	334.20	288.48

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	929	10.50	9.29	277.11	265.86	870.21	847.72
UR	1583	17.89	15.83	733.84	700.72	875.77	856.90
LL	5588	63.14	55.88	204.30	185.51	197.71	155.74
LR	750	8.47	7.50	585.45	562.82	357.19	321.02

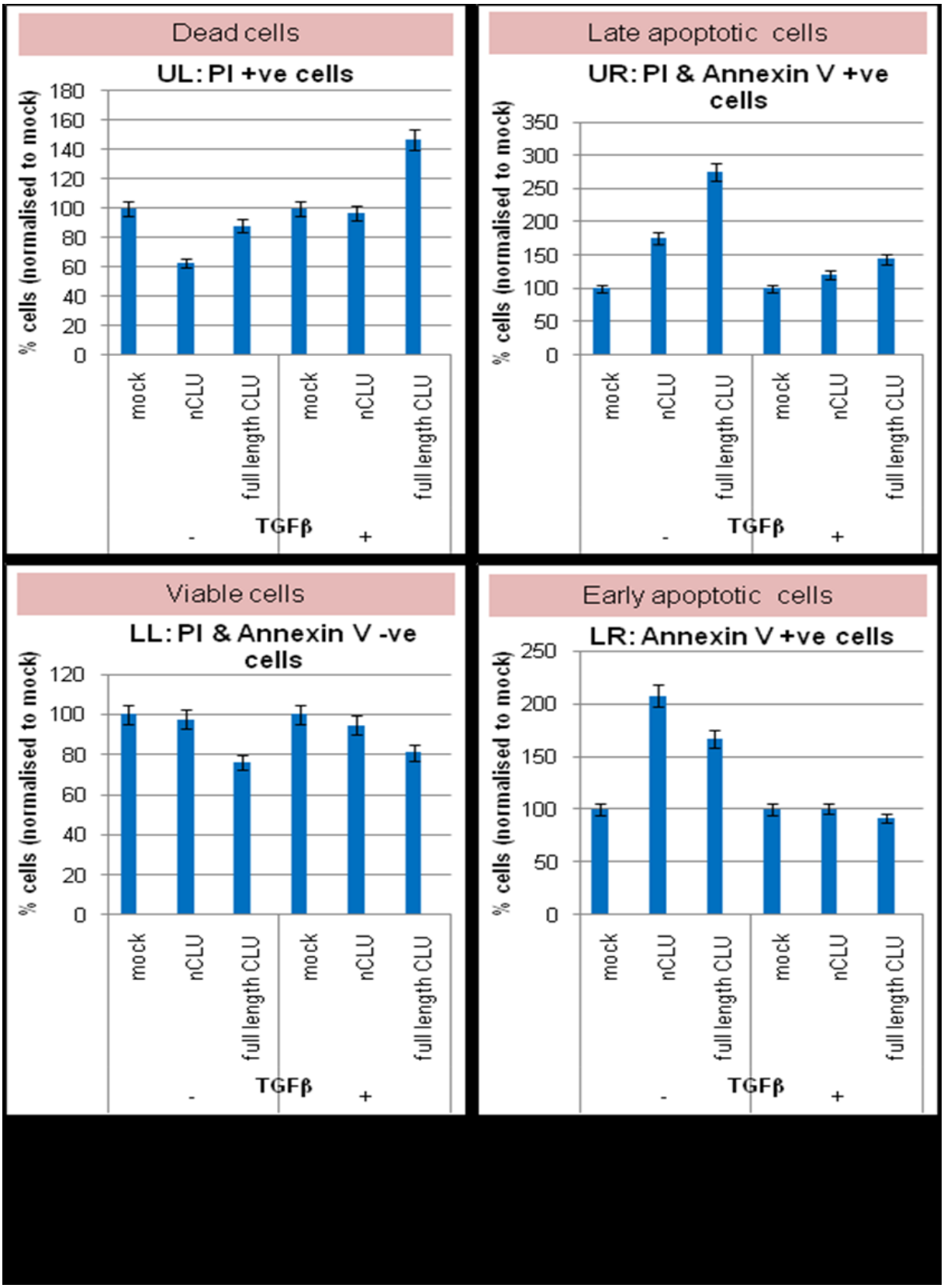
Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	1299	14.17	12.99	279.05	268.49	860.49	838.96
UR	1537	16.76	15.37	722.42	687.84	910.75	894.55
LL	5856	63.86	58.56	207.72	191.40	224.52	184.69
LR	478	5.21	4.78	586.37	561.23	361.72	332.23

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	904	10.11	9.04	285.45	275.51	884.19	863.70
UR	1936	21.64	19.36	759.35	725.72	906.40	889.81
LL	5343	59.73	53.43	204.43	183.59	184.45	138.27
LR	762	8.52	7.62	575.21	554.72	335.36	301.03

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	1347	19.93	17.96	283.15	271.98	798.98	779.75
UR	1762	26.07	23.49	737.53	701.71	950.60	939.58
LL	3369	49.84	44.92	198.01	181.80	279.78	238.63
LR	282	4.17	3.76	645.99	612.17	353.09	299.86

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	1367	15.40	13.67	285.11	274.34	861.32	839.77
UR	2287	25.76	22.87	752.89	718.61	907.38	890.88
LL	4536	51.09	45.36	210.25	190.99	229.57	184.75
LR	688	7.75	6.88	614.16	586.45	349.81	308.87

Figure 5.10. Annexin V staining of C666-1 cells following transfection with CLU. A represents C666-1 cells transfected with mock vector or CLU and B transfection followed by TGFß stimulation. Annexin V (FL1) and propidium iodide (PI) (FL3) scattering allows gating to determine viability of cells and stage of apoptosis in the overall cell population. PI positive cells represent the dead cell population (upper left quadrant), Annexin-V positive cells are early apoptotic cells (lower right quadrant), PI and Annexin-V positive cells are late apoptotic cells (upper right quadrant) and unstained cells are viable cells (lower left quadrant). Bold figures in the upper right and lower right quadrants show the percentages early and late apoptotic cells.


Dead cells
UL: PI +ve cells
% cells (normalised to mock)
180
160
140
120
100
80
60
40
20
0
mock
nCLU
full length CLU
mock
nCLU
full length CLU
-
TGFβ
+
Late apoptotic cells
UR: PI & Annexin V +ve cells
% cells (normalised to mock)
350
300
250
200
150
100
50
0
mock
nCLU
full length CLU
mock
nCLU
full length CLU
-
TGFβ
+
Viable cells
LL: PI & Annexin V -ve cells
% cells (normalised to mock)
120
100
80
60
40
20
0
mock
nCLU
full length CLU
mock
nCLU
full length CLU
-
TGFβ
+
Early apoptotic cells
LR: Annexin V +ve cells
% cells (normalised to mock)
250
200
150
100
50
0
mock
nCLU
full length CLU
mock
nCLU
full length CLU
-
TGFβ
+

5.3. Regulation of NF-kB activity by CLU

The up-regulation of NF-kB signalling is a common feature of malignant disease. NF-kB induced gene expression has been shown to contribute significantly to the pathogenesis of inflammatory disease. Santilli *et al.* proposed an explanation for the pro-apoptotic activity of CLU through an involvement in the regulation of NF-kB activity (Santilli, Aronow et al. 2003). Ectopic expression of CLU strongly inhibited NF-kB activity in human neuroblastoma cells and murine embryonic fibroblasts by stabilising inhibitors of NF-kB (IkBs). I therefore first measured NF-kB activity following the expression of CLU in the HEK 293 and C666-1 cell lines.

NF-κB reporter assays were carried out using the Dual-Luciferase® Reporter activities of firefly and *Renilla* luciferases. NF-κB reporter assays are routinely used to monitor the activity of NFκB-regulated signal transduction pathways in cultured cells through use of an NFκB reporter construct and control constructs. The NFκB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the NFκB transcriptional response element (TRE). Photon emission is brought about through the ATP/ Mg^{2+}/O_2 dependent oxidation of beetle luciferin, via a luciferyl-AMP intermediate that generates a luminescent signal for firefly luminescence which when quenched simultaneously initiates the *Renilla* luciferase reaction. NF-κB reporter assays are carried out following co-transfection of NF-κB reporter plasmid and with a renilla plasmid and promoter activity calculated following normalization against internal renilla values.

To minimise the possibility of variability in this assay and to enable statistical analysis to be carried out on data, each individual transfection experiment was repeated on 6 occasions, each of which were all assayed in triplicate at separate times, with each individual sample per assay also being carried out in triplicate.

5.3.1. Impact on NF-kB activity of CLU over-expression in 293 cells

I co-transfected the CLU plasmids described with an NFkB luciferase reporter plasmid and measured luciferase activity. Basal activity in HEK 293 cells was determined by dividing the observed luminescence in cells transfected with the reporter plasmid with that observed in cells transfected with an empty vector. In order to exclude the impact on NF-kB activity of non-specific stress responses, the CLU plasmids were titrated from concentrations of 1ug to 31.3ng. The results show that at 12 hours post transfection of HEK 293 with nCLU there is a 50% reduction in NF-kB activity, as measured by luciferase reporter assay, which was statistical significant, P-value <0.001 (Figure 5.12). This reduction in activity had returned to baseline by 48 hours, with a slight increase in activity at 48 hours. However, at 12 hours post transfection of HEK 293 with full length CLU there is an increase in NF-κB activity with a return to baseline by 48 hours (Figure 5.13). Utilising these findings I have used plasmid concentrations of 1µg and 100ng, and measured NF-kB activity from 6 hours post transfection in subsequent analysis of the C666-1 cell line (Figure 5.14).

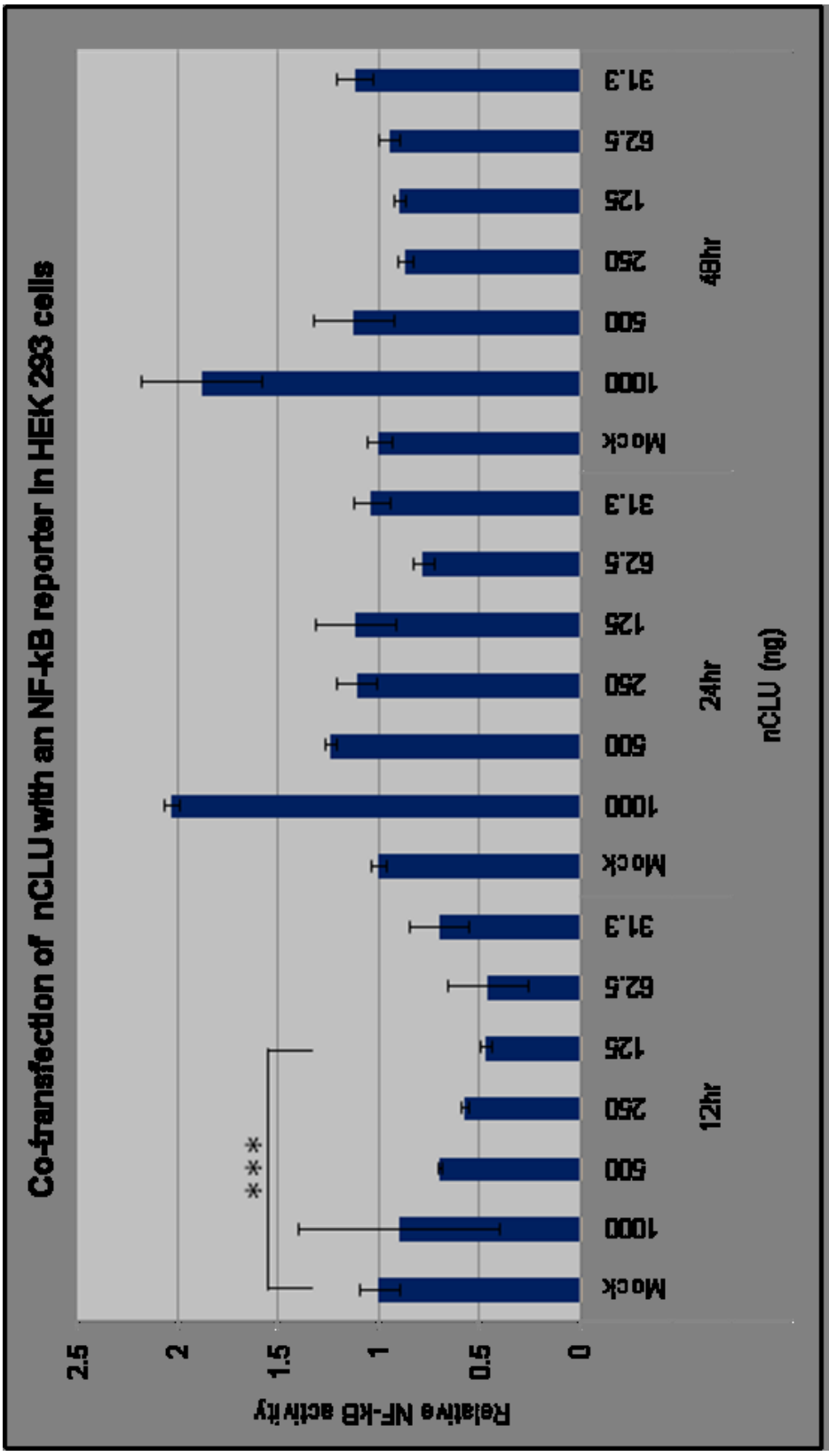


Figure 5.12: Luciferase reporter assay of NF-kB activity following nCLU transfection

12 hours post transfection of HEK 293 with nCLU there is a 50% reduction in NF-kB activity, that was statistical significant, P-value <0.001 (***). This reduction in activity had returned to baseline by 48 hours, with a slight increase in activity at 48 hours.

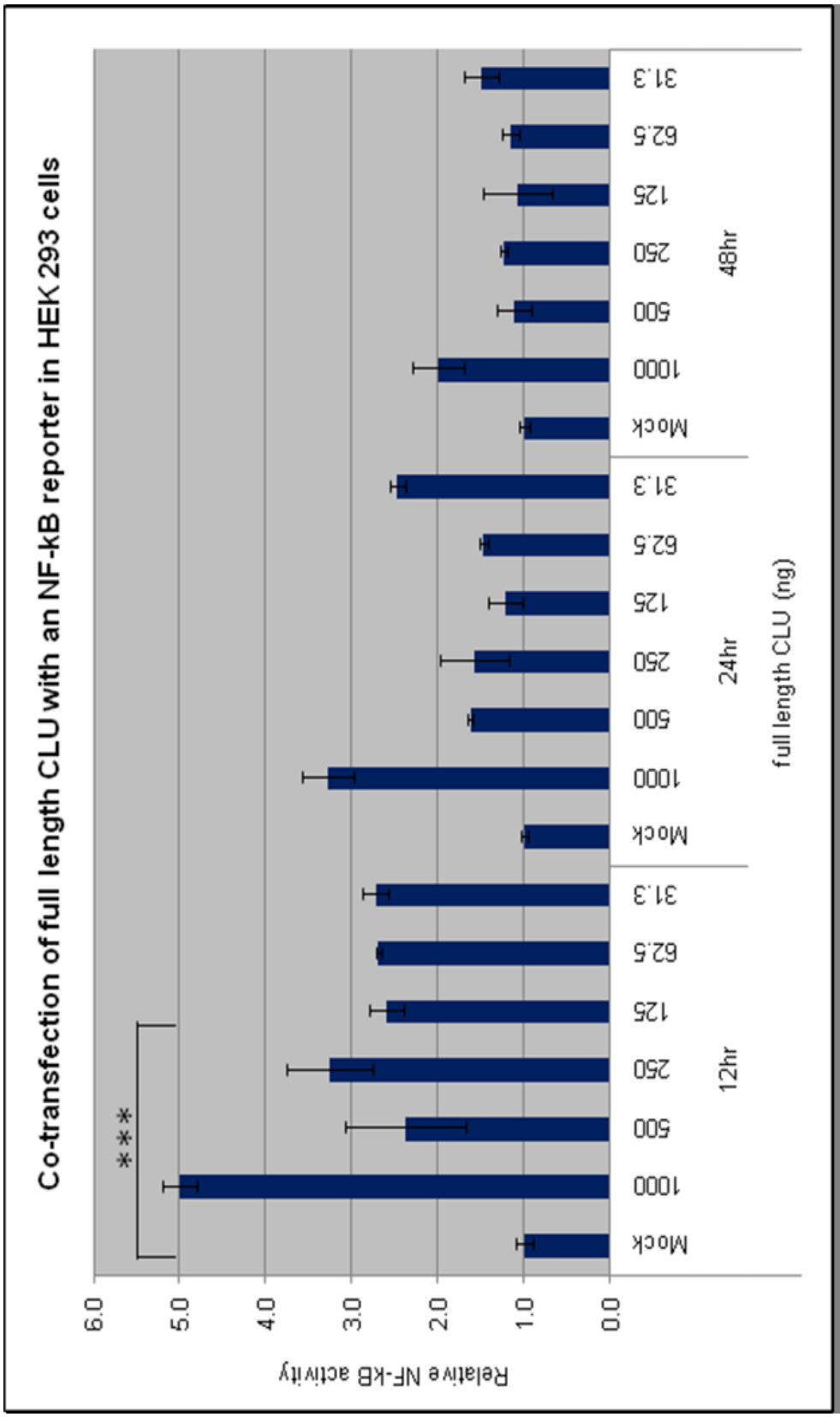


Figure 6.13: Luciferase reporter assay of NF-kB activity following full-length CLU transfection
12 hours post transfection of HEK 293 with full length CLU there is an increase in NF-kB activity, that was statistical significant, P-value <0.001 (***); with a return to baseline by 48 hours

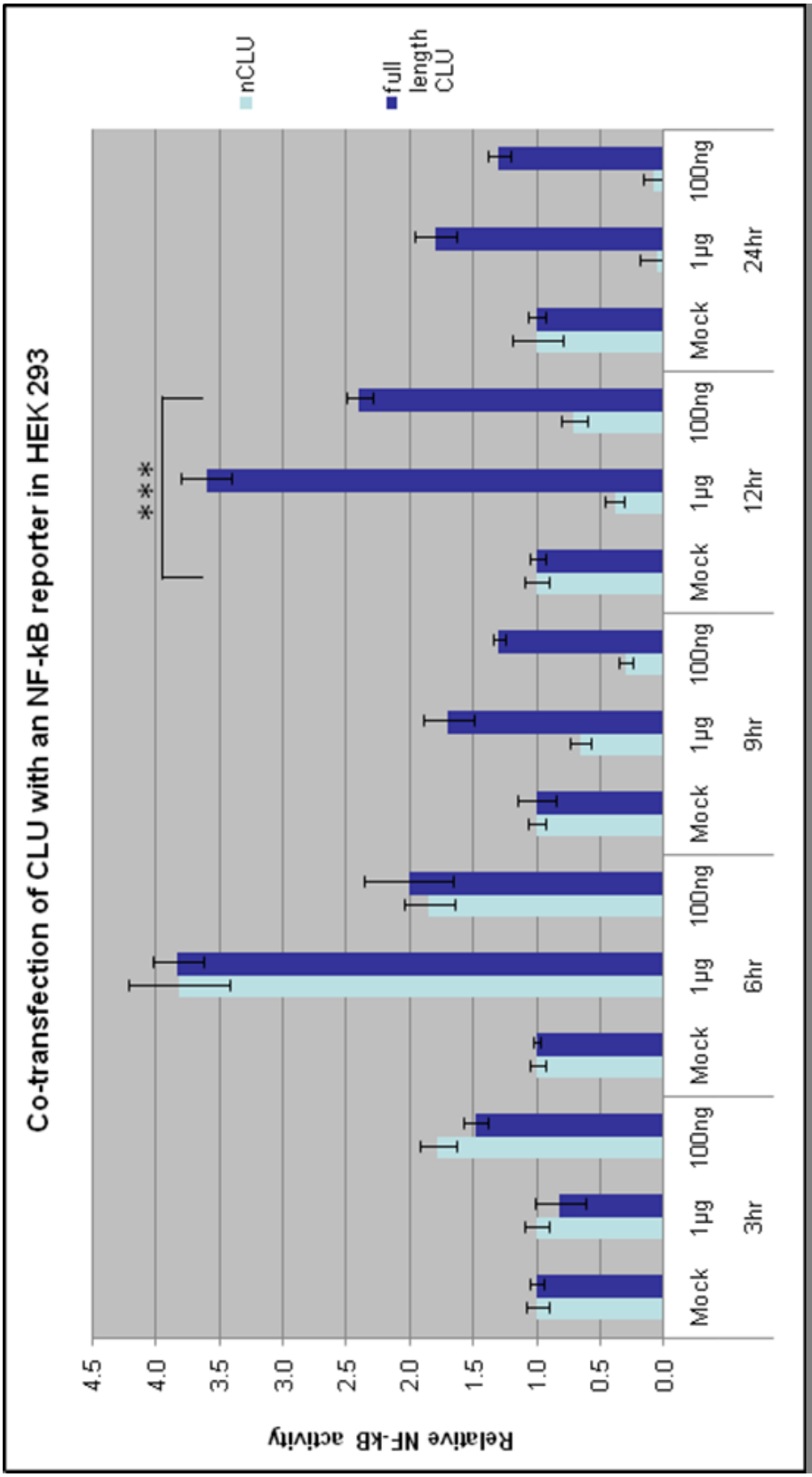


Figure 5.14: Luciferase reporter assay shows stimulation of NF-kB activity 3-24 hours following transfection of CLU.

12 hours post transfection of HEK 293 with nCLU there is a 50% reduction in NF-kB activity, this reduction in activity had returned to baseline by 48 hours, with a slight increase in activity at 48 hours. 12 hours post transfection of HEK 293 with full length CLU there is an increase in NF-kB activity with a return to baseline by 48 hours. Both of which were statistical significant, P-value <0.001 (***).

5.3.2. Impact of clusterin expression on NF-kB activity in C666-1 cells

I next examined the impact of nCLU and full length CLU expression on NF-kB activity in C666-1 cells. Figure 5.15 shows that both nCLU and full length CLU down-regulated NF-kB activity in these cells from 12 hours post transfection and were statistical significant, P-value <0.001. Since in HEK 293 cells the impact of CLU on NF- kB activity was short lived, with a return to baseline by 48 hours post transfection, the time-course was extended to 96 hours post-transfection (Figure 5.16). There is some evidence to suggest that NF-kB activity is returning to baseline following 24 hours despite it not being fully reached. The luciferase reporter assays were carried out in triplicate and repeated on 6 occasions in order to verify these results.

5.3.3. IkBα stabilisation

The activity of NF-kB is suppressed through interaction with the inhibitory molecule IkB which blocks the translocation of the to p55/p60 heterodimer to the nucleus and its transcriptional activity. Phosphorylation of IkB by IkB kinases (IKKs) marks the latter for degradation, which results in the release and translocation of the active NF-kB heterodimer (Pajak and Orzechowski 2006). CLU has been shown to induce IkBα stabilisation by inhibiting E3 ubiquitin ligase binding to phosphorylated IkBα, resulting in decreased NF-kB activity. Following transfection of CLU into HEK 293 and C666-1 cells, expression of the IkBα protein was examined by restripping of the Western blot (Figures 5.2 and 5.6). The results showed an increase in the expression of IkBα in cells transfected with CLU up to 24 hours post transfection, and provides a possible mechanism for the down regulation of NF-kB activity by CLU in these cell lines.

IkB is able to suppress NF-kB activity by blocking its nuclear translocation, thereby rendering it inactive in the cytoplasm. Having observed suppression of NF-kB by CLU though stabilisation of IkBα, I sought to determine whether this would inhibit p50 and p65 nuclear translocation.

Western blotting for p50 and p65 was carried out to confirmed the results obtained by luciferase reporter assays and to determine whether suppression of NF-kB by CLU though stabilisation of IkBα would inhibit p50 and p65 nuclear translocation. Western blotting of nuclear and cytosolic extracts showed an increase in cytoplasmic p50 and p65 post transfection with nCLU and a concurrent decrease in their nuclear localisation. Transfection with full length CLU showed little, if any, change in p50 and p65 localisation (Figure 5.17).

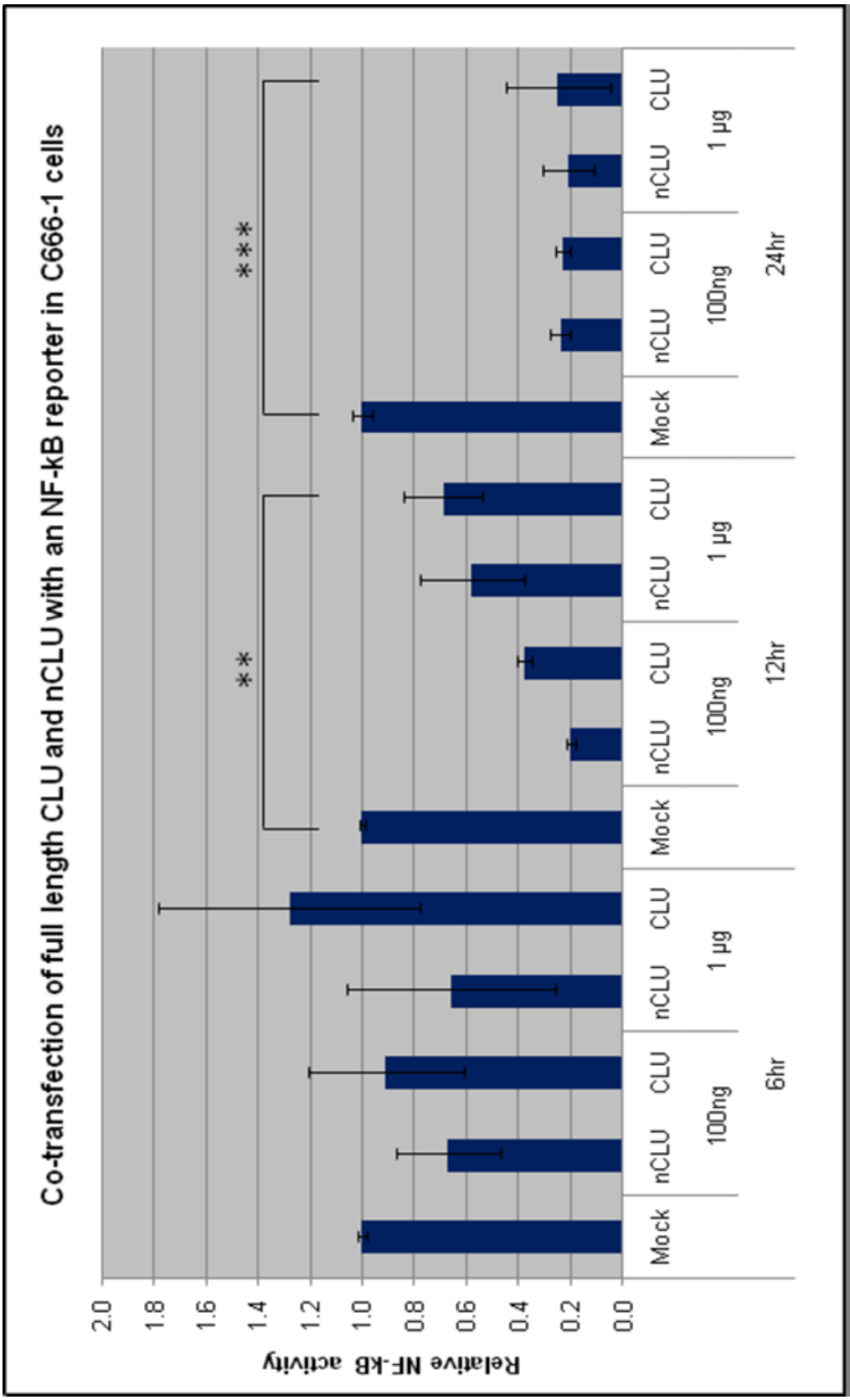


Figure 5.16: Luciferase reporter assay of NF-kB activity in C666-1 transfected with CLU

Both nCLU and full length CLU down-regulated NF-kB activity in these cells from 12 hours post transfection. Both of which were statistical significant, P-value <0.001 (***) and <0.01 (**).

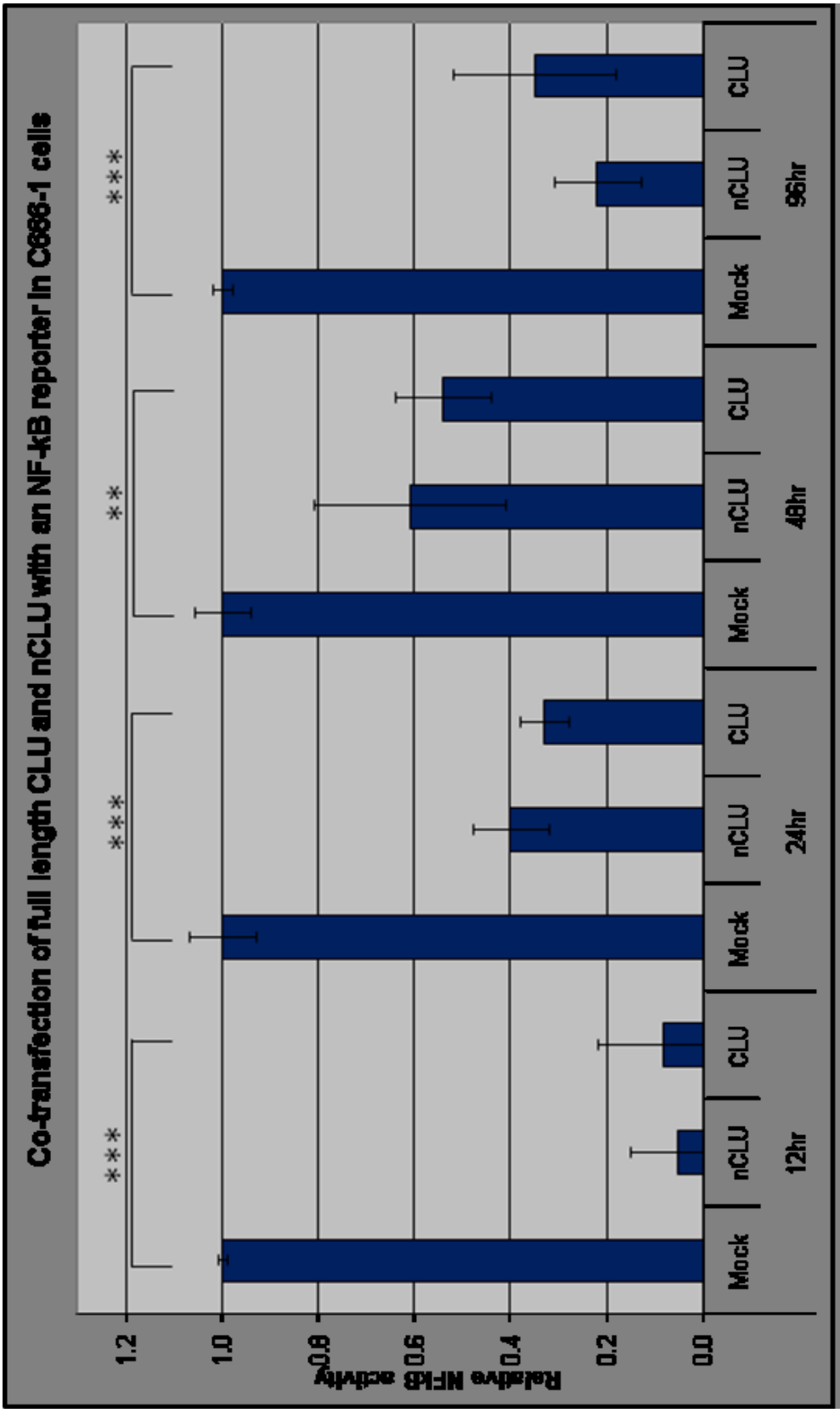


Figure 5.16: Luciferase reporter assay of NF-kB activity in C666-1 transfected with CLU at 12-96hr

nCLU and full length CLU down-regulate CLU at 12 hours post transfection of C666-1 which was statistical significant, P-value <0.001 (***). These results also suggest that NF-kB activity is returning to baseline from 24 hours despite it not being fully reached.

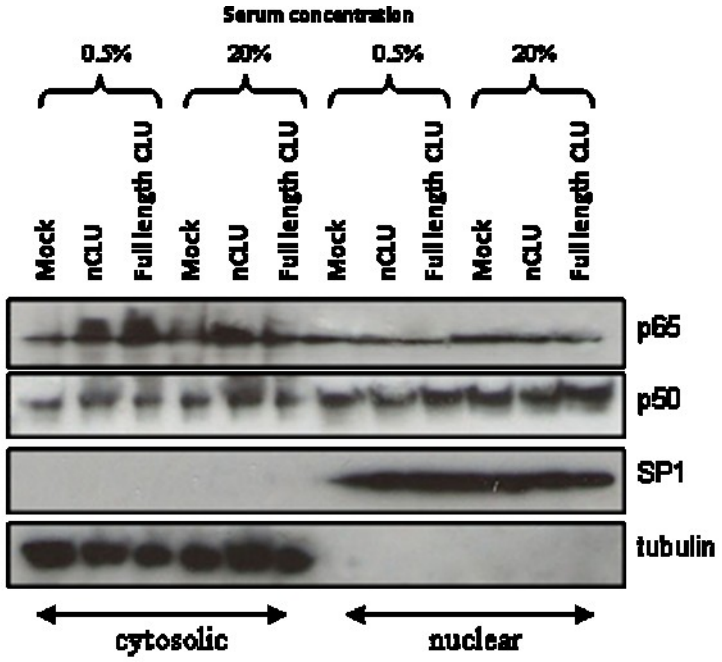


Figure 5.17: Western blotting of nuclear and cytosolic extracts for p50 and p65.

Western blotting of nuclear and cytosolic extracts showed an increase in cytoplasmic p50 and p65 post transfection with nCLU and a concurrent decrease in their nuclear localisation. Transfection with full length CLU showed little, if any, change in p50 and p65 localisation.

5.4. Knockdown of CLU in HEK293 cells

Following the observation that transient transfection of CLU down-regulated NF-kB activity through stabilization of IkBα, I set out to ascertain whether reduction of basal CLU levels through siRNA knockdown would have the converse effect of up-regulating NF-kB activity. I first transfected the HEK 293 cell line with siRNA targeted to full length CLU in order to optimize my knock down conditions. Scrambled siRNA was used as a negative control to ensure any effects seen were specific to knockdown. Both of these siRNA sequences were provided by Prof. Bettuzzi. Expression of transcript variant 1 was reduced post-transfection with CLU siRNA, but not with scrambled siRNA or lipofectamine alone (Figure 5.18). Transcript variant 2 could not be detected in these cells.

5.5. Knockdown of full length CLU in HeLa cells

I next attempted to knockdown CLU expression in HeLa cells which had previously been stably transfected with an NF-kB reporter plasmid to avoid any problems with co-transfecting siRNA with a plasmid. Expression was measured at 24, 48 and 72 hours post transfection with siRNA, scrambled siRNA and empty vector.

5.5.1. RNA expression following knockdown of full length CLU in HeLa

I evaluated the expression of CLU transcript variants 1 and 2 at 24, 48 and 72 hours post-knockdown using RT-PCR and GAPDH as a loading control (Figure 5.19). Transcript variant 1

was knocked-down from 24 hours post transfection with CLU siRNA, but not with scramble siRNA. Transcript variant 2, which unlike in the 293 cell line is weakly expressed in HeLa cells, was also found to be knocked-down from 24 hours post transfection. These data were confirmed using Q RT-PCR, showing statistically significant 90% knockdown at the RNA level at 24 hours, P-value <0.001(Figure 5.20).

5.5.2. Assessment of a possible interferon response

Recent publications have shown that siRNA knockdown may invoke an interferon response, especially if duplexes are longer than 23bp and cell viability is affected (Reynolds, Anderson et al. 2006). To address this issue the interferon response genes, ISG 20 and OAS 1 were analysed by Q RT-PCR for all siRNA knockdowns of CLU to check that no interferon response was observed. An interferon response is that of 10-fold difference in expression or higher, however changes do not seem to be significant (Figure 5.21A and 5.21B), indicating that no such response occurred in the course of these experiments.

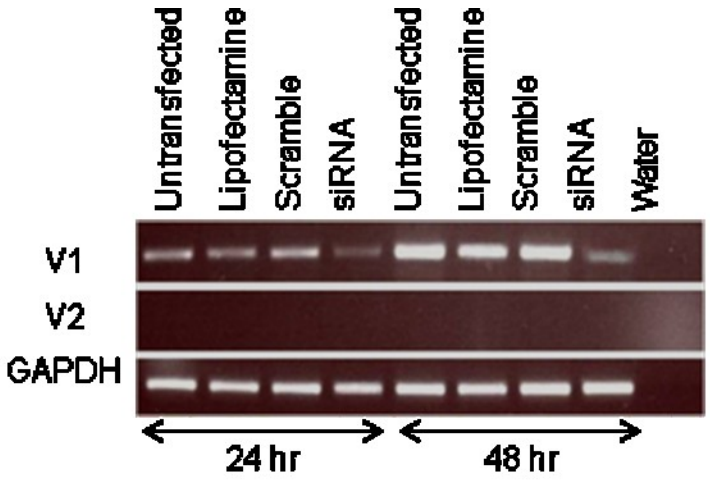


Figure 5.18: CLU expression in HEK 293 by RT-PCR

RT PCR shows the expression of CLU transcript variants 1 and 2 in 293 cells following knockdown with CLU siRNA; GAPDH was used as a loading control. The siRNA duplex reduced the level of transcript variant 1 at 24 and 48 hours post treatment

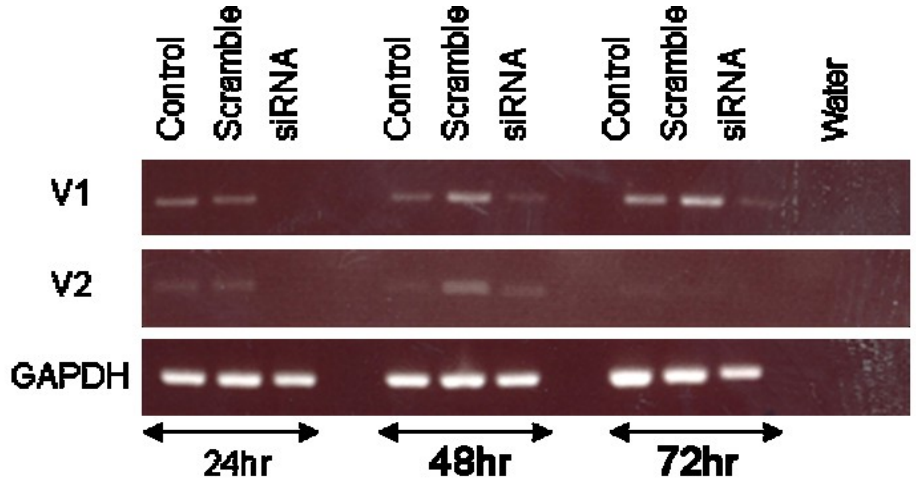


Figure 5.19: CLU expression HeLa cells by RT-PCR

Transcript variant 1 was knocked-down from 24 hours post transfection with CLU siRNA, but not with scramble siRNA. Transcript variant 2 is weakly expressed in HeLa cells and was also found to be knocked-down from 24 hours post transfection.

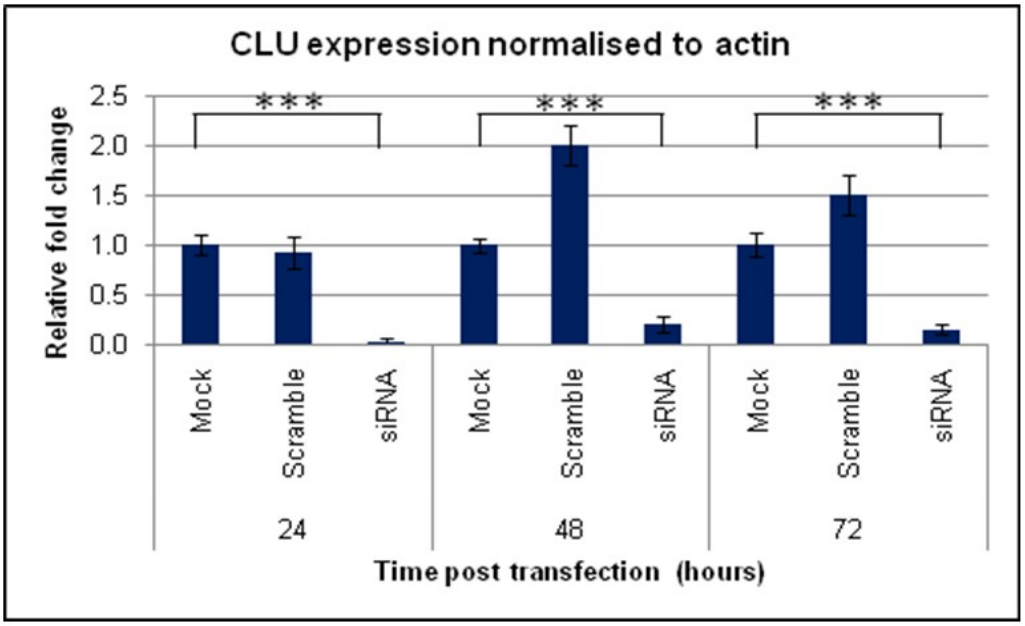


Figure 5.20: CLU expression in HeLa cells by quantitative RT-PCR

CLU siRNA resulted in 90% knockdown at the RNA level at 24 hours that was statistical significant, P value <0.001 (***).

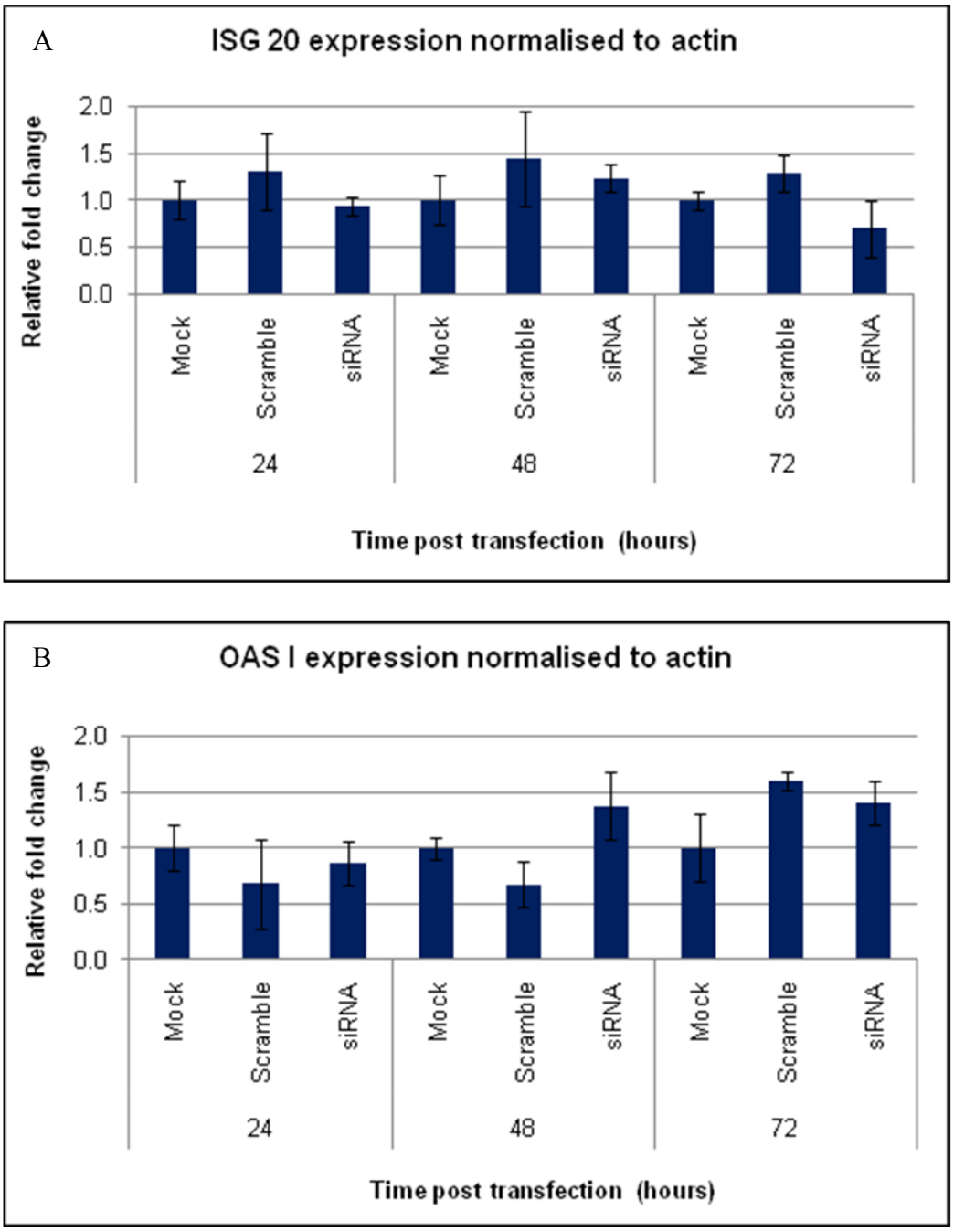


Figure 5.21 A and B: ISG 20 and OAS 1 expression in HeLa cells by quantitative RT-PCR

Small fold changes in ISG20 (**A**) and OAS1 (**B**) of less than 2.5 fold were observed in siRNA treated cells compared with untransfected cells indicating that no such response occurred in the course of these experiments.

5.5.3. Protein expression following knockdown of full length CLU in HeLa cells

I next examined CLU expression at the protein level following knockdown in the HeLa cell line. I investigated the expression of CLU in lysates prepared from HeLa cells at 24, 48 and 72 hours post transfection with mock vector, scrambled siRNA and CLU siRNA using Western blotting and two antibodies, β-actin was used as a loading control for all cell lysates (Figure 5.22). A 60kDa protein, corresponding to the cytoplasmic precursor of secreted CLU, is clearly seen to be knocked-down from 24 hours post transfection with CLU siRNA but not with scrambled siRNA. A 45kDa protein is not expressed in control cells. However, it can be detected using both antibodies 12 hours post transfection with scrambled siRNA. This apparent induction of the nuclear isoform of CLU may reflect a non-specific stress response.

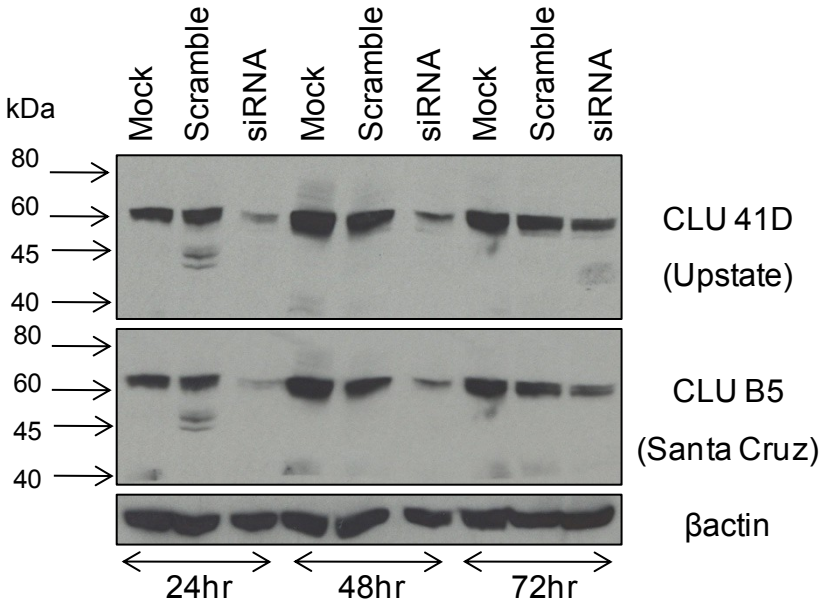


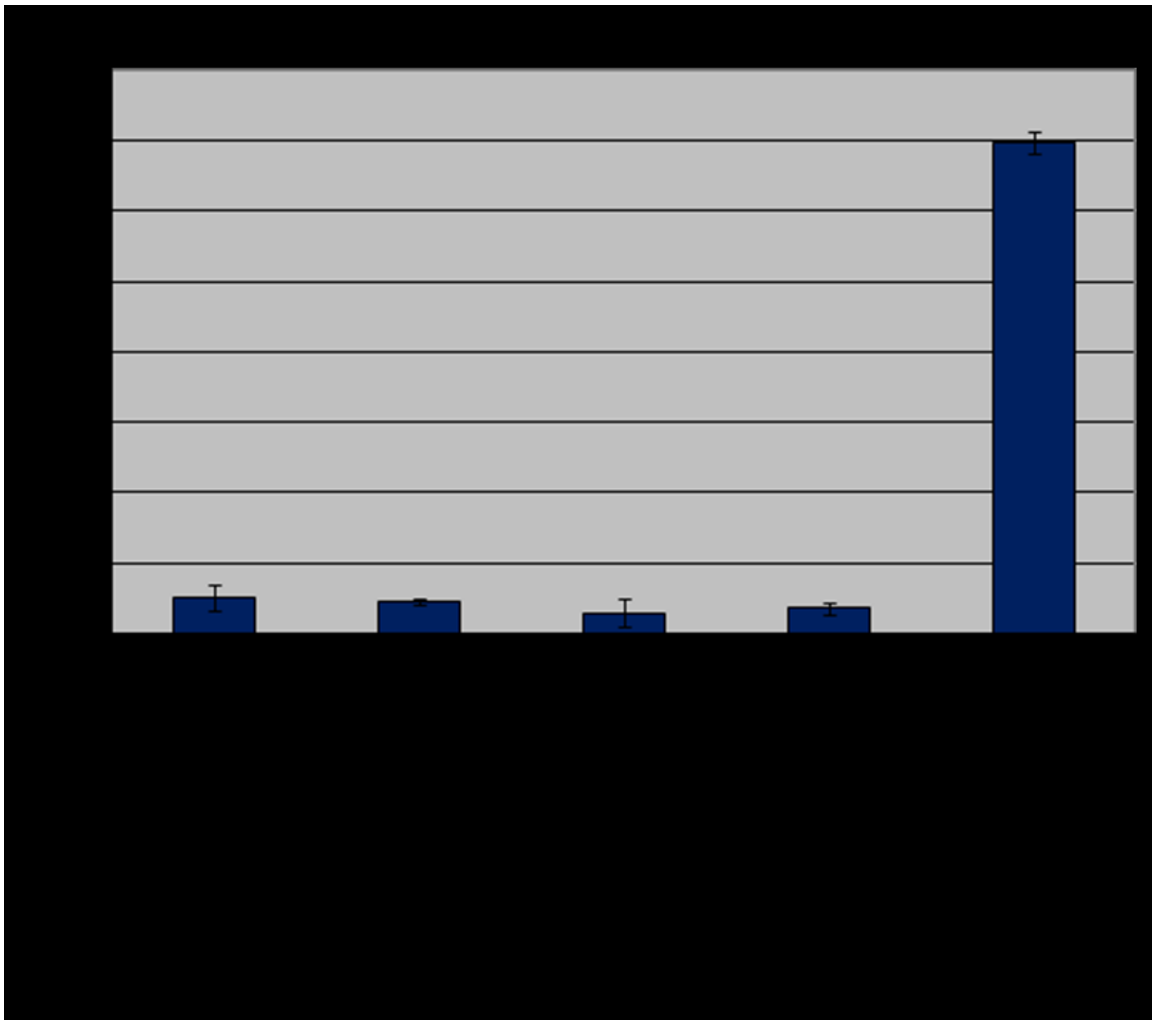
Figure 5.22: CLU expression in HeLa cells by Western blotting

A 60kDa protein, corresponding to the cytoplasmic precursor of secreted CLU, is clearly seen to be knocked-down from 24 hours post transfection with CLU siRNA but not with scrambled siRNA. A 45kDa protein is not expressed in control cells.

5.5.4. Impact on NF-kB activity of CLU knockdown in HeLa cells

I next set out to establish whether the knockdown of CLU in HeLa cells would have the predicted effect of up-regulating NF-κB activity. I first measured NF-κB activity following knock-down of CLU in a HeLa cell line stably expressing an NF-kB reporter plasmid. Since TNFα is known to induce NF-κB activity I assessed NF-kB activity with and without TNFα stimulation. Stimulation of NF-κB activity by TNFα in the HeLa cell line was tested at 10 minutes, 30 minutes, 1 hour and 3 hours and stimulation, demonstrating that 3 hour incubation of HeLa cells with TNFα is sufficient to induce NF-κB (Figure 5.23).

A luciferase reporter assay for NF-κB activity was carried out at 24, 48 and 72 hours post knockdown of CLU in HeLa cells (figure 5.24). At 24 hours following knockdown of CLU there is a 1.6 fold increase in NF-κB activity compared with mock transfected cells and cell transfected with scrambled siRNA, which is mirrored also at 48hours following knockdown. No effect was seen using scrambled siRNA at these time-points, although at 72 hours post transfection both CLU siRNA and scrambled siRNA had similar effects on NF-κB activity. This might suggest a non-specific stress response. These experiments were also performed following 3 hour TNFα stimulation of the HeLa cell line. The effects of CLU knockdown on NF-κB activity are very similar to those seen by TNFα stimulation. Therefore, the regulation of NF-κB activity by CLU does not appear to be TNF dependent.



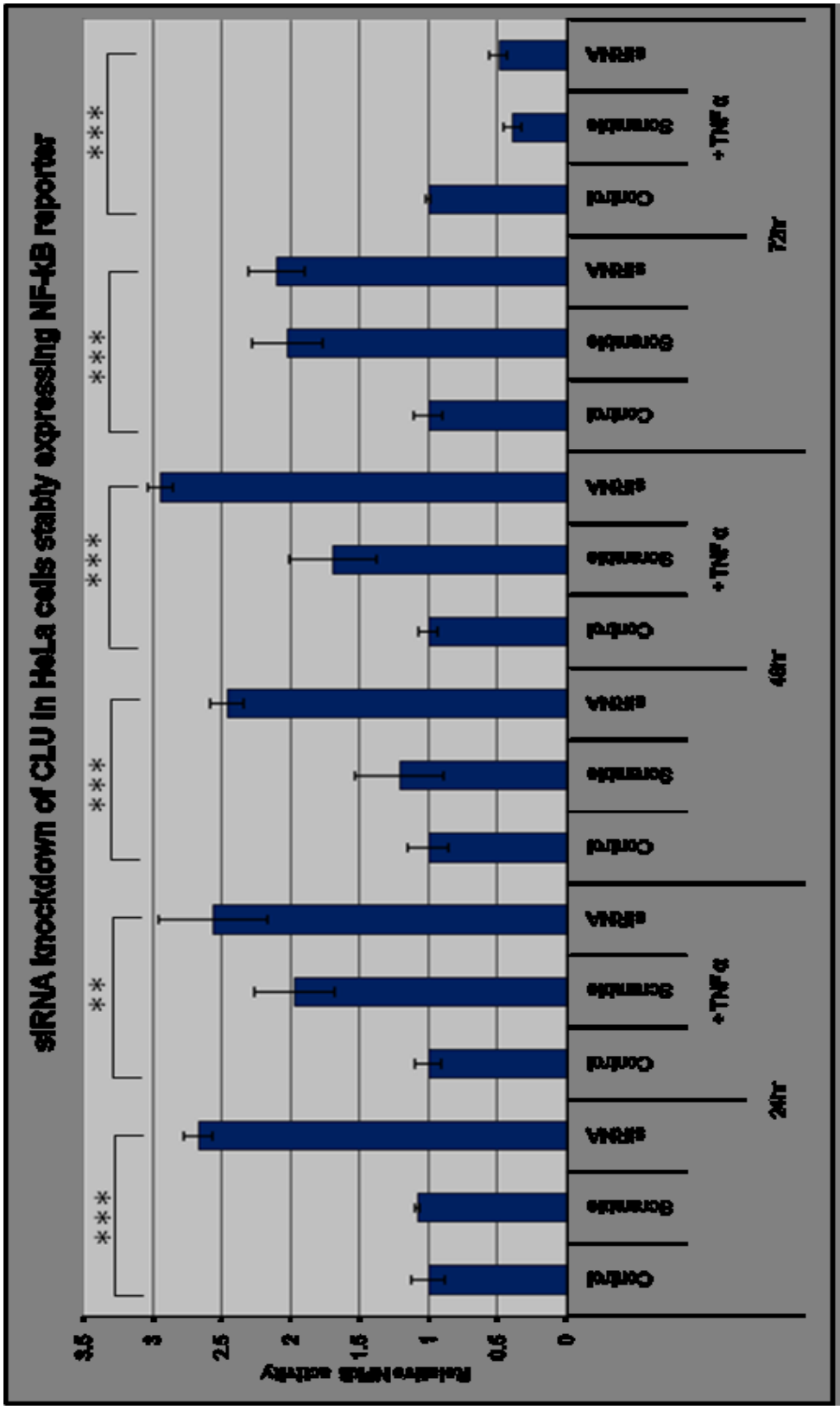


Figure 6.24: Luciferase reporter assay of NF-kB activity following siRNA knockdown of CLU in HeLa

Ludiferase reporter assay shows stimulation of NF-kB activity 24 hours following knockdown of CLU; the regulation of NF-kB activity by CLU does not appear to be TNF dependent. Knockdown ofCLU was statistical significant, P-value <0.001 (***).

5.6. Summary

The defect in the C666-1 cell line has successfully been repaired by transiently transfecting both nCLU and full length CLU into the cell line. Expression of both transcripts has been demonstrated at the RNA level from 6 hours post transfection and this was also translated in the full length and truncated proteins. Decreased viability and proliferation were observed following transient transfection of C666-1 with nCLU, with an increase in the number of proliferating cells following transfection with full length CLU. There was no convincing increase in viability following transfection with full length CLU, which I would have expected to see give its anti-apoptotic function. The effects on cell proliferation when expressed as raw OD values are not convincing of any effect, compared with when this data is expressed as a percentage of that observed in the mock transfected cells. There was an increase in the number of early apoptotic cells and late apoptotic/necrotic cells following transfection with both nCLU and full length CLU. However, these results are not entirely convincing, especially given that no change was observed following stimulation with TGFβ as might have been expected due to the fact that this induces the nuclear translocation of CLU and apoptosis. Theses apoptosis assays were carried out 48 hours post transfection when the effect of nCLU is masked by that of sCLU and so an earlier time point may in fact have been more appropriate to study the levels of induced apoptosis by nCLU.

Both nCLU and full length CLU down-regulated NF-κB activity from 12 hours post transfection of C666-1 and there is some evidence to suggest that NF-κB activity is returning to baseline from 24 hours. A possible mechanism for the down regulation of NF-κB activity by CLU in C666-1 is through stabilization of the IkB molecule, and the results did indeed show an increase in the expression of IkB in cells transfected with CLU up to 24 hours post transfection. As proof of principle, endogenous levels of CLU were knocked down in the HeLa cell line, showing 90% knockdown at the RNA level which was translated at the protein level at 24 hours post transfection with siRNA, without generating an interferon response. Knockdown of CLU resulted in a TNF independent increase in NF-κB activity of 1.6 fold and lasted for up to 48 hours post knockdown.

Chapter 6

Discussion

6.1: Identification and initial analysis of candidate genes in cervical neoplasia

Cervical cancer carcinogenesis includes a number of genetic and epigenetic changes that result in numerous cellular abnormalities, with the contribution of the viral HPV oncogenes E6 ad E7 affecting cellular proliferation. A large number of genes have been identified to be tumour suppressors in cervical neoplasia, including p16 and p53, whose function is inhibited by E6 and E7 and results in apoptosis inhibition and impeded DNA repair. The pathogenesis of the disease also involves a number of epigenetic changes, in particular DNA methylation of genes, such as RASSF1A, involved in cell proliferation and differentiation resulting in gene expression changes (Kang, Kim et al. 2007; Kisseljov, Sakharova et al. 2008).

The initial aim of this thesis was to identify novel tumour suppressor genes in cervical neoplasia. As a starting point for these investigations, genes which had been identified from a microarray experiment looking at expression changes post demethylation of 4 cervical cancer cell lines were narrowed down to a viable group of candidate genes. Detailed analysis of the results of these experiments combined with literature searches highlighted seven genes that were potentially epigenetically regulated in cervical cancer. The approach taken was successful and provided a large number of genes potentially epigenetically regulated in cervical cancer and used three replicates of four cervical cancer cell lines to allow statistical analysis. The microarray gave promising results, revealing a number of genes which have been previously identified to be tumour suppressors in cervical neoplasia, including p53, p16, DAPK and CADM1 (Anton, Horky et al. 2000; Steenbergen, Kramer et al. 2004; Jeong, Youm et al. 2006; de la Cruz-Hernandez, Perez-Cardenas et al. 2007). To take into consideration confounding factors such as infecting HPV type the cell lines used included a

HPV negative cell line (C33a), a HPV 18 positive cell line (HeLa) and HPV 16 positive cell lines (CaSki and SiHa). The two HPV 16 positive cell lines were used as these have different viral loads and transcription activities, with CaSki having more than 600 integrated HPV-16 copies and SiHa having only about 2 (Rajeevan, Swan et al. 2006; Roberts, Ng et al. 2008).

The genes that were selected for further investigation were DKK3, KLF4, TIMP1, AKAP12, RNASET2, CLU and CADM1, which were found to be increased in a post-treatment sample compared to at least one pre-treatment sample, following demethylation of cervical cancer cell lines. These were chosen if they were either up-regulated by the viral oncogenes HPV E6/E7; or up-regulated following HPV integration; or up-regulated in invasive cancer compared with normal tissue (Nees 2001, Duffy 2003, Alawazi 2002). To confirm that these were viable candidate genes to study further, baseline expression levels were evaluated in a range of cervical cell lines and compared with normal human foreskin keratinocytes, as this was the best control available to me at the time of the study.

DKK3 is a secretory glycoprotein and Wnt antagonist that had shown tumour-specific DNA hypermethylation in prostate cancer, breast cancer and acute lymphoblastic leukaemia (Roman-Gomez, Jimenez-Velasco et al. 2004; Lodygin, Epanchintsev et al. 2005; Veeck, Bektas et al. 2008). DKK3 baseline expression in C33a was comparable to that of NHFK and it was not expressed in the three HPV positive cervical cell lines HeLa, CaSki and SiHa. This is not surprising because since the undertaking of this thesis the Wnt pathway has been shown to be activated in cervical carcinogenesis, facilitated through DKK3 negatively regulating beta catenin (Lee, Jo et al. 2009). HPV is thought to provide the initial hit and activation of

canonical Wnt pathway providing the second hit required for malignant transformation (Pahnke, Mix et al. 2004; Uren, Fallen et al. 2005; Perez-Plasencia, Vazquez-Ortiz et al. 2007). In this study DKK3 was methylated in the three HPV positive cervical cell lines HeLa, CaSki and SiHa and hemi-methylated in C33a and was up-regulated post-demethylation of HeLa and two other HPV 18 positive cell lines. These findings are in accordance with a paper just published showing methylation of the DKK3 promoter in cervical cancer cell lines (Lee, Jo et al. 2009).

KLF4 is a zinc-finger protein and an inhibitor of the cell cycle, blocking G1/S progression and suppressing cell proliferation through p21 (Shields, Christy et al. 1996; Chen, Johns et al. 2001), and is down-regulated as a consequence of hypermethylation of its promoter and LOH (Wei, Gong et al. 2005). KLF4 has not been shown to be associated with cervical cancer or HPV infection. KLF4 was expressed at a lower level in SiHa and C33a compared with NHFK, HeLa and CaSki, which may be explained by the fact that it can act both as a tumour suppressor and oncogene through its regulation of p53 and p21 expression, both of which have been implicated in cervical cancer (Rowland, Bernards et al. 2005). I found no evidence for the up-regulation post-demethylation of HeLa, and KLF4 was unmethylated in all the cervical cell lines examined. The results obtained by RT-PCR and MSP contradict those obtained by the original gene expression microarray, which may be because the demethylation of candidate genes also included the use of trichostatin A, which is an acetylating agent. Acetylation has been shown to be important for KLF4-mediated transactivation, with KLF4 differentially modulating histone H4 acetylation at the promoters of target genes. Evans *et al.* suggest that depending on whether KLF4 interacts with co-activators (e.g. p300 and CREB-

binding protein) or co-repressors (e.g. HDAC3) it is able to function both as a transcriptional activator and a repressor (Evans, Zhang et al. 2007).

RNASET2, a T2-Rnase glycoprotein, is able to bind actin *in vitro* (Smirnoff, Roiz et al. 2006). Deletion at this locus has been demonstrated in a number of malignancies, including ovarian (Cooke, Shelling et al. 1996; Lin and Morin 2001), breast (Devilee, van den Broek et al. 1991; Devilee, van Vliet et al. 1991), stomach (Queimado, Seruca et al. 1995), uterine (Chappell, Lydon et al. 1997), kidney (Morita, Ishikawa et al. 1991), colon (Honchel, McDonnell et al. 1996) and liver (De Souza et al., 1995). RNASET2 is expressed at comparable levels in NHFK and in the HPV 16 positive cell lines CaSki and SiHa, but expression is not detectable in the HPV 18 cell line HeLa, and very high levels of expression were detected in the HPV negative cell line C33a. RNASET2 has not been associated with cervical cancer carcinogenesis, however it has been shown to be a tumour suppressor in ovarian cancers, which was not regulated by DNA methylation (Acquati, Morelli et al. 2001). This is in agreement with my data showing that RNASET2 is unmethylated in cervical cancer cell lines; however it is contradicted by its up-regulation post-demethylation of HeLa in which it was also not expressed.

TIMP1 is an inhibitor of matrix metalloproteinases (MMPs) that has variable X-chromosome inactivation in females, which has been suggested to be regulated by acetylation of histone H3 (Anderson et al., 2005). TIMP1 was equally expressed in all of the four cervical cell lines compared with NHFK, which is contradictory to preliminary data from one group has suggested that TIMP1 is up-regulated in cervical cancer (Vazquez-Ortiz, Pina-Sanchez et al.

2005). However, other matrix metalloproteinases, especially MMP2 and MMP9 have been associated with cervical cancer (Davidson, Goldberg et al. 1999; Mitra, Chakrabarti et al. 2003), with MMP2 increased in CIN and invasive disease but TIMP2 down-regulated in cervical cancer (Branca, Ciotti et al. 2006). Although TIMP1 was methylated in all 3 cervical cell lines its regulation is thought to be through histone acetylation and not methylation. This would be in line with its up-regulation post-demethylation of HeLa and two other HPV18 positive cell lines, as these were also treated with the acetylating agent trichostatin A. Nonetheless, methylation of the TIMP1 promoter has been correlated with X chromosme instability and low expression, with TIMP1 methylation having been demonstrated in bladder cancer (Anderson and Brown 2002; Veerla, Panagopoulos et al. 2008).

AKAP12, an anchoring protein controlling cell signalling, adhesion and differentiation (Gelman, Lee et al. 1998; Lin, Nelson et al. 2000), has not previously been reported to be methylated in cervical tissue but methylated forms have been reported in colorectal and gastric cancers (Kwak, Ma et al. 2004; Lau, Sham et al. 2006; Mori, Cai et al. 2006). AKAP12 is expressed weakly but at a comparable level to that of NHFK in HeLa and SiHa, highly expressed in C33a and expression was not detectable in CaSki. This variation in expression is unexpected because although AKAP12 it has not been correlated with cervical cancer it has been shown plays an important role in differentiation and metastasis at other sites. AKAP12 was unmethylated in HeLa and C33a, and hemi-methylated in CaSki, which correlates with their baseline expression. Although AKAP12 was found to be unmethylated by MSP the up-regulation post-demethylation of HeLa and HPV18 positive foreskin keratinocytes is supported by studies demonstrating AKAP12 to be an epigenetically regulated tumour suppressor gene at sites such as lung cancer (Jo, Whang et al. 2006).

CADM1 is a cell adhesion molecule involved in cell-cell interactions (Gomyo, Arai et al. 1999). It has been shown to be inactivated by promoter methylation and LOH in non-small cell lung cancer and other sites (Murakami 2005; Murakami 2005). CADM1 expression in C33a is comparable to that in NHFK. It is not expressed in the three HPV positive cervical cancer cell lines HeLa, CaSki and SiHa, which is consistent with the data suggesting that it is epigenetically silenced in HPV positive cervical cell lines (Steenbergen, Kramer et al. 2004; Lee, Jo et al. 2009). It is up-regulated post-demethylation of HeLa and two other HPV 18 positive cell lines, with methylation all cervical cell lines. All these data are in agreement with that of other groups showing that CADM1 gene silencing via promoter hypermethylation is a frequent event in the progression from high-risk HPV-containing, high-grade CIN lesions to invasive cervical cancer (Steenbergen, Kramer et al. 2004; Overmeer, Henken et al. 2008).

CLU is a stress inducible glycoprotein regulating of apoptosis (Pajak and Orzechowski 2006) that is hypermethylated in prostate cancer (Rauhala, Porkka et al. 2008). CLU is expressed at a higher level in all of the cervical cell lines compared with NHFK, especially in C33a, which is suggested of an oncogene, not a tumour suppressor gene. These results are in agreement with data published since the undertaking of this thesis demonstrating CLU over-expression in invasive disease compared with normal cervical epithelium (Park, Yeo et al. 2006). However, another recent study has reported its down-regulation (Choi, Kim et al. 2007) and a further study also shows that there is no change in CLU expression in squamous and adenocarcinomas relative to disease free controls (Abdul-Rahman, Lim et al. 2007). The detection of methylation in all cervical cell lines and up-regulation post demethylation of

HeLa and HPV18 positive foreskin keratinocytes does not correlate with baseline expression, but is not surprising given the vast literature on the role of CLU in the pathogenesis of prostate cancer, illustrating it is an epigenetically silenced tumour suppressor gene (Rauhala, Porkka et al. 2008).

Although we might have expected consistent expression levels across cell lines for strong candidate genes, these results show a varying pattern of expression between cell lines for most genes at baseline, notably with the exception of CLU, which was expressed consistently strongly in all cancer cell lines and only weakly in normal human foreskin keratinocytes, and TIMP1, which was expressed strongly in all cell lines. The fact that the baseline expression of CLU is identical in all cervical cell lines and correlates with a recently published study would suggest this to be a strong candidate for further investigation, even though it is suggestive of an oncogenic function and not a tumour suppressor gene. Although this study set out to identify an epigenetically regulated tumour suppressor gene, it was felt that it was just as worthy to investigate a potential oncogenic role in cervical neoplasia. However, it is important to recognise that the mixed pattern of expression seen in these experiments may have been due to several variables. In particular, these various cell lines had different HPV statuses, which may well have potentially influenced their methylation status. Equally, when cell lines were demethylated they were also treated with the acetylating agent trichostatin A and so any expression changes seen may not solely be due methylation (see section 6.16.4).

With the exception of KLF4, validation of candidate genes confirmed their up-regulation post demethylation of HeLa, although with differing degrees of intensity. KLF4 and RNASET2

were not methylated in the cervical cancer cell lines and so this stage not viable candidates. AKAP12 showed hemi methylation and DKK3, TIMP1, CLU and CADM1 showed high levels of methylation by MSP and warrant further investigation as epigenetically regulated candidate genes.

Methylation Specific PCR was used to determine the methylation status of candidate genes in cervical cancer cell lines. This is a very sensitive technique, showing the methylation status of CpG dinucleodies in the candidate gene promoter within designed PCR primers. However, the technique is thought to be biased towards the detection of even low levels of methylation, which could potential give false positive results. Despite this, the approach taken is a means of initially determining gene methylation status in a series of candidate genes rapidly before further validation using other techniques such as bisulphite genomic sequencing.

6.2. Further validation of candidate genes in disease progression models

Having confirmed the baseline expression and methylation status of candidate genes in cervical cancer cell lines the W12 longitudinal model of disease progression was used to further examine changes in candidate gene expression. As in the cervical cancer cell lines examined methylated forms for KLF4 and RNASET2 cannot be detected in the W12; thus strengthening the conclusion that these are unlikely to be epigenetically regulated and hence not suitable candidate genes for further validation. AKAP12 showed variable methylation is cervical cancer cell lines, but since it was hemi-methylated in HPV16 positive CaSki the absence of any methylation in pre and post integration passages of W12 was unexpected. I might have expected to see methylation of AKAP12 in late but not early passages of W12,

with methylation being associated with viral integration since when it is methylated in CaSki there are more than 600 integrated HPV-16 copies present; however this was not the case. The candidate genes that were methylated in the cervical cancer cell lines, DKK3, CADM1, TIMP1 and CLU, were also methylated in W12. Furthermore, these genes showed an increase in methylation with serial passage of the cell line as it becomes increasingly dysplastic. These genes at this stage show great potential as epigenetically regulated genes in cervical neoplasia, strengthened by their increase in methylation in W12. However, no accompanying down-regulation of candidate genes at the RNA level was observed with serial passage and increasing methylation status. It is possible that this observed methylation may precede transcriptional changes which will only become apparent in passages of the W12 beyond those tested here.

6.3 Re-evaluation of potential candidate genes

At this point in the investigation, since KLF4, RNASET2 and AKAP12 were all unmethylated and showed no change in expression in cervical cell lines they did not warrant further validation. DKK3, CADM1, TIMP1 and CLU showed notable promise as potential epigenetically regulated genes in cervical cancer given that they were methylated in cervical cell lines and increasingly in the disease progression model. However, on reflection, looking at their basal levels of expression in cervical cancer cell lines, CLU and TIMP1 were not down-regulated compared with normal keratinocytes, whereas DKK3 and CADM1 were, suggesting that the methylation observed in CLU and TIMP1 might not be detrimental to their transcriptional control in cervical neoplasia.

6.4. Investigation of candidate genes in cervical cancer, CIN and normal tissue

In order to determine whether the candidate gene expression seen *in vitro* recapitulates the situation in patients, the expression of candidate genes that were found to be methylated in cervical cell lines was next examined in patient samples. Normal cervix was compared with CIN and invasive disease to identify whether epigenetic regulation of candidate genes was an early event in pre-invasive disease that could potentially be used as a diagnostic indicator for treatment before the disease becomes invasive, making it harder to eradicate. This provides a more realistic insight as it is known that cell lines undergo *de novo* methylation during *in vitro* cell culture and acquire methylation marks not associated with primary tumours (Jones and Laird 1999; Pantoja, de Los Rios et al. 2005). This is important given that two of the candidates, CLU and TIMP1 showed no correlation between methylation and transcription silencing in cell lines but DKK3 and CADM1 did.

These studies revealed that TIMP1 was also expressed at the RNA level in CIN as it was in cell lines, whereas CLU which as expressed at the RNA level in cell lines was not detected in patients with CIN. This is in agreement with the recently published study by Choi *et al.* demonstrating down-regulation of CLU in RNA extracted from cervical cancer patients compared with that extracted from normal cervical epithelium, thus CLU may still have potential as a candidate gene (Choi, Kim et al. 2007). The remaining genes showed very little or no expression in patient samples, adding to their worth as potential candidate genes.

At the time of this study, commercial antibodies were available to DKK3, TIMP1, AKAP12 and CLU and so paraffin fixed sections of normal cervix, CIN I to III and invasive disease

were stained for protein expression. DKK3 expression was not interpretable due to poor quality antibodies and the one published study of DKK3 expression in cervix only looked at RNA expression not protein expression. TIMP1 and AKAP12 showed no difference in expression between normal cervix and CIN, thus are not suitable candidates for epigenetically regulated genes in cervical cancer. It was surprising that the down-regulation of candidate genes at the RNA level did not translate to change at the protein level between normal cervix and CIN or invasive disease. CLU on the other hand was up-regulated in CIN compared to normal cervical epithelium and then down-regulated in invasive disease compared with CIN. This shift in protein expression provides a possible explanation for the contradictory studies regarding CLU expression in cervical neoplasia. The study by Park *et al.* showed up-regulation of CLU in cervical cancers compared with normal cervical epithelium by immunohistochemical staining, which is what was found in the invasive samples examined in this study (Park, Yeo et al. 2006).

CLU is down-regulated during the onset of cervical abnormality, possibly induced by HPV infection, and this lack of CLU then increases the susceptibility to tumourigenesis after carcinogenic challenge. Hence, CLU can be considered to be a tumour attenuator acting predominantly at early stages of neoplastic growth. It is probable that this down-regulation observed is of the nuclear isoform of the protein, as some studies discussed have indeed suggested. What is not known in these studies is whether the intracellular localization of the protein is cytoplasmic CLU or a pre-cursor for the nuclear isoform, not yet translocated to the nucleus. It would have been informative at this stage to have stained cervical sections for markers of proliferation and apoptosis given the opposing role of CLU in regulating apoptosis depending upon subcellular localisation.

6.5. Further validation of CLU as a target gene in cervical neoplasia

Having identified CLU as potential tumour suppressor gene, it was necessary to confirm the results of previous RT-PCR using quantitative RT-PCR, to confirm the changes in protein expression in a wider cohort of cervical samples and with different CLU antibodies. Validation of expression using Q RT-PCR highlighted the importance of using a consistent housekeeping gene across all samples. Having validated a number of housekeeping genes and selected B2M because it showed least variability between samples, quantitative analysis of demethylation experiments confirmed those obtained using end point PCR. Had I used a different housekeeping gene for validation of expression changes by QPCR a completely different pattern of expression would have emerged. CLU transcriptional changes of were validated in serial passage of the W12 cell line showing CLU not to be down-regulated during serial passage of W12 cells, and so is not associated with the observed methylation.

Having demonstrated that CLU was up-regulated in CIN compared with normal cervix using a commercially available antibody from Vector, the same cohort was examined with two further CLU antibodies and confirmed the increased staining for CLU in high grade CIN compared with normal cervical epithelium and its down-regulation in invasive cancer. In order to validate the specificity of the CLU antibodies, the expression of CLU at the protein level in cell lines was investigated using western blotting. This confirmed the expression of CLU proteins confirmed with a transfection control and showed increased expression in HeLa, CaSki and SiHa when compared with C33a and normal human foreskin keratinocytes, as was seen at the RNA level. Western blotting of W12 passage 15 and 56 cell lysates showed no change in CLU expression between the passages and confirmed results obtained by

immunohistochemistry of cell lines. Data obtained by western blotting is further strengthened by Park *et al.* demonstrating up-regulation of CLU in cervical cancer cell lines (Park, Yeo et al. 2006).

The starting point of pursuing CLU as a candidate tumour suppressor gene in the pathogenesis of cervical neoplasia was the finding that CLU was up-regulated in the initial demethylation experiment performed in my laboratory. CLU was found to be methylated by MSP in cervical cancer cell lines and this was confirmed by pyrosequencing, with 50% of the cervical cell lines showing high levels of methylation of the CLU promoter. However, although 20 fold up-regulation of CLU was seen post-treatment of the HeLa cell line with 5-Aza-2'-deoxycitidine and TSA, the cell line showed low levels of methylation between 5 and 15% by BGS and pyrosequencing. Additionally, when pyrosequencing was carried out on the demethylated HeLa, no significant change in methylation status was detected; this would suggest that the substantial up-regulation post-treatment of HeLa is more likely to be due to acetylation by the Trichostatin A. In the W12 cell line model, there was variation in the methylation status of CLU with a distinct up-regulation of CLU between passages 9 and 11 proceeded by a significant decrease in methylation in passage 12; these changes in the methylation status of the CLU promoter may be related to the loss of episomes and viral integration.

In DNA extracted from cervical smears from women with CIN the majority showing an increase in methylation status correlating with disease progression from CIN I to CIN II and CIN III. All of the methylation frequencies seen in cervical smears were much lower than

those in the cervical cell lines, with the exception of the HeLa cell line, suggesting that cell lines do not accurately represent the gene methylation frequencies observed *in vivo*. Results are consistent with previous evidence suggesting that cell lines can acquire specific DNA methylation defects as a result of immortalisation and continuous culture that are unrelated to the tumour origin (Jones et al, 1990; Pantoja et al, 2005). Cell lines contain *de novo* methylation events not present in primary tumours and have higher methylation frequency for specific genes than primary tumours, but are still useful indicators of gene methylation status. However, the methylation status of the CLU promoter was only examined in CIN and not in invasive disease, and had we examined invasive tumour samples the methylation frequencies could have been much higher, especially given the expression of CLU was very low or lost in squamous and adeno-carcinoma samples examined. Alternatively, it is possible that high levels of methylation could be a feature of a small subset of cervical tumours.

6.6. Investigation of the expression of clusterin in oral carcinoma

In cervix, there was a down-regulation of CLU in pre-invasive disease and an up-regulation in invasive disease compared with normal cervical tissue. The potential for a role for CLU in oral cancer was identified in a microarray showing down-regulation of CLU in oral cancers and compared with cancer-free controls, which was subsequently confirmed by Q RT-PCR. The down-regulation of CLU in oral cancers is consistent with what is observed in CIN, but not invasive cervical cancer. There are no studies published of human CLU expression in oral carcinoma, however one recent study used serum proteomics in mice bearing orthotopic human oral squamous cell carcinomas, which identified a disparity in expression of mouse CLU between control and cancer-bearing mice (Bijian, Mlynarek et al. 2009).

To validate and further investigate these results, I used Q RT-PCR and western blotting in oral cancer cell lines. However, my results, which showed a high level of CLU expression at both the RNA and protein levels, were contrary to expression levels in seen in oral cancers by microarray and Q RT-PCR in the original experiments. This suggests that the NHFK control available at the time of this study may not have been a representative control for these samples or that these cell lines are not representative of tumour samples from patients. Immunohistochemical staining of cytospins prepared from the same cell lines were consistent with those seen by western blot and Q RT-PCR, with strong 3+ staining in the oral cancer cell line SCC4.

Contrary to these findings and in agreement with microarray results, immunohistochemical staining of oral cancers showed a down-regulation or loss of CLU in oral cancer. Where CLU expression was detected, it was the necrotic cells that stained positive for CLU, which is not surprising given the role of CLU in the regulation of apoptosis.

As was observed in cervical cancer cell lines, different housekeeping genes used for normalisation of Q RT-PCR data resulted in different expression patterns and so selecting the correct housekeeping gene is essential. Protein expression data is more reliable as it is not subject to the same sensitivity issues when comparing gene expression to housekeeping gene expression. This is of course dependent upon the reliability of CLU antibodies, the specificity of which was confirmed following transfection of CLU proteins into 293 cells, which showed

the 60kDa and 40kDa CLU proteins that are well document (Park, Yeo et al. 2006; Chayka, Corvetta et al. 2009).

6.7. Investigation of the epigenetic regulation of expression of clusterin in oral carcinoma

SNP analysis revealed a possible deletion encompassing the CLU locus on chromosome 8p21 which may play a role in the down-regulation of CLU in oral cancer. As epigenetic regulation, specifically DNA methylation, was demonstrated to play a role in cervical cancer carcinogenesis, I evaluated whether this phenomenon governed CLU expression at other sites of cancer, in this case oral carcinoma. All oral cancer cell lines were methylated by MSP, which was confirmed by pyrosequencing. Interestingly, SCC4 which appeared to have the highest level of CLU expression at the RNA and protein level of all the cell lines also had the lowest level of methylation of the 7 cell lines, suggesting that methylation is regulating gene expression in these cell lines to some extent. Although not shown for CLU, methylation has been shown to regulate expression of the cell lines used in this study in a variety of gene promoters, such as E-cadherin (Nakayama, Sasaki et al. 2001), with up-regulation of the p16ink4a gene following demethylation (Timmermann, Hinds et al. 1998; Lee, Kim et al. 2004).

MSP showed hemi-methylation of the CLU promoter in oral cancers that was comparable to that of methylation in disease-free oral epithelium. The presence of both unmethylated and methylated products, hemi-methylation, could be due to a mixed cell population with some

cells being methylated while others are not, or mixture of methylated and unmethylated alleles within these tumours reflecting that the population as a whole is heterogeneous. It is also possible that in the tumour samples that adjacent normal cells may be contributing to the level of methylation detected and micro-dissected tumours would have provided a more accurate comparison. Pyrosequencing identified low levels of methylation in a different cohort of oral cancers. Methylation frequencies were much lower in both cohorts of oral cancers using MSP and pyrosequencing than in oral cancer cell lines, suggesting that methylation is unlikely to be the sole regulator of reduced CLU expression in oral cancer. Problems inherent with studying methylation in cell lines are discussed in methodological considerations (section 6.16.4.1).

6.8. Investigation of the expression of clusterin in nasopharyngeal carcinoma

Contemporaneous in-house microarray experiments showed that CLU was down-regulated in NPC cell lines, and in 19 (76%) of 25 NPC biopsies compared with cancer free controls. A review of the literature also revealed down-regulation of CLU in two published arrays of primary NPC, one of which included whole tissue samples, the other, micro-dissected epithelium (Shi 2006, Sriuranpong 2004). I therefore set out to validate these results to evaluate CLU as a potential tumour suppressor gene in NPC.

In NPC cell lines, I was only able to confirm the down regulation of CLU in one of the cell lines with high level of CLU expression in others. This was not reflective of the expression I observed in NPC tumours, in which I confirmed down regulation of CLU in 10/12 tumours, confirming initial microarray and Q RT-PCR down-regulation. Q RT-PCR normalised against

B2M, showed CLU to be down regulated in only one cell line (C666-1), and up regulated in four others (CNE 1, SUNE 1, NPC TWO 1 and HONE 1). Western blotting and immunohistochemical staining of cytospins confirmed that C666-1 does not express any of the CLU proteins. The 60kDa and 40kDa CLU proteins are present in other all cell lines. The difference in expression of CLU between C666-1 and Ad-AH could be because Ad-AH is an adenocarcinoma cell line not squamous carcinoma.

C666-1 was the only cell line in which the expression of CLU was representative of NPC tumours, which can be explained by the fact that it is the only NPC cell line consistently carrying the Epstein-Barr virus **(**EBV**)** in long-term culture (Cheung, Huang et al. 1999). A number of other tumour suppressor genes have been demonstrated to be only down regulated in this cell line model, but not in EBV negative cell lines, such as ATM and RASSF1A (Chow, Lo et al. 2004; Bose, Yap et al. 2009). However, in these studies *in vitro* infection with a recombinant EBV resulted in tumour suppressor gene down-regulation, whereas the opposite was found with regards to CLU expression.

6.9. Investigation of the regulation of expression of clusterin in nasopharyngeal carcinoma by EBV and its latent genes

As the C666-1 model is the only EBV positive cell line, and because in cervical cancer HPV possibly may play a role in clusterin expression, I decided to further investigate clusterin expression in a cell line model following infection with a recombinant EBV.

CLU RNA was significantly up-regulated following infection with EBV and ΔLMP2A EBV when compared with the parental Ad-AH cell line. This was mirrored at the protein level by both immunohistochemical staining and western blotting where compared with the parental cell line, the 60kDa and 40kDa CLU proteins are over-expressed following infection with EBV and ΔLMP2A EBV. Expression of the latent genes EBNA1, LMP2A, LMP2B and LMP1 in Ad-AH did not show any change in the expression of CLU at the RNA level compared with the parental cell line. This was surprising as I would have expected a down-regulation following transfection with LMP2A and an up-regulation following transfection with LMP1, given that LMP1 has been known to have oncogenic properties during latent infection in NPC.

LMP1 functions as a constitutively active tumour necrosis factor receptor activating signalling pathways such as NF-kappaB, MAPK, JNKand the JAK/STAT pathway (Tsao, Tramoutanis et al. 2002). It is the CTAR region of LMP1 that is able to directly activate multiple signalling pathways such as NF-kB, with the goal of preventing apoptosis. It has been shown that inhibition of LMP1 can increase the sensitivity of tumour cells to chemo and radiotherapy (Mei, Zhou et al. 2007). Studies have suggested that LMP1 acts in early stages of carcinogenesis to down-regulated tumour suppressor genes such as INK4a, induce anti-apoptotic genes such as bcl-2 and promote more aggressive disease progression (Murono, Yoshizaki et al. 1999; Macdiarmid, Stevenson et al. 2003). LMP2A increases the invasiveness of NPC cells and has been shown to down-regulate both LMP1 and NF-kB (Pegtel, Subramanian et al. 2005).

Although RNA expression of CLU was not altered following transfection of Ad-AH with latent genes, it was found to be up-regulated following transfection with LMP1 at the protein level. Equally the 60kDa CLU protein was down-regulated following transfection of Ad-AH cells with LMP2A and LMP2B, which was not seen at the RNA level. Interesting the down-regulation of the 60kDa CLU protein was accompanied by an induction of the 45kDa CLU protein that was not expressed in the parental cell line; this corresponds to the molecular weight of nuclear CLU. This correlates with the role of LMP2 in the pathogenesis of NPC where a recent study showed LMP2 down-regulated in the majority of NPC with a concurrent induction of apoptosis mediators FasL, Fas and IL-10 (Ogino, Moriai et al. 2007). Animal studies have shown that down-regulation of LMP2 prevents the proteolytic processing required for NF-kB activation and prevents it from increases the susceptibility to apoptosis induced by TNF-α (Hayashi and Faustman 2000). In this scenario it is possible that the reduction in active NF-kB induces expression of nuclear CLU to induce apoptosis.

6.10. Investigation of the epigenetic regulation of expression of clusterin in nasopharyngeal carcinoma

I also wished to confirm whether there were any epigenetic mechanisms of clusterin regulation in NPC, as there had been some evidence for this mechanism in oral cancer. Although infection of the Ad-AH cell line with EBV appeared to up-regulate CLU, this did not correlate with methylation of the CLU promoter as there was also an increase in promoter methylation. Pyrosequencing showed that there actually was an increased level in methylation following infection of the parental line with EBV and its latent genes, suggesting methylation of CLU promoter is not regulating the expression of CLU in these transfected Ad-AH cells.

Pyrosequencing and bisulphite genomic sequencing of NPC cell lines showed only the EBV positive C666-1 to have a very high level of methylation of 84%. All other NPC cell lines still showed methylation that was greater than that observed in normal keratinocytes, ~20% compared with 10%, however this was not as high as that of C666-1.

A study by Bakos *et al.* in C666-1 investigated DNA methylation and *in vivo* protein-DNA interactions at the latency promoters Qp and Cp. This revealed that the active, unmethylated Qp was marked with strong marks of cellular transcription factors and the viral protein EBNA 1 which were not detectable in the methylated and silenced Cp promoter. The authors concluded that the epigenetic marks at Qp and Cp in C666-1 cells of epithelial origin resemble those of group I Burkitt's lymphoma cell lines not nasopharyngeal cell lines (Bakos, Banati et al. 2007).

Pyrosequencing of NPC tumours show a mean methylation of 23%, with a range from 10 to 49%, and 3 out of 10 tumours having high levels of methylation; however, this did not correlate with CLU expression in all cases. Methylation frequencies were much higher in NPC cell lines than in NPC tumours, which is discussed in methodological considerations (section 6.16.4.1).

A SNP analysis showed a deletion at the CLU locus on 8p21 in 2 of 14 nasopharyngeal cancers from which epithelium had been removed by micro-dissection and in C666-1 there was a possible deletion encompassing the CLU locus (8p21), which was subsequently

confirmed using genomic PCR, a 7.6mb deletion with loss of one CLU allele in C666-1. This allelic loss was accompanied by methylation of the remaining CLU allele in C666-1 as determined by MSP, bisulphite genomic sequencing and pyrosequencing. Following demethylation of C666-1 RT-PCR showed transcriptional up-regulation of CLU, with a reduction in methylation from 77% to 51% as determined by pyrosequencing. Silencing of tumour suppressor genes, such as TIG1 by promoter hypermethylation has previously been observed in the NPC cell line C666-1 (Kwong, Lo et al. 2005). However, LOH of one allele with epigenetic silencing of the remaining allele has not been demonstrated before in NPC cell lines, nor has it been demonstrated for CLU at any other sites.

6.11. Phenotypic consequences of expression of clusterin in a nasopharyngeal carcinoma cell line model

I next wished to examine the phenotypic effects of re-introducing clusterin into the C666-1 cell line which did not express it. This is because the lack of expression of clusterin in this cell line recapitulates what we see *in vivo* in NPC tumours, which also makes it a particularly useful model in which to study the phenotypic effects of each individual CLU isoform, given the baseline absence of expression of both.

HEK 293 were used to optimise transfection experiments as these cells are easy to transfect and so ideal to ensure CLU plasmids express the correct CLU proteins. CLU expression was seen at 12, 24 and 48 hours following transfection with full length and nuclear CLU. Transfection with full length CLU produced the characteristic 60kDa and 40kDa cytoplasmic

CLU proteins, and after 25 hours the 45kDa protein. The 45kDa protein but not the 60kDa protein is detectable 12 hours following transfection with nCLU. This is consistent with the possibility that the full length plasmid is also able to generate nuclear CLU by translocating to the nucleus and hence it not being detectable until 24 hours post transfection. It was important to carry out a number of timepoints to avoid missing the optimum or earliest time to see differences in transcript expression. Expression of the 60kDa CLU protein observed using full length CLU plasmids was equivalent to that reported by other groups at other sites (Bettuzzi, Scorcioni et al. 2002; Scaltriti, Bettuzzi et al. 2004; Moretti, Marelli et al. 2007).

GFP transfection showed a transfection efficiency of 70% in C666-1 cells, and RNA expression post transfection with full length and nCLU showed expression of two transcripts from 6 hours post transfection. Western blotting of whole cell lystates showed the predicted 60kDa and 40kDa cytoplasmic proteins post transfection with full length CLU, but not with nCLU. In contrast to HEK 293 a 45kDa protein is detectable following transfection with nCLU but not full length CLU, which is in agreement with transfection studies in prostate cancer cell lines (Bettuzzi, Scorcioni et al. 2002; Scaltriti, Bettuzzi et al. 2004; Moretti, Marelli et al. 2007). Western blotting of nuclear and cytosolic extracts confirmed that this 45kDa CLU protein was nuclear in localisation. It was essential to confirm this localisation given that CLU functions as a pro-apoptotic protein when in the nucleus rather than an anti-apoptotic protein when it resides in the cytoplasm (Caccamo, Scaltriti et al. 2003; Leskov, Klokov et al. 2003).

As I would have expected, decreased viability and proliferation was observed following transient transfection of C666-1 with nCLU, with an increase in the number of proliferating cells following transfection with full length CLU. These results are in agreement with published data regarding transient transfection of CLU (Scaltriti, Santamaria et al. 2004; Caccamo, Scaltriti et al. 2005). There was an increase in the number of early apoptotic cells and late apoptotic/necrotic cells following transfection with both nCLU and full length CLU, an effect which is ameliorated following TGFβ stimulation. TGFβ stimulates nuclear translocation of CLU (Reddy, Jin et al. 1996) so I would have expected this to increase the number of apoptotic cells. This result was unexpected – I would have expected to see an increase in the number of apoptotic cells following stimulation of nCLU transfected C666-1 with TGFβ. This is possibly because apoptosis assays were carried out 48 hours post transfection when the effect of nCLU is masked by that of sCLU and so an earlier time point would have been more appropriate to study the levels of TGFβ induced apoptosis by nCLU.

6.12. Regulation of NF-kB activity by CLU

NF-kB is a transcription factor constitutively expressed in many cancer cell types that plays a pivotal role in numerous cellular processes including cell survival, stress response, immunity, cell motility, proliferation, and transformation (Cortes Sempere, Rodriguez Fanjul et al. 2008), with the up-regulation of NF-kB signalling a common feature of malignant disease. Santilli *et al.* (2003) proposed an explanation for the pro-apoptotic activity of CLU through an involvement in the regulation of NF-kB activity, demonstrating that ectopic expression of CLU strongly inhibited NF-kB activity in human neuroblastoma cells and murine embryonic fibroblasts by stabilising inhibitors of NF-kB (IkBs).

Luciferase reporter assays following co-transfection of CLU with a NF-kB reporter construct were optimised in HEK 293 prior to carrying out in C666-1 cells, due to their ease of manipulation in cell culture. This showed down regulation of NF-kB post transfection of nCLU and an increase in NF-kB activity post transfection of full length CLU. In C666-1 both nCLU and full length CLU down-regulated NF-kB activity in these cells from 12 hours post transfection. Luciferase reporter assay for measuring NF-kB activity are notorious for showing variability between experiments and so these experiments were repeated 6 separate occasions with replicates on each occasion for each transfection to confirm that these data were statistically significant. Western blotting for p50 and p65 was carried out to confirm the results obtained by luciferase reporter assays and to determine whether suppression of NF-kB by CLU though stabilisation of IkBα would inhibit p50 and p65 nuclear translocation. Western blotting of nuclear and cytosolic extracts showed an increase in cytoplasmic p50 and p65 post transfection with nCLU and a concurrent decrease in their nuclear localisation. Transfection with full length CLU showed little, if any, change in p50 and p65 localisation. A more reliable assay for measuring NF-kB activity would have been to use the commercially available TransAM assay p50 and p65 ELISA kits, which were not used due to funding restraints of the project.

The activity of NF-kB is suppressed through interaction with the inhibitory molecule IkB which blocks the translocation of the to p55/p60 heterodimer to the nucleus and its transcriptional activity. Phosphorylation of IkB by IkB kinases (IKKs) marks the latter for degradation, which results in the release and translocation of the active NF-kB heterodimer (Pajak et al, 2006). CLU has been shown to induce IkBα stabilisation by inhibiting E3 ubiquitin ligase binding to phosphorylated IkBα, resulting in decreased NF-kB activity.

Following transfection of CLU into HEK 293 and C666-1 cells there was an increase in the expression of IkBα, supporting the published data in other cell lines as to the mechanism for the down regulation of NF-kB activity by CLU in these cell lines (Santilli, Aronow et al. 2003; Pajak and Orzechowski 2006). Devauchelle *et al.* showed that CLU induces IKBα stabilisation by inhibiting E3 ubiquitin ligase binding to phosphorylated IkBα (Devauchelle, Essabbani et al. 2006). Loss of CLU expression in cells that depend on NF-kB activity for chemoresistance, proliferation, or invasion could lead to tumour progression. Data presented in this thesis suggests that CLU is a tumour suppressor gene required to regulation of tumourigenic signals emanating from the NF-kB pathway.

Proof of principal experiments carried out in HEK 293 cells and HeLa cells showed that knockdown of CLU using siRNA resulted in increased NF-kB activity. These results are in agreement with a study in rheumatoid arthritis showing that CLU knock-down by siRNA promoted IL6 and IL8 production, which are transcriptionally regulated by NF-kB (Devauchelle, Essabbani et al. 2006). Santilli *et al.* showed that in the absence of CLU IkB stability is reduced leading to a TNF dependent increase in NF-kB activity and increased transcription of NF-kB target gene cIAP and down modulation of p53 (Santilli, Aronow et al. 2003). However, the effects of CLU knockdown on NF-κB activity in HeLa cells were not affected by TNFα stimulation. Recent publications have shown that siRNA knockdown may invoke an interferon response, especially if duplexes are longer than 23bp and cell viability is affected (Reynolds, Anderson et al. 2006). Q RT-PCR for interferon response genes ISG 20 and OAS post siRNA knockdowns of CLU ruled out the possibility of induction of an interferon response.

6.13. Novel findings with regard to the pattern of expression of CLU in cervical, oral and nasopharyngeal cancer compared with normal epithelium

I have demonstrated the down-regulation of CLU in biopsies taken from patients with oral and nasopharyngeal cancers, which has previously not been published. I was also able to show down regulation of CLU in cervical cancers, consistent one other study (Choi, Kim et al. 2007). However, over-expression of CLU has also been noted in two other studies of patients with cervical cancer (Park, Yeo et al. 2006); (Watari, Ohta et al. 2008). The reason for this discrepancy is not clear, but interestingly I found that CLU was up-regulated in CIN. An in-house SNP analysis revealed a possible deletion encompassing the CLU locus on chromosome 8p21 which may play a role in the down-regulation of CLU in oral cancer. It has previously been shown that LOH contributes to the pathogenesis of oral cancer and studies have shown this to include 8p and 11q22.2~q22.3 as frequent sites of loss of heterozygosity in head and neck SCC (Niu, Xin et al. 2005). This suggests that LOH might be a phenomenon affecting a number of tumour suppressor genes in this region and is not restricted to CLU, although in our analysis LOH of other TSGs at this locus were not identified.

6.14. Novel findings with regard to the contribution of epigenetic regulation to the transcriptional control of CLU

I have shown for the first time, using a variety of techniques, the presence of methylated form of CLU in cell lines and in whole tissue samples taken from patients with oral, nasopharyngeal and cervical cancer. In the NPC cell line C666-1 I was able to shown that loss of one CLU allele was accompanied by methylation of the remaining allele. I was also able to

show re-expression of CLU following treatment with the pharmacological demethylating agent, 5-Aza-2'-deoxycitidine. Although CLU was shown to be methylated in a number of cervical cancer cell lines, and its expression to be up-regulated following treatment of these lines with 5-Aza-2'-deoxycitidine, I could find no compelling evidence to suggest that the presence of methylated forms of CLU in cervical material predicted the risk of progression to high grade CIN in a cohort of untreated women referred for investigation of minor cytological abnormality. Of course, it is possible that high levels of methylation are a feature of only a small subset of cervical tumours.

6.15. Novel findings with regard to the contribution of CLU to the regulation of NF-kB activity

It has previously been postulated that CLU could be a tumour suppressor protein required to control tumourigenic signals emanating from the NF-kB pathway (Santilli, Aronow et al. 2003). In suppressing NF-kB activity through stabilisation of IkBs CLU could induce tumour cells to undergo apoptosis and become more responsive to chemo-and radiotherapy (Kumar, Shirley et al. 2004). However, these experiments were undertaken in normal and transformed cells that already expressed CLU, and moreover this was only in an inflammatory context. I was able to show for the first time, using a cell line model in which CLU was silenced that the ectopic expression of CLU downregulated NF-kB activity. Unlike the situation in HEK 293 cells, both nCLU and full length CLU reduced NF-kB activity at 12 and 24 hours post transfection of C666-1 with CLU. This reduction in NF-kB activity was accompanied by the stabilisation of IkB determined by western blotting.

The regulation of NF-kB by CLU was confirmed following the knockdown of CLU in HeLa cells. These results are in accordance with those of Savkovic *et al.* who showed that down-regulation of CLU resulted in a loss of IkB stability and a TNF dependent increase in NF-κB activity, with a consequential effect on NF-kB target genes (Savkovic, Gantzer et al. 2007).

6.16. Methodological considerations

Although my results provide some insight into the regulation of CLU activity in cervical, oral, and nasopharyngeal cancers as well being more specifically defined in the C666-1 cell line, there are a few potential drawbacks to some of the methods and models that I have used which may have impacted upon my results and therefore the strength of the conclusions I have been able to draw within the context of this study.

6.16.1. Use of 5-Aza and TSA

The starting point for my thesis was the identification of candidate tumour suppressor genes from a demethylation experiment in which the cervical cell lines, HeLa, CaSki, SiHa and C33a has been treated with the demethylating agent 5-Aza-2'-deoxycitidine. 5-Aza-2'-deoxycitidine (5-Aza) is frequently used in the laboratory and its derivatives have been tested in clinical trials. TSA is an acetylating agent which has no demethylating activity alone, although when used in combination with 5-Aza-2 it has an additive effect compared with using 5-Aza alone.

Genes up-regulated by 5-Aza also may include genes known to poses tumour-promoting properties consistent with an oncogene function, such as sCLU. This indicates that 5-Aza may activate genes that promote a malignant phenotype, which may be of relevance when considering therapeutic interventions for cancer patients based on 5-Aza reactivation of epigenetically inactivated TSGs. Furthermore, 5-Aza is cytotoxic, and members of the interferon pathway are commonly up-regulated by 5-Aza treatment of cancer and normal cells

indicating a stress response (Karpf, Lasek et al. 2004). DNA methylation may not be confined to just one gene but have a wider effect on transcriptional activity. Several other chromosomal regions also appear to contain clusters of genes upregulated by 5-Aza in close proximity to one another (Ghoshal and Bai 2007). It is possible that DNA methylation spreads over large sections and can simultaneously regulate the expression of multiple genes clustered together. When using microarray analysis to identify genes in this context it is important to remember that this technique is limited towards the detection of specific transcripts with corresponding probe sets on the array, may be biased towards detection of only abundantly expressed transcripts, and is unlikely to be capable of detecting all gene reactivation events.

6.16.2. Methylation analysis

The analysis of promoter methylation status presents a number of difficulties. Firstly, the definition of a CpG island is arbitrary and has been defined by *in silico* analysis of the known gene sequence using the CpG plot programme (www.ebi.ac.uk/emboss/cpgplot). In some cases, more than one CpG island is predicted to occur near the transcriptional start site of the gene, so the criteria used to define a CpG island were gradually increased until only one island remains and the sequence of this island was then used to design MSP primers for methylation analysis. This approach will have biased analysis towards those islands which were the most CpG rich, but these were not necessarily the ones most likely to be methylated or play a potential role in controlling transcription. It is known that more than one CpG island can be present in a gene and control the expression of alternative transcripts. For example, the RASSF1 gene contains 2 CpG islands, one at the 5' end controlling isoform A expression, the other located downstream of this island and controlling RASSGF1 isoform C expression

(Chow, Lo et al. 2004). In rats it has been show that methylation of CLU also occurs in a CpG island 14.5kb upstream from the transcription start site and is responsible for gene silencing (Rosemblit and Chen 1994; Lund, Weisshaupt et al. 2006).

There are also limitations with the detection methods used to analyse the methylation status of candidate genes. MSP was used to assess CpG island DNA methylation, and while this is a very sensitive technique, it is limited by the fact that it can only provide information based on a small number of CpG residues recognised by the methylated and unmethylatetd DNA specific PCR primers. However, methylation of specific CpG residues rather than entire CpG islands can be integral to transcriptional control. The technique of MSP is naturally biased towards the detection of methylated forms and the PCR for unmethylated forms is not as sensitive as that for the methylated, detecting methylated DNA even when only 0.1% of alleles are methylated (Herman, Graff et al. 1996; Liu and Maekawa 2003). MSP is not a quantitative method and small changes in methylation status will be easily missed, thus further analysis using a quantitative method such as bisulphite genomic sequencing is required before conclusions can be drawn. Bisulphite sequencing provides more detailed analysis of methylation across CpG islands, and can simultaneously assess the methylation of up to ~40 CpG sites. Cloning of PCR products into plasmids before bisulphite sequencing, sequencing of clones (BGS) would have given an idea of the relative proportions of methylated and unmethylated DNA present. However, the approach taken is a means of initially determining gene methylation status in a series of candidate genes.

MSP is useful as an initial screen as it is a quick method for qualitative analysis, however for a quantitative analysis of multiple adjacent CpG sites bisulphite genomic sequencing is frequently used, which is highly labour intensive. There are several other technologies available for the detection of methylation in both cell lines and tumours, including COBRA, SnuPE, SnaPmeth, MethyLight, Mass spectrometry and global methylation by MeDIP. In this study, the methylation initially detected was validated using the relatively new technology of pyrosequencing, which has many advantages over pre-existing techniques. It provides consistent, individual quantification of multiple CpG sites with a range of analyses, from single CpGs to global methylation. There are built in controls to confirm complete bisulfite modification and it is very high throughput with the potential to analyse up to 96 samples in parallel in 1 hour after their amplification by PCR (Biotage, 2007).

To address these issues, methylation of the CLU promoter was initial confirmed by MSP and then validated extensively using bisulphite genomic sequencing in order to determine not only the level of methylation but also the frequency of methylation at each individual CpG dinucleotide in the CLU promoter and adjacent CpG rich region. For high throughput analysis of the frequency of methylation of CLU in multiple cell lines and DNA from tumours I used pyrosequencing, which had the advantage of the built in controls for efficient bisulphite modification.

6.16.3. Q PCR confirmation of changes in gene expression

RT-PCR is used to study transcriptional regulation *in vitro*; gene expression in normal and tumour tissue; cDNA array validation; and pathogen detection e.g. viral/ bacterial contamination and genotyping. Previously gene expression was analysed by northern blotting which is cumbersome, slow and not very sensitive. Semi-quantitative RT-PCR is a more modern technique that is quicker, more sensitive, but not quantitative and potentially inaccurate as only the end point of the reaction. There are also multiple error points and using scanning densitometry to normalise expression on an agarose gel is notoriously inaccurate and subjective. Most studies of gene expression are now validated by Q RT-PCR that is very sensitive with the potential to detect a single copy of a gene and is high through-put (Terra, Murta et al. 2007). One of the major problems with validating gene expression by Q RT-PCR is finding a suitable housekeeping gene for normalisation of data sets (McNeill, Miller et al. 2007). It is essential to test a number of housekeeping genes to determine which one shows least variation in cell types or data sets. There is a requirement to identify housekeeping type genes that show sample independent stability and studies have found that newly identified genes were more stably expressed in individual samples with similar ranges. Thus, statistical analysis of microarray data can be used to identify new candidate housekeeping genes showing consistent expression across tissues and diseases.

I attempted to address this problem by validating 4 commonly used housekeeping genes in my dataset, but continually found differences when expression values were normalised to each of the genes. I then went on to study protein levels in order to define the gene which showed most reliable expression. My results indicated that B2M was the most appropriate gene to use

in this case. However, even with this gene results were variable, and where a one cycle difference in expression is sufficient to create a fold-change in final expression level readings this is a matter of concern. It may in future be more appropriate to identify genes expressed on microarrays which are unchanged between specific cancer cell lines and use these as internal controls in appropriate experiments.

6.16.4. Use of cell lines as models in which to identify and characterise epigenetically inactivated genes in squamous cancer

Through continuous culture, cell lines provide a resource of unlimited amounts of high quality tumour-derived DNA. However, there are notable and well-documented problems with their reliability as perfect models of the events which take (or have taken) place in primary tumour cells. In this thesis, the cervical, oral and nasopharyngeal cell lines that i used were not all reflective of the tumour environment and other studies have shown that cell lines grown in culture show changes in gene expression from the tissues they are derived from.

6.16.4.1. Cell lines for the study of hypermethylation events

Cell lines are commonly used for the initial screening of genes for evidence of methylation in cancer, and provide invaluable tools for functional assays in which to investigate the role of aberrantly methylated genes. Cell lines are known to undergo *de novo* methylation during *in vitro* cell culture and acquire methylation marks not associated with primary tumours, suggesting that their use in methylation studies should be viewed with caution (Jones and Laird 1999; Pantoja, de Los Rios et al. 2005). Most types of primary mammalian cells

proliferate for only a short number of doublings before entering into a stable proliferative arrest, so *de novo* epigenetic events in immortilised cells may occur as part of a stress response or mechanism of adaption to the culture environment.

Results obtained in this study suggest that the cell lines i used do not accurately represent the gene methylation frequencies observed *in vivo*. Results are consistent with previous evidence suggesting that cell lines can acquire specific DNA methylation defects as a result of immortalisation and continuous culture that are unrelated to the tumour origin (Jones and Buckley 1990; Jones, Wolkowicz et al. 1990; Jones, Wolkowicz et al. 1990; Pantoja, de Los Rios et al. 2005). Cell lines contain *de novo* methylation events not present in primary tumours and have higher methylation frequency for specific genes than primary tumours. However, finding an appropriate cell line model, such as the NPC cell line C666-1, is a useful indicator of gene methylation status. It must be noted that in this study the methylation status of the CLU promoter was only examined in CIN and not in invasive disease, and had we examined invasive tumour samples the methylation frequencies could have been much higher, especially given the expression of CLU was very low or lost in squamous and adeno-carcinoma samples examined. Alternatively, it is possible that high levels of methylation could be a feature of a small subset of cervical tumours.

6.16.4.2. Cell lines for the study of transcriptional silencing

Once evidence of DNA methylation has been established in primary tumours, it is next important to determine whether methylation leads to epigenetic inactivation of the

corresponding transcript(s). This is because methylation of certain CpG islands downstream from the promoter of mammalian genes which did not block transcription have been previously identified (Jones et al 1999), demonstrating that gene hypermethylation does not always lead to transcriptional inactivation. Direct evidence of methylation-dependent silencing comes from the culture of cell lines with demethylating agents such as 5-Aza-2'-deoxycitidine, which can be used to reactivate genes transcriptionally silenced by methylation in cell lines.

In the W12 cell line model of disease progression changes in methylation status of CLU and other candidate tumour suppressor genes was identified, yet there was no compelling evidence of transcriptional down-regulation on serial passage of W12. However, in all other cell line models demethylated in this study; HeLa, C666-1, HPV18 positive NHFK and NHCK, methylation of CLU was accompanied by transcriptional downregulation. The C666-1 cell line may be a more useful indicator of gene methylation status *in vivo*.

6.16.4.3. Cell lines for functional analysis

The use of cell lines as models in which to investigate gene function may be limited as it is becoming increasingly apparent that tumours are a heterogeneous mix of different cell types, which may influence each other through paracrine signalling pathways and interactions with each other and with the extracellular matrix (Lyons, Lobo et al. 2008). In contrast, cell lines consist of clonal populations of cells that lack interactions with other cell types when grown *in vitro* and have been selected for their ability to grow under non-physiological conditions.

Studies have shown that cell lines grown in culture show changes in gene expression from the tissues they are derived from. Genes differing were associated with higher proliferation rates and disrupted tissue organisation *in vitro*, as many genes involved in processes such as cell cycle progression and energy metabolism were upregulated, whilst cell adhesion molecules and membrane signalling proteins were downregulated (Sandberg and Ernberg 2005; Sandberg and Ernberg 2005).

6.17. Summary

Clusterin is a multifunctional glycoprotein widely expressed as two isoforms. One isoform, sCLU is secreted, cytoplasmic and anti-apoptotic, the other nCLU is nuclear and pro-apoptotic (nCLU). Pre-nuclear CLU translocates to nucleus in response to various cytotoxic stimuli. nCLU is down-regulated in colon cancer and its over-expression in a prostate cancer cell line causes G2-M arrest. The pro-apoptotic function of CLU may be due to its interaction with XRCC6, a component of the DNA double strand break repair complex. HRAS suppresses clusterin expression through promoter hypermethylation.

I have investigated the regulation and expression of CLU at three sites of squamous cancer; the oral cavity, cervix and nasopharynx. Transcriptional downregulation of CLU was demonstrated in nasopharyngeal cancer (NPC) and oral cancer, and downregulation of CLU was also shown at the protein level at these sites. I have shown loss of one CLU allele and methylation of the other in the NPC cell lines C666-1. I have gone on to repair this defect in this NPC cell line and found that CLU overexpression of the nuclear isoform of CLU resulted

in reduced proliferation and decreased cell viability. I have also shown using luciferase reporter assays that the overexpression of both isoforms of CLU in C666-1 cells and their knockdown in HeLa cells regulates NF-kB activity. Further evidence that CLU may regulate NF-kB activity was suggested by the finding that its overexpression in C666-1 resulted in the stabilisation of IkBα.

Although sCLU is now considered a promising therapeutic target because of its anti-apoptotic function, with an antisense oligonucleotide currently undergoing clinical evaluation, my results suggest that further consideration needs to be given to the possible tumour suppressor function of nCLU.

Chapter 7

Future Work

The mechanism of production of nCLU is still a matter of much debate. According to Leskov *et al*. nCLU is the result of alternative splicing events, however it has been suggested by Caccamo *et al*. that nCLU is produced by post-translational modification of the cytoplasmic precursor (Leskov, Klokov et al. 2003; Caccamo, Desenzani et al. 2006). It has also been seen that not only nCLU is able to enter the nucleus but full length cytoplasmic CLU, which is more stable, also has the ability to translocate to the nucleus (Pajak and Orzechowski 2006). The antisense oligonucleotide in clinical trials for breast and prostate cancer, OGX-011 is targeted to exon II, in a region common to both CLU isoform 1 and isoform 2 mRNAs. This strategy is effective for depletion of the secreted/cytoplasm CLU but if nCLU is produced by alternative splicing, skipping exon II, this would have no effect on the pro-apoptotic activity of nCLU. Thus tumour cells would be depleted of the pro-survival effects of sCLU with a concurrent accumulation of pro-apoptotic nCLU. This ASO strategy is tenuous given that clinical results obtained show that OGX-011 by itself has not been shown to actually induce apoptosis.

It has been shown that the turnover of CLU is tightly regulated by ubiquitin-proteasome mediated degradation, with CLU half life being 115 mins (Rizzi, Caccamo et al. 2009). In prostate cancer cells proteasome inhibition by MG132 resulted in an accumulation of the nuclear form of CLU, committing cells to caspase-dependent death. Therefore, the use of proteosome inhibitors or gene therapy to deliver an expression cassette driving the production of a truncated form of CLU lacking the leader export sequence would be worth exploring in combination with the anti-sense oligonucleotide.

My work has suggested a number of additional lines of enquiry. However, in the first instance it would be crucial to generate other models for the study of the impact of CLU down-regulation. Although I was able to use the C666-1 cell line, which recapitulated the changes seen *in vivo,* these experiments would have been facilitated had I access to an inducible system for the expression of CLU. It would also be important to study the phenotypic and transcriptional consequences of deregulation of CLU in untransformed cells. Towards this end CLU knockdown experiments should be conducted in normal and transformed keratinocytes. Gene expression arrays performed pre and post knock-down of CLU would help to determine the transcriptional consequences of deregulated CLU expression. It would also be of interest to examine, in normal and in transformed cells, the impact of environmental stresses, for example heat shock in cells in which the expression of CLU had been ablated.

The development of such cell line models would allow for a more detailed examination of the regulation of NF-kB by CLU in disease-free and in abnormal transformed cells. Such an examination should include other assays for NF-kB activity which take into consideration the specific NF-kB subunits and both the canonical and non-canonical pathways. In order to determine the mechanism of regulation of NF-kB by CLU, it would be useful to identify CLU binding partners. Of course the interpretation of these findings would be dependent upon a clear understanding of the physiological roles of nCLU and sCLU, and their contribution to disease pathogenesis

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