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Mechanisms of glucocorticoid-induced apoptosis in small cell lung cancer

A thesis submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Life Sciences

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Abbreviations

ACTH:	adrenocorticotropic hormone
CTD:	c-terminal domain
DBD:	DNA-binding domain
Dex:	dexamethasone
DMEM:	Dulbecco's modified eagle medium
FBS:	foetal bovine serum
FKBP52:	FK506 binding protein 52
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gc:	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper
GMCO:	a chloramphenicol acetyltransferase reporter gene construct
GR:	glucocorticoid receptor
GRE:	glucocorticoid response element
Hc:	hydrocortisone
HD:	hinge domain
HPA:	hypothalamic-pituitary-adrenal
LBD:	ligand-binding domain
nSCLC:	non-small cell lung carcinoma
NTD	N-terminal domain
POMC:	pro-opiomelanocortin
RPMI:	Roswell park memorial institute
SCLC:	small cell lung carcinoma
TAT:	tyrosine aminotransferase
Tet:	tetracycline
TRE3tkCAT:	a phorbol ester reporter gene construct
wtGR:	wild type glucocorticoid receptor

Abstract

Eleanor Platt – University of Manchester
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"Mechanisms of glucocorticoid-induced apoptosis in small cell lung cancer."

Small cell lung cancer (SCLC) tumours are very aggressive. Patients often present with metastases at diagnosis and prognosis is very poor. SCLC cell lines have been shown to be resistant to glucocorticoids due to impaired glucocorticoid receptor (GR) expression (Gaitan *et al*, 1995; Ray *et al*, 1996). Restoration of GR expression in these cells triggers apoptosis *in vitro* (Sommer *et al*, 2007) and *in vivo* (Sommer *et al*, 2010). It is possible that loss of GR expression plays a role in SCLC pathogenesis and therefore GR could be considered as a novel tumour suppressor gene for SCLC.

Over-expression of exogenous GR restores glucocorticoid sensitivity in SCLC cells and triggers glucocorticoid-induced apoptosis, both *in vitro* (Sommer *et al*, 2007) and *in vivo*. (Sommer *et al*, 2010). The mechanisms underlying regulation of GR expression and glucocorticoid-induced apoptosis in these cells are not fully understood. This thesis aims to investigate the regulation of GR in relation to glucocorticoid-induced apoptosis in SCLC cell lines.

Quantitative PCR data showed that GR gene expression was not altered by glucocorticoid treatment in SCLC cell line DMS-79 cells. Levels of GR protein were seen to be very low in these cells and were not affected by an increase in cell number / density or treatment with low concentrations of glucocorticoids. A decrease in GR protein levels was observed following treatment of DMS-79 cells with very high concentrations of dexamethasone or hydrocortisone, however GR protein levels returned to that of untreated cells following removal of glucocorticoids. GR protein levels were also found to be very low *in vivo*, as demonstrated by immunohistochemistry of DMS-79 cell xenografts. This suggests that DMS-79 cells tightly regulate GR protein levels in order to evade glucocorticoid-induced apoptosis.

A number of approaches to increase GR protein levels in DMS-79 cells were investigated, with a view to conferring glucocorticoid sensitivity to these cells. DMS-79 cells did not show auto-upregulation of GR from promoter 1A following treatment with glucocorticoids and attempts to stably transfect these cells with a construct containing GR under the control of a tetracycline-inducible promoter were unsuccessful. Further investigation into approaches to increase GR protein levels should be undertaken since understanding the mechanisms underlying glucocorticoid-induced apoptosis may provide insight into novel therapeutic approaches for SCLC patients.

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Declaration

No portion of this work referred to in this thesis has been submitted in support of an application for another degree of qualification at this or any other university or other institute of learning.

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Chapter 1: Introduction

1.1. <u>Secretion of POMC in neuroendocrine tumours</u>

1.1.1. POMC and the HPA axis

The hypothalamo-pituitary-adrenal (HPA) axis regulates the secretion of glucocorticoids from the adrenal glands in response to physiological or psychological stressors (Figure 1.01). This neuroendocrine pathway is present in almost all organisms. Pro-opiomelanocortin (POMC) and adrenocorticotropic hormone (ACTH) play a role in the regulation of the HPA axis.

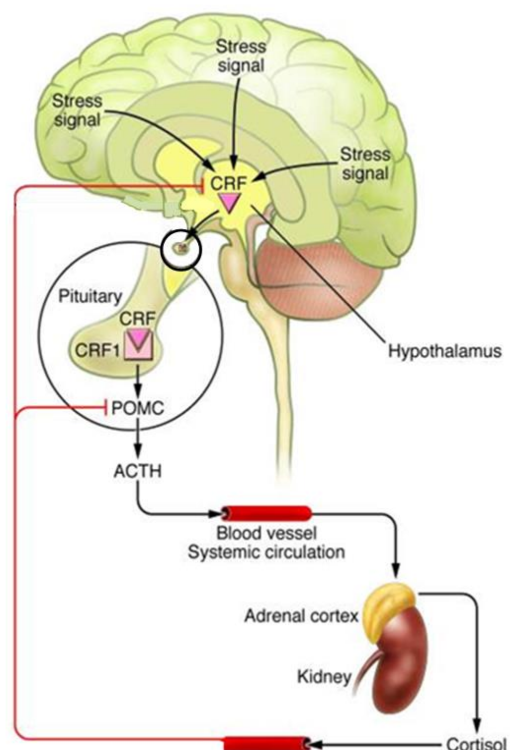


Figure 1.01: An overview of the HPA axis. Corticotropin-releasing factor (CRF) is secreted by the hypothalamus and binds to corticotropin-releasing factor receptor 1 (CRFR1) triggering secretion of pro-opiomelanocortin (POMC) from the pituitary. POMC is cleaved to produce adrenocorticotropic hormone (ACTH) which enters the circulation and binds to the ACTH receptor (ACTH-R), leading to glucocorticoid production and secretion from the adrenal cortex. [Figure adapted from Slominski, 2007].

Stress causes the secretion of corticotropin-releasing factor (CRF) from the hypothalamus which is detected by binding to the CRF receptor type 1 at the membrane of corticotroph cells in the anterior pituitary (Vale *et al*, 1983). This binding stimulates the synthesis of POMC, which, following proteolytic cleavage by prohormone convertase 1/3 (PC1/3) leads to the

production of ACTH and β-endorphins. ACTH is released into the bloodstream and transported to the cortex of the adrenal glands, where it binds to its receptor, ACTH-R. This stimulates production and secretion of glucocorticoids, typically cortisol in humans, from adrenocortical cells. Glucocorticoids in turn play a role in the regulation of the HPA axis, since secretion of high levels of glucocorticoids from the adrenal cortex inhibits the secretion of POMC and ACTH (Kovacs *et al*, 1986).

1.1.2 Ectopic ACTH Syndrome

In healthy individuals, ACTH is secreted into the circulation from the pituitary gland only, although other tissues such as skin have been shown to express ACTH precursors such as POMC (Ray *et al*, 1994). In patients with ectopic ACTH syndrome, non-pituitary tumours such as small cell lung cancer (SCLC) tumours secrete ACTH. The production of ACTH precursors in SCLC and other non-pituitary tumours may indicate a neuroendocrine progenitor phenotype in these cells (White and Clark, 1993). Ectopic ACTH syndrome is characterised by expression and inefficient processing of POMC, leading to high levels of ACTH precursors and low levels of processed ACTH molecules (Stewart *et al*, 1989). POMC expression in cells of non-pituitary tumours is resistant to glucocorticoid-induced down-regulation, resulting in Cushing's syndrome.

1.2. Small cell lung cancer

Lung cancers may be classed into distinct groups according to their cellular characteristics; small cell lung cancer (SCLC) which accounts for approximately 20% of all lung cancer patients and non-small cell (nSCLC), which includes adenocarcinomas, squamous cell and large cell lung cancer. SCLC is by far the most aggressive of these, with less than 15% of patients surviving more than 5 years following diagnosis (Jemal *et al*, 2006). The majority of

patients present with metastases at diagnosis and although initially sensitive to treatments such as chemotherapy, SCLC invariably relapses with cells resistant to treatment.

Tobacco smoking greatly increases the risk of developing lung cancer, however there are a number of other environmental risk factors including inherited genetic mutations and exposure to ionising radiation or pollutants such as asbestos. SCLC tumours are typically derived from the hormone-secreting cells of the neuroendocrine system. Malignant proliferation of SCLC cells relies on autocrine signalling *in vivo* and many SCLC tumours secrete neuropeptides such as chromogranin A (Borglum *et al*, 2007), pro-gastrin releasing peptide (pro-GRP) (Miyake *et al*, 1994) and pro-opiomelanocortin (POMC) (White *et al*, 1989).

1.2.1. POMC in SCLC cell lines

Neuropeptide secretion is also seen in SCLC cell lines and can therefore be used to study the significance of these proteins in SCLC cells. Expression and secretion of ACTH and related precursor proteins has been analysed in different human SCLC cell lines. Stewart *et al* (1989) used an immunoradiometic assay (IRMA) to detect ACTH, precursor peptides POMC and pro-ACTH in the supernatant medium of SCLC cell lines (see Figure 1.02). All cell lines tested were isolated from patients with SCLC, with the exception of CORL32 which, although derived from a patient with squamous cell cancer, shows a number of SCLC cell characteristics.

Previous findings suggested that SCLC cells *in vitro* secrete high levels of ACTH. Ellison *et al* (1976) and Sorenson *et al* (1981) used a radioimmunoassay to detect ACTH precursors in chromatographically separated fractions. This technique relied largely on efficient binding of the antiserum to high molecular weight ACTH precursors however, since this was not investigated using POMC and pro-ACTH standards, these findings are somewhat unreliable.

Stewart *et al* (1989) used two-site immunoradiometric assays (IRMA) based on monoclonal antibodies to quantitate the level of ACTH and ACTH precursors in SCLC cell lines. This technique is more robust than radioimmunoassay analysis of fractions, since the assay for precursors requires two antibodies, increasing the specificity for ACTH precursors. Of the 18 SCLC cell lines tested, 10 were shown to secrete significantly higher levels of ACTH precursors such as POMC and pro-ACTH than ACTH. This suggests that there is minimal processing of ACTH precursors in SCLC cells.

Cell line	ACTH precursors (pmol)	ACTH (pmol)	Molar ratio
COR L24	426	2.6	164:1
COR L27	596	3.4	175:1
COR L31	1200	12.4	97:1
COR L32	< 2.6	< 0.8	-
COR L42	1017	5.2	196:1
COR L47	< 2.6	< 0.8	-
COR L51	< 2.6	< 0.8	-
COR L88	135	< 0.8	> 135:1
COR L99	44	< 0.8	> 44:1
COR L103	1405	9.0	156:1
NCI H82	< 2.6	< 0.8	-
NCI H128	37	1.5	25:1
NCI H209	< 2.6	< 0.8	-
NCI N417	< 2.6	< 0.8	-
GLC-1	< 2.6	< 0.8	-
GLC-1-M13	198	1.4	141:1
HC12	97	< 0.8	> 97:1
HX149	< 2.6	< 0.8	-

Table 1: Amount of ACTH and ACTH precursor proteins in supernatant medium of SCLC cell lines. Shaded data indicates concentrations of ACTH and ACTH precursors were too low to detect. [Figure adapted from Stewart *et al*, 1989].

1.2.2. Glucocorticoid regulation of POMC in SCLC cells

A number of POMC-expressing SCLC cell lines show no inhibition of POMC in response to glucocorticoid treatment, suggesting these cells are resistant to the action of glucocorticoids (Clark *et al*, 1990). Farrell *et al*, (1993), analysed the glucocorticoid sensitivity of 5 SCLC cell lines and found that none of the cell lines tested showed significant inhibition of POMC or

ACTH expression upon treatment of cells with glucocorticoids. Only one of the three SCLC cell lines tested showed a significant reduction in POMC expression following incubation of cells with 2μM dexamethasone. These findings indicate that the glucocorticoid resistance seen in some SCLC cell lines is due to the impairment rather than the absence of normal mechanisms which inhibit POMC and ACTH expression. In order to identify the cause of glucocorticoid resistance in SCLC cell lines, it is important to understand the structure of the GR protein present in these cells and the pathways in which this nuclear receptor functions.

1.3. Glucocorticoids and the glucocorticoid receptor

In humans, the glucocorticoid cortisol is produced by the zona fasciculata of the adrenal gland and secreted in response to stress or low glucocorticoid levels in the blood. Cortisol secretion is regulated by the HPA axis which in turn is regulated by diurnal rhythms, with the highest levels of cortisol present in the blood in the morning and lowest during the night (Walker *et al*, 2010). Over 90% of cortisol in the blood is bound to proteins such as serum albumin or corticosteroid binding protein, however glucocorticoid receptor (GR) can only bind free cortisol.

1.3.1. Physiological actions of glucocorticoids

The effects of glucocorticoids are mediated by binding to GR, a ubiquitously expressed intracellular nuclear receptor which functions as a ligand-dependent transcription factor to regulate glucocorticoid-dependent gene expression. In the absence of glucocorticoids, GR is held within the cytoplasm in an inactive heterocomplex including heat shock proteins (HSP70 and HSP90) and immunophilin FKBP52 (Cadepond *et al*, 1991). GR is dissociated from this complex upon glucocorticoid binding and GR homodimers form via interaction of the C-terminal domains. Active GR then translocates to the nucleus where it can either activate or

repress gene transcription.

GR dimers binds to short palindromic sequences called glucocorticoid response elements (GREs), located within the promoter regions of many genes. Interactions with co-activator proteins such as histone acetyltransferases (HATs) open the chromatin structure and drive transcription. GR can also interact with other transcription factors such as c-Myb to direct gene expression (Geng & Vedeckis, 2005). Ligand-bound GR is able to repress transcription of genes, through binding to negative glucocorticoid response elements (nGREs) within the DNA. This triggers recruitment of co-repressor proteins such as histone deacetylases (HDACs) which close the chromatin structure and prevent gene transcription.

Upon glucocorticoid binding, GR protein becomes hyperphosphorylated. This phosphorylation triggers ubiquitination of lysine residues within the GR protein by ubiquitin-conjugating enzymes (UBCs). This marks the GR protein for degradation by the proteasome; a multi-subunit protein complex which breaks down proteins into small peptide fragments (Wallace & Cidlowski, 2001).

1.3.2. Structure of the glucocorticoid receptor

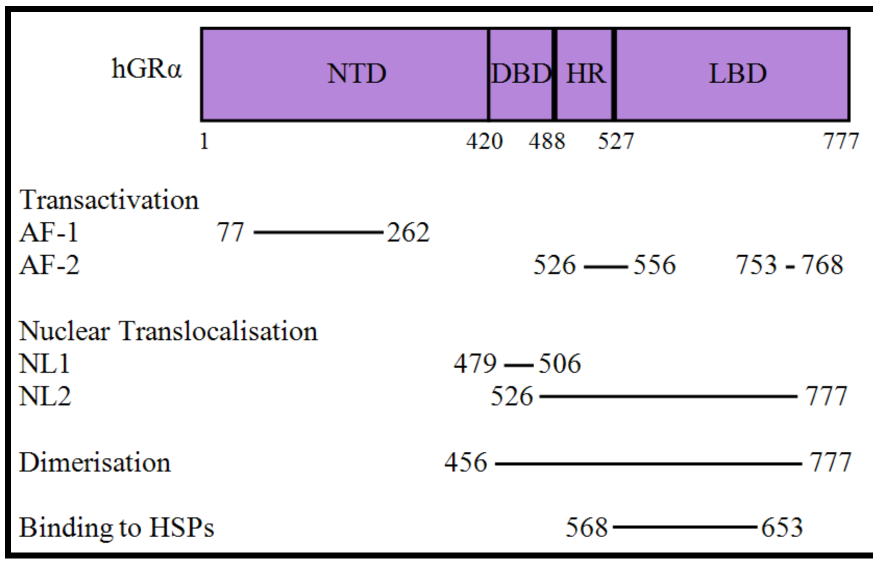


Figure 1.02: Functional domains of the GRα isoform. The N-terminal domain (NTD) of GR consists of amino acids 1-420, the DNA-binding domain (DBD) amino acids 421-488 and the ligand-binding domain (LBD) amino acids 528-777. [Figure adapted from Nicolaides *et al,* 2010].

The human GR gene has been shown to contain 10 exons, with the 3' region of exon 5, exons 6-8 and the 5' region of exon 9α encoding the ligand-binding domain (Encio & Detera-Wadleigh, 1991). Binding of glucocorticoids to the ligand binding domain of GR stimulates receptor dimerisation and translocation to the nucleus. This complex then binds to glucocorticoid response elements (GREs) of promoter sequences in the DNA, leading to transactivation of anti-inflammatory genes such as lipocortin 1 and calpactin binding protein. The tyrosine residue located at position 735 of GR has been shown to play a central role in this process, since mutation of this residue alone has been shown to reduce transactivation of the mouse mammary tumour virus (MMTV) promoter following incubation of cell lines with cortisol (Ray *et al*, 1999).

The glucocorticoid receptor gene (NR3C1) is a member of the nuclear receptor family expressed at varying levels in almost all cell types. The human GR gene is located on chromosome 5 and consists of over 160kbp of DNA, including promoter regions, alternative non-coding exons and eight coding exons (Kino & Chrousos, 2004). Methylation of these

alternative GR promoters plays a central role in the regulation of GR, which appears to be largely at the transcriptional level in humans. The varying level of GR expression in different cells may be due to a glucocorticoid response element identified in one of the promoter regions of the NR3C1 gene, allowing expression of GR to be up-regulated or down-regulated (Strahle *et al*, 1992).

1.3.3. GR isoforms

In healthy tissues, the human GR gene can undergo alternative splicing to produce two distinct but homologous GR isoforms; GRα, a 94kDa protein located primarily in the cytoplasm which acts as a ligand-dependent transcription factor and GRβ, a 97kDa protein which is unable to bind glucocorticoid agonists and prevents transcription by GRα in a dominant negative manner (Bamberger *et al*, 1995). As shown in Figures 1.04 and 1.05, both GRα and GRβ can be divided into distinct regions; the N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR) and ligand-binding domain (LBD). The NTD of GRα (amino acids 77-262) is essential in the initiation of GR effects on transcription through interactions with other proteins such as transcription factors, while the highly conserved DBD region (amino acids 420-480) plays a role in the binding of the receptor to GRE DNA sequences. The hinge region provides structural flexibility to GR and is involved in receptor dimerisation and binding to GREs. The LBD (amino acids 481-777 of GRα) regulates the binding of glucocorticoids to GR, triggering receptor dimerisation and translocation to the nucleus (Nicolaides *et al*, 2010).

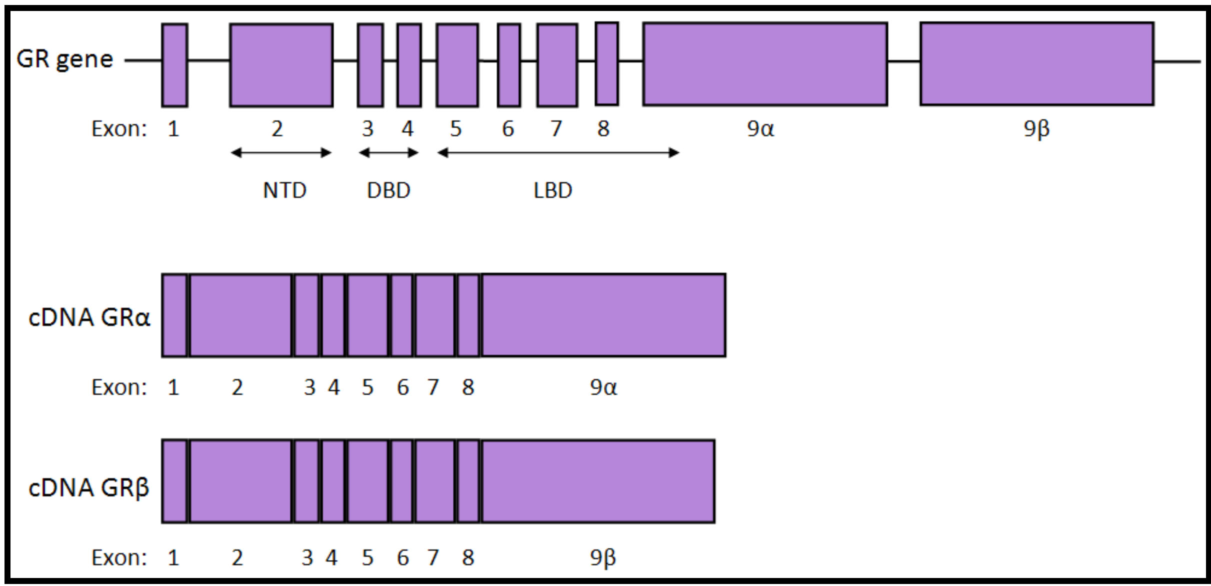


Figure 1.03: The structure of the human glucocorticoid receptor gene and the constituent exons of GRα and GRβ isoforms. Exon 2 encodes the N-terminal domain (NTD) of the GR protein, exons 3-4 encode the DNA-binding domain and exons 5-9 encode the hinge domain and ligand-binding domain (LBD). The GR gene contains an alternative splice acceptor site at the 3' end of exon 8 leading to the formation of alternative GR isoforms.

A number of other GR isoforms have been identified, including GRγ which contains an additional amino acid in the DBD region of the protein (Ray *et al*, 1996) and GRδ (also referred to as GR-P), a truncated form of the protein with a molecular weight of 74kDa, identified in SCLC cell line DMS-79 cells (Gaitan *et al*, 1995).

1.3.4. Alternative GR promoters

Expression of the GR gene is regulated by upstream sequences; 9 untranslated first exons thought to be under the control of separate promoters, located in the proximal and distal promoter sites (Figure 1.05). For example, regulation of the GRα isoform relies heavily on promoter 1C, whilst promoter 1B is involved in the regulation of GRδ (GR-P) expression (Russcher *et al*, 2007). GR mRNA transcripts identified in different tissues have been shown to contain alternative first exons, involved in the regulation of GR expression levels (Turner *et al*, 2005, Presul *et al*, 2007). This variation in first exon usage is not seen in the GR protein since the translational start codon (ATG) is located in exon 2 (Zong *et al*, 1990).

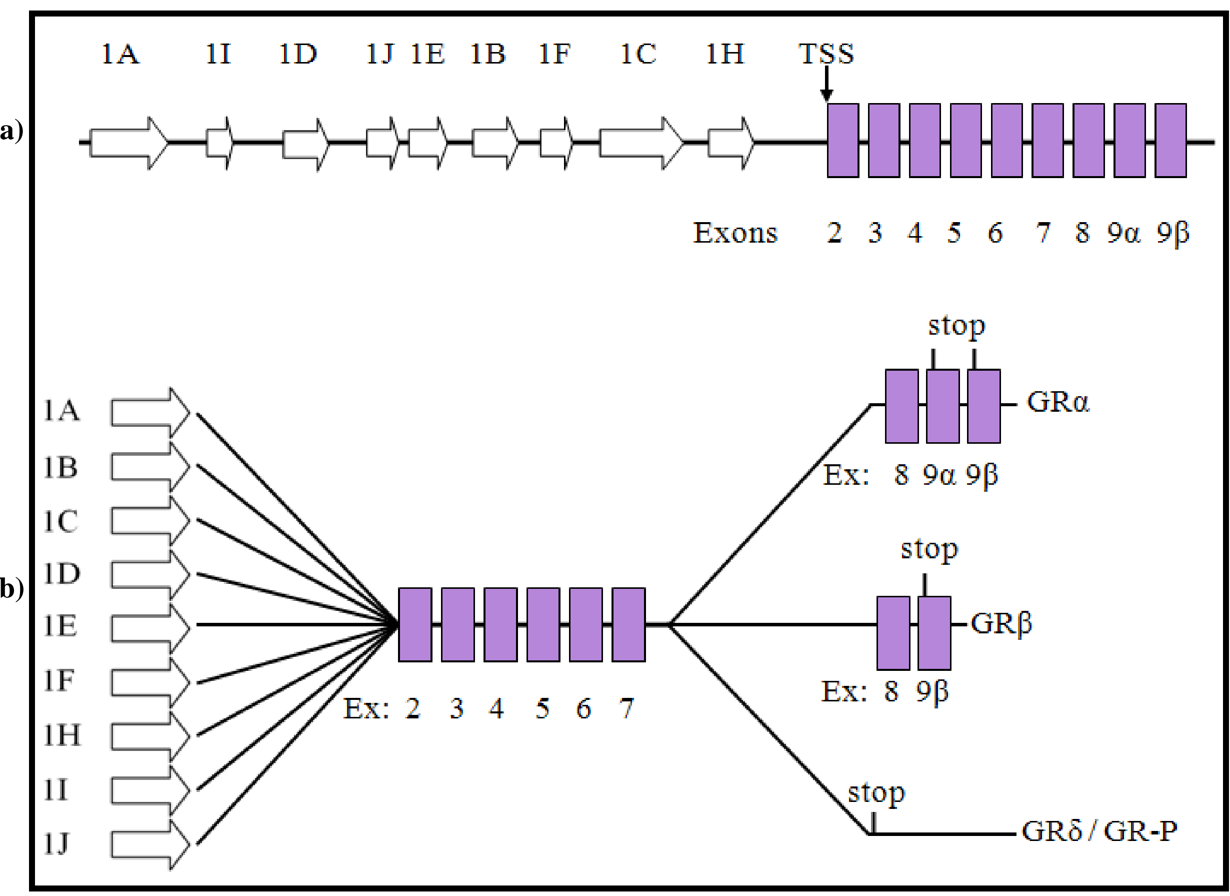


Figure 1.04: a) Alternative first exons of the GR gene located upstream of the translation start site (TSS) **b)** Constituent exons of GR isoforms α, β and δ. [Figure adapted from Turner *et al*, 2010].

With the exception of exons 1A and 1I, all the alternative first exons and their promoters are located in a CpG island, upstream of the GR gene (Turner *et al*, 2010) and methylation patterns in these promoters vary widely between individuals (Turner *et al*, 2008). CpG islands are DNA sequences of 300-3,000bp which contain a high frequency of CpG dinucleotides, present in approximately 40% of human gene promoters. Although methylated CpG islands are present throughout the genome, those in promoters are typically unmethylated when the regulated gene is expressed; suggesting that methylation of CpG islands plays a role in the inhibition of gene expression.

Following binding of GR to glucocorticoids, active GR is able to bind to glucocorticoid response elements (GREs) in DNA and alter expression of target genes. GR regulates its own expression, with high levels of GR leading to inhibition of GR expression in most cell types. Geng *et al*, (2008) identified three glucocorticoid response units (GRUs) in GR promoters at positions -4559→-4525, -3947→-3924 and -3423→-3378, upstream from the translation start site. These GRUs contain a GR binding site along with overlapping c-Myb and Ets binding sites, therefore binding of c-Myb and Ets is mutually exclusive. Binding of GR and c-Myb to GRUs is thought to up-regulate GR expression, while binding of GR and Ets is thought to down-regulate GR expression (Geng *et al*, 2008). Further investigation into the mechanisms underlying this regulation of GR is required, since up-regulation of GR expression may be able to restore GR protein levels and thereby confer glucocorticoid sensitivity to SCLC cells.

1.4. Glucocorticoid resistance in SCLC cells

Glucocorticoids such as cortisol are involved in negative feedback of the HPA axis, inhibiting synthesis of POMC and ACTH. However this mechanism of regulation is not present in the majority of SCLC cell lines tested since although these cells typically show high levels of

POMC, this expression is characteristically resistant to glucocorticoid repression. This was thought to be due to mutation of the POMC gene or steroid metabolism by enzymes such as 11 β-hydroxysteroid dehydrogenase (11β-HSD) which converts biologically inactive cortisone to active cortisol, or mutation of the GR (Parks *et al*, 1998). However it has since been shown that the cause of glucocorticoid resistance varies between cell lines.

1.4.1. Glucocorticoid resistance in the SCLC cell line DMS-79

SCLC cell lines are each derived from a single patient. DMS-79 cells are resistant to glucocorticoids and this resistance is thought to be due to a mutation within one allele of the GR gene, leading to the production of a truncated GR isoform GRδ (GR-P) which lacks part of the ligand binding domain and is therefore unable to bind glucocorticoids (Gaitan *et al*, 1995). GRδ has been shown to share exons 1-7 with GRα (nucleotides 1-2155), however the remaining DNA sequence contains an in-frame termination codon, leading to the production of a truncated GR protein. Transfection of this GRδ isoform and wild type GR (wtGR) into control COS cell lines showed no evidence of a dominant negative effect (Gaitan *et al*, 1995) and infection of DMS-79 cells with wtGR DNA was shown to restore GR signalling in these cells (Sommer *et al*, 2007). This decrease in wtGR expression is therefore thought to provide the basis of glucocorticoid resistance in DMS-79 cells. Promoter 1C of the GR gene has been shown to be methylated in these cells, leading to a further decrease in GR gene expression (Kay *et al*, 2011).

1.4.2. Glucocorticoid resistance in the SCLC cell line COR L24

COR L24 cells express GR with normal binding affinity for dexamethasone, however these cells are also glucocorticoid resistant (Ray *et al*, 1994). Transfection of these cells with a positive reporter gene (GMCO, a chloramphenicol acetyltransferase reporter gene under the control of the murine mammary tumour virus positive GRE) and a negative reporter gene

(TRE$_3$-tkCAT, tandem repeats of the phorbol ester response element under the control of a thymidine kinase promoter) constructs showed that this resistance is not due to a defect in the POMC gene in these cells, but the failure of endogenous glucocorticoid signalling. Therefore glucocorticoid resistance in these cells was thought to be caused by mutation of GR outside of the ligand binding domain (LBD) or the presence of a dominant negative GR allele. However, Ray *et al* (1994) showed that transfection of COR L24 cells with GR-expressing plasmids is able to restore wtGR signalling in these cells. This was demonstrated by the co-transfection of COR L24 cells with plasmids containing wtGR and either positive (GMCO) or negative ((TRE)3-tkCAT) reporter genes. COR L24 cells containing the GMCO reporter gene were shown to induce expression of chloramphenicol acteyltransferase (CAT) following incubation with hydrocortisone, whereas cells containing the (TRE)3-tkCAT reporter gene demonstrated a decrease in CAT expression following hydrocortisone incubation. This indicates that expression of wtGR is able to restore glucocorticoid signalling in COR L24 cells.

Sequencing of the GR gene in COR L24 has identified 2 distinct mutations; a single nucleotide polymorphism triggering an amino acid substitution at position 363 (Asp→Ser) in one allele and a trinucleotide insertion site in the DNA binding domain of the other allele, leading to the introduction of an additional arginine residue at position 453 (Ray *et al*, 1996). Both mutations lead to an alteration in the amino acid structure of GR in these cells. This loss of wtGR expression is therefore thought to play a role in the glucocorticoid resistant phenotype of COR L24 cells.

1.4.3. Low level expression of glucocorticoid receptor in SCLC cell lines

The mechanism of glucocorticoid resistance in different SCLC cells varies between cell lines, however low level GR expression has been observed in many SCLC cell lines. A number of

CpG islands in the GR gene are methylated, which may play a role in inhibition of transcription factors and therefore decrease GR expression

Reversal of this hyper-methylation, through incubation of SCLC cells with 5' azadeoxycytidine, induces up-regulation of GR synthesis (Kay *et al*, 2011). Promoter 1C of the GR gene is thought to play an important role in this process, since increased methylation coincides with decreased GR synthesis in DMS-79 cells. Epigenetic regulation of genes plays an important role in cancer pathology. Hyper-methylation of CpG islands in promoter regions of a number of tumour suppressor genes prevents their expression in cancer cells (Lin *et al*, 2010).

1.5. <u>The role of GR in apoptosis of SCLC cells</u>

1.5.1. Glucocorticoid regulation of apoptosis in SCLC cells *in vitro*

A number of small cell lung cancer cell lines are resistant to glucocorticoid signalling, due to low levels of GR expression or mutation of the GR gene. Sommer *et al* (2007) infected SCLC cell lines with a retroviral construct expressing wtGR. This showed that restoration of wtGR signaling confers glucocorticoid sensitivity to these cells and triggers apoptosis upon dexamethasone treatment, as characterised by increased Annexin V binding and changes in nuclear morphology.

GR regulation also plays a role in apoptosis of a number of different cell lines, for example osteosarcoma cells lines. GRα isoforms, generated through initiation of translation at alternative start codons have been shown to differentially regulate anti-apoptotic genes such as Bcl-xL and survivin (Gross *et al*, 2011). In osteosarcoma cells, the GRα-D isoform lacking amino acids 98-335 localises to the nucleus and is therefore unable to interact with pro-

apoptotic proteins, such as p65, in the cytoplasm. These findings may indicate that N-terminal GRα isoforms play a role in resistance to glucocorticoid-induced apoptosis observed in certain cancer cells.

1.5.2. Glucocorticoid regulation of apoptosis in SCLC cells *in vivo*

Restoration of wtGR expression also induces apoptosis of SCLC cells *in vivo* (Sommer *et al*, 2010). This study used mice with human SCLC cell xenografts which synthesise ACTH precursors such as POMC, resulting in high corticosterone levels. Following development, these tumours were injected with wtGR-expressing adenoviruses. Sommer *et al* (2010) shows that driving wtGR expression in SCLC cell lines confers glucocorticoid sensitivity to these cells *in vivo* and induces apoptosis. Furthermore apoptosis of untransfected SCLC cells was significantly increased, suggesting the presence of a bystander killing effect. Over-expression of GR in SCLC cells regulates expression of a number of genes, many of which are involved in pro-apoptotic pathways (Sommer *et al*, 2010).

1.5.3. Glucocorticoid-induced mechanisms of apoptosis

Apoptosis, or programmed cell death, is the process by which cells commit suicide. This mechanism is distinct from necrosis, premature cell death in response to acute cellular injury induced by stress. Apoptosis is characterised by membrane blebbing, DNA fragmentation and chromatin condensation.

Apoptosis of SCLC cells is induced by binding of activated GR to specific DNA sequences. Mouse models containing mutations in the dimerisation domain of the GR gene ($GR_{dim/dim}$) have been generated, in which transactivation of glucocorticoid-responsive genes does not occur. Reichardt *et al* (2001) demonstrated that induction of apoptosis in thymocytes (haematopoetic progenitor cells) following treatment with dexamethasone relies on GR binding to specific DNA sequences, triggering expression of glucocorticoid responsive genes.

A similar pathway may exist in SCLC cells, however further investigation into the apoptotic pathways in these cells is required.

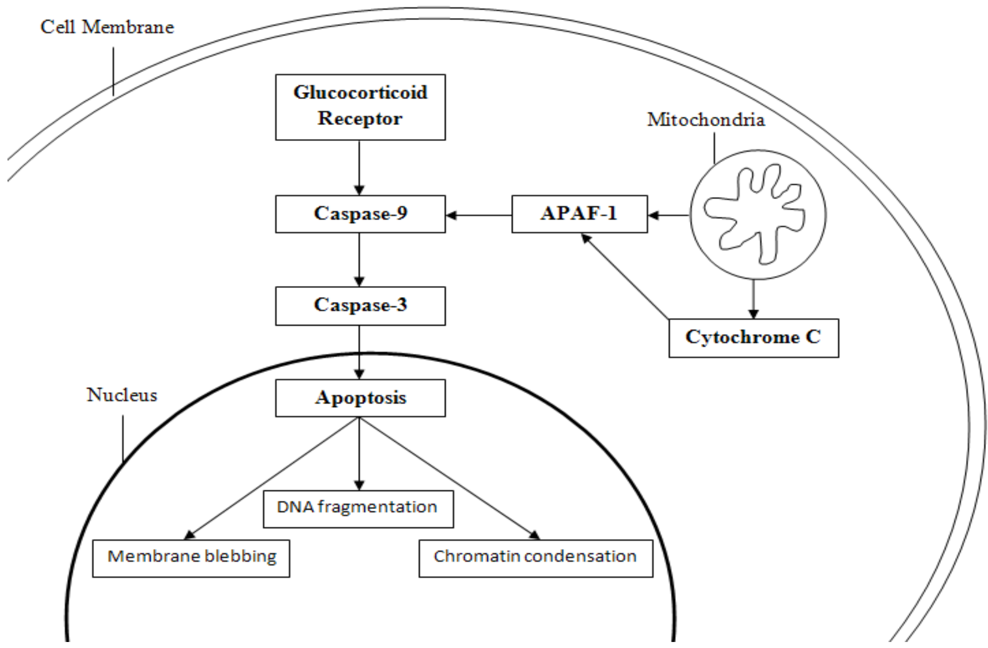


Figure 1.05: Glucocorticoid-induced apoptotic pathways. [Figure adapted from Oberholzer *et al*, 2001].

Apoptotic signaling may act through targeting mitochondrial function, by inducing the formation of pores in the mitochondrial membrane. This may cause mitochondrial swelling or release of factors such as second mitochondrial-derived activators of caspases (or SMACs) and cytochrome C into the cytosol. SMACs trigger apoptosis by binding to and inactivating proteins known as inhibitors of apoptosis (IAPs) and therefore activating caspases. Cytochrome C acts through binding to apoptotic protease activating factor 1 (Apaf-1), ATP and inactive pro-caspase-9 to trigger the formation of the apoptosome (Fig. 1.06). Pro-caspase-9 is then cleaved to caspase 9 and this activates caspase-3, triggering apoptosis. Retroviral expression of a GR-eYFP transgene in SCLC cells induces activation of caspase 3, indicating activation of apoptotic pathways (Kay *et al*, 2011).

1.6. Project overview

All SCLC cell lines investigated have been insensitive to glucocorticoids. The cause of glucocorticoid insensitivity in the panel of SCLC cell lines varies between cells, although a number of cell lines contain mutations in the GR gene leading to impaired GR expression. These SCLC cell lines show mutations in only one GR allele, therefore there is a potential for glucocorticoid signaling in these cells. However recent studies have suggested that decreased GR expression in the panel of SCLC cell lines may also be due to methylation of alternative GR promoters. This has been demonstrated in the DMS-79 SCLC cell line, whereby inhibition of methyltransferases showed an increase in GR protein levels due to reduction in methylation of the ubiquitous 1C GR promoter (Kay *et al*, 2011).

Glucocorticoid treatment induces apoptosis in other cell lines; previous studies in acute lymphoblastic leukemia (ALL) cells have demonstrated upregulation of GR promoters 1C and 1D in response to glucocorticoids and increased sensitivity to glucocorticoid-induced apoptosis (Geng & Vedeckis, 2011). This auto-upregulation of GR relies on glucocorticoid response units (GRUs) in DNA which potentially bind activated GR and other transcription factors.

Over-expression of GR in SCLC cell lines induces apoptosis, both *in vitro* and *in vivo* (Sommer *et al*, 2007; Sommer *et al*, 2010). It is therefore important to further investigate methods through which these cells can be induced to undergo apoptosis, since little research has been conducted into this field in SCLC cells. The mechanisms through which SCLC cells undergo apoptosis following induction of wtGR expression are not fully understood; therefore further investigation into the mechanisms underlying this process is necessary. Previous investigations have relied on retroviral or adenoviral infection of GR into SCLC cell lines,

however expression of this exogenous GR gene is brief. Furthermore, investigation into the initial downstream targets of GR has been hampered by the time taken for SCLC cell lines to recover from transfection. These problems could be overcome by stable integration of wtGR gene under the control of a tetracycline-inducible promoter into SCLC cell lines. In this system, expression of GR would be repressed by tetracycline-repressors bound to the constitutively active promoter. Treatment of cells with tetracycline would then lead to removal of the tetracycline repressors and induce constitutive expression of GR.

The aims of this study were:

1) To characterise the expression of GR in SCLC cell lines.

2) To examine the effects of glucocorticoids on GR mRNA and protein levels in SCLC cell lines.

3) To investigate the stable integration of wtGR under the control of a tetracycline-inducible promoter in order to have a regulated over-expression of GR in SCLC cells to investigate the downstream mechanisms of induction of apoptosis

4) To investigate mechanisms to increase the level of endogenous GR expression in SCLC cell lines and thereby trigger glucocorticoid-induced apoptosis in these cells.

Chapter 2: General Methods

2.1. Cell culture

Adherent HeLa lacZeo/TO (a kind gift from Dr. Anthony Tighe), stably integrated with the GR expression vector and A549 cells were cultured in Dulbecco Modified Eagles Medium (DMEM) with high glucose (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS) (Seralab, West Sussex, UK).

SCLC cell line DMS-79 were cultured in RPMI with L-Glutamine (Gibco) supplemented with 10% FBS, 10 nM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (Gibco). These cells grow as spheroids in suspension and a confluent culture was identified by a high number of spheroids and an increase in culture medium pH, as indicated by a change in the colour of the medium. Cells were pipetted repeatedly during passage to decrease the size of spheroids and stimulate mitosis.

All cells were incubated at 37°C, 5% CO_2 atmosphere (Sanyo Electric Co Ltd, Watford, UK). Antibiotics zeocin and blasticidin (Invitrogen Ltd, Paisley, UK) were used in the culture of HeLa lacZeo/TO cells (50 nM zeocin, 4 nM blasticidin). Blasticidin and hygromycin (Duchefa Biochemie, Suffolk, UK) were used in the culture of HeLa GR cells (4 nM blasticidin, 100 nM hygromycin). All cells incubated with antibiotics were treated 24 hours after passage.

2.1.1. Viable cell counts

Viable cell counts of all cell lines were conducted using trypan blue exclusion (Sigma, Dorset, UK). Suspended cells were mixed in a 1:1 ratio with trypan blue to stain dead cells. The cells were then counted using a haemocytomer and the total cell number calculated.

2.2. <u>Sample collection</u>

2.2.1. Suspension cells

Samples of cells grown in suspension, including SCLC cell line DMS-79 cells, were collected as cells approached 90% confluency or at set time points. A viable cell count was carried out using the trypan blue exclusion method. The cells were then pelleted at 1,000rpm for 5 minutes, the resulting supernatant discarded and the pellets washed twice with phosphate buffered saline (PBS). Cell pellets were stored at -80°C.

2.2.2. Adherent cells

Adherent cell lines were collected as cells approached 90% confluency or at set time points. Culture media was aspirated and the cell monolayer washed with PBS. StemPro Accutase (Gibco) was then pipetted onto the monolayer and incubated at 37°C for 15 minutes to dissociate cells from the culture flask. Cells were re-suspended to the starting concentration using culture media and a viable cell count was conducted using the trypan blue exclusion method. Finally the cells were pelleted at 1,000rpm for 5 minutes, the resulting supernatant was discarded and the pellets washed twice with PBS. Cells pellets were stored at -80°C.

2.3. <u>Western blot</u>

Protein was extracted from cell samples by re-suspension of cell pellets in 50µl of protein extraction buffer (1M Tris HCl buffer pH 8, 0.1% Triton X100, 5M EDTA, 100nM Trehalose and Protease Inhibitor (Roche)). The samples were then incubated on ice for 30 minutes and centrifuged at 15,000rpm at 4°C for 15 minutes to pellet the cell debris. The resulting supernatant was collected in a clean microcentrifuge tube and the pellet was discarded. Protein concentration of all samples was determined using Nanodrop measurement at absorbance

280nm, and normalised with protein extraction buffer to give a concentration of 1mg/ml protein in all samples.

Samples were diluted in 2x loading buffer (Sigma) and heated at 95°C for 5 minutes to denature the protein. A total of 30µl (15 µg protein) was loaded into the wells of 4-12% Novex Bis-Tris gels (Invitrogen) along with a chemichrome protein standard ladder (Sigma). The gel was run for 80 minutes at 150V in MOPS buffer. The bands were then transferred to a nitrocellulose membrane (Whatman, UK) at 60V for 90 minutes in methanol transfer buffer.

The nitrocellulose membranes were blocked in 1% milk powder in TBS-T (Tris buffered saline, 0.1% Tween 20) for 2 hours. The membranes were probed for GR and α-tubulin using a 1:2000 dilution of mouse anti-GR antibody which binds to GR amino acids 176-289 (clone 41/glucocorticoid receptor, BD Biosciences, Oxford, UK) and 1:2000 dilution of mouse anti-α-tubulin (Santa Cruz, Heidelberg, Germany) overnight at 4°C. Membranes were then washed in 0.25% milk powder in TBS-T for 30 minutes and subsequently incubated with a 1:5000 dilution of goat anti-mouse-IgG conjugated to horseradish peroxidase (HRP) for 1 hour (Invitrogen). The nitrocellulose membranes were then washed again in 0.25% milk protein in TBS-T for 30 minutes and incubated with chemiluminescence detection kit (Thermo Scientific, UK) for 2 minutes. Finally, the membranes were exposed to X-ray XAR films (Kodak) and the bands visualised using a Compact X2 automatic developer (X-OGraph, Wiltshire, UK).

2.4. Gene expression studies

2.4.1. cDNA synthesis

RNA was extracted from cell samples using QIAgen RNeasy Mini Kit with the recommended QIAshredder tubes (QIAgen, West Sussex, UK). RNA was eluted from the columns in 50µl nuclease free water and the RNA concentration of each sample was measured by Nanodrop measurement at 230nm. cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAgen) which includes a gDNA wipeout buffer to prevent genomic DNA carryover. The reverse transcription reaction mix containing 1µl primer mix, 1µl reverse transcriptase (RT) and 4µl RT buffer was then added to the RNA sample and tubes were incubated at 42ºC for 15 minutes then at 95ºC for 3 minutes.

2.4.2. Primer design

Primers were designed to create a PCR product of approximately 100bp and were tested for efficiency before use (Eurofins MWG, London, UK).

	Primer sequences (5'-3')
Glucocorticoid receptor:	CCAACGGTGGCAATGTGAAAT
	CCAAGGACTCTCATTCGTCTCTT
Pro-opiomelanocortin:	CCCCTACAGGATGGAGCACTT
	GATGGCGTTTTTGAACAGCGT
Glucocorticoid-induced leucine zipper:	TGTGGATGAGGGATGAACAA
	ACCCGCTACAGACAAGCTTT
GR exon 1A:	TTTAAATGGCAGAGAGAAGGAGAAA
GR exon 1B:	GCCGGCACGCGACTCC
GR exon 1C:	GGGAACTGCGGACGGTG
GR exon 1D:	ACAACCTTTCCCAGAGTC
GR exon 1E:	CGTGCAACTTCCTTCGAGT
GR exon 1F:	TAGCGAGAAAAGAAACTGG
GR exon 1H:	TGACAGCCCGCAACTTGGA
GR exon 1I:	CCATCTACATGAGAGAG
GR exon 1J:	AACTTGGACGCGGGCC
GR exon 2:	CCTGAGCAAGCACACTGCTG
Glyceraldehyde 3-phosphate dehydrogenase:	GAAGGTGAAGGTCGGAGTCA
	AATGAAGGGTCATTGATGG

2.4.3. Reverse transcription polymerase chain reaction (RT-PCR)

20µl of RT-PCR mix was added to all samples and PCR conditions were as shown below.

1. Initial denaturation and enzyme activation - 4 minutes at 95ºC
2. Denaturing - 20 seconds at 95ºC
3. Annealing - 30 seconds at 55ºC
4. Elongation - 30 seconds at 72ºC
5. Repeat steps 2, 3 & 4 for 40 cycles.
6. Extension - 5 minutes at 72ºC
7. Hold at 4ºC

The resulting PCR products were loaded onto a 2% agarose gel containing 0.05% ethidium bromide and run at 90V for 45 minutes in Tris-Acetate ETDA (TAE) buffer. Bands were then visualised under a UV light at 245nm.

2.4.4. Quantitative real time polymerase chain reaction (qRT-PCR)

96 well plates were used for qRT-PCR reactions and all samples were run in duplicate. qRT-PCR was performed using QuantiTect SYBR Green PCR Kit (QIAgen) and all samples contained 4µl cDNA, 10µl SYBR Green, 0.2µl forward primer, 0.2µl reverse primer and 5.6µl RNase free water. qRT-PCR conditions were as shown below.

1. Initial denaturation and enzyme activation: 10 minutes at 95ºC
2. Denaturing: 15 seconds at 95ºC
3. Annealing and elongation: 1 minute at 60ºC
4. Repeat steps 2 & 3 for 40 cycles
5. Melt curve: 15 seconds at 95ºC
6. 1 minute at 60ºC
7. 15 seconds at 95ºC

2.5 DAB staining of DMS-79 xenograft sections

DMS-79 cells were collected for xenograft preparation during exponential growth phase and washed 3 times in PBS. Cells were counted using trypan blue exclusion and resuspended in serum-free media at $1x10^8$ cells/ml. DMS-79 cell suspensions were mixed with an equal volume of matrigel (Scientific Laboratory Supplies, UK) to generate a solution containing

$5x10^7$ cells/ml. A total of $5x10^6$ cells were then injected subcutaneously into the flank of each nude (nu/nu) female mouse. All procedures involving animals were conducted under the UK Home Office Animals (Scientific Procedures) Act of 1986. Blood samples were taken from each mouse via tail nick before the injection of cells, on day 13 post-injection and subsequently every 7 days. The mice were culled using CO_2 and cervical dislocation when tumours reached $1000mm^3$. The xenografts were then excised and half of the tumour was frozen in liquid nitrogen and stored at -80ºC, while the other half was fixed in 10% formalin for 24 hours before being embedded in paraffin wax. The wax embedded DMS-79 xenografts were sliced into 5µm sections using a Microm HM 330 microtome (Leica); consecutive sections were transferred onto slides and dewaxed as follows.

Solution	Time
Xylene	5 minutes
Xylene	3 minutes
100% IMS	2 minutes
100% IMS	2 minutes
70% IMS	2 minutes
Distilled water	2 minutes
Distilled water	2 minutes
Distilled water	2 minutes

Table 2: Protocol used to dewax xenograft sections. Wax embedded DMS-79 xenografts were sliced into 5µm sections and dewaxed in preparation for antigen retrieval.

The xenograft sections were then soaked in citrate buffer (pH=3) and place in a water bath at 95°C for 30 minutes. The slides were washed with PBS 3 times at room temperature and DAB staining carried out. Peroxidase block (Dako) was applied to slides for 20 minutes followed by 3 PBS washes for 3 minutes each. Serum block (PBS with 20% goat serum, 1% BSA, 0.2% Triton-X100) was then applied to slides for 60 minutes. Serum block was then removed and GR primary antibody (BD Biosciences, clone 41/glucocorticoid receptor) was prepared in serum block solution at a 1:25 dilution. Primary antibody was applied to slides for 60 minutes followed by 3 PBS washes for 3 minutes each. Anti-mouse HRP-conjugated secondary

antibody (Dako) was applied to the slides for 30 minutes followed by a further 3 PBS washes for 3 minutes each. Chromogen was then added to DAB substrate and applied to the slides for 10 minutes, followed by 3 PBS washes for 3 minutes each. Slides were washed in distilled water and Gills haematoxylin applied to slides for 2 minutes to counterstain nuclei. Finally the sections were dehydrated using the protocol below and mounted in pertex.

Solution	**Time**
Haematoxylin	2 minutes
Distilled water	1 minute
Bluing solution	1 minute
Water	2 minutes
70% IMS	2 minutes
100% IMS	2 minutes
100% IMS	2 minutes
Histoclear	3 minutes
Xylene	3 minutes
Xylene	3 minutes

Table 3: Protocol used to counterstain and dehydrate slides. DAB stained DMS-79 xenograft sections were counterstained and dehydrated in preparation for mounting in pertex.

2.6. Cloning

DH5α *Escherichia coli* (*E. coli*) cells were cultured in LB Broth (Sigma, Dorset, UK) and incubated at 37°C, 5% CO_2 for 16 hours. Cells were then streaked onto ampicillin selection plates (100µg/ml) and incubated overnight at 37°C. A single colony was isolated, re-suspended in LB Broth (Sigma) with 100µg/ml ampicillin and incubated at 37°C for 16 hours. The remaining colonies on the ampicillin selection plate were stored at 4°C.

2.7. Plasmid preparation

The Flp-In TRex system of expression relies on the stable integration of 4 plasmids into the host cell line and confers tetracycline-inducible expression of the gene of interest. The pFRT/lacZeo plasmid contains a Flp recombinase target (FRT) site which binds Flp recombinase, (an enzyme that cleaves DNA) and a lacZ-zeocin fusion gene which confers zeocin resistance to the cells. The pcDNA6/TR plasmid contains a bacterial tetracycline (Tet) repressor operon which controls expression of the gene of interest and a blasticidin resistance gene. The gene of interest is cloned into the pcDNA5/FRT plasmid under the control of the cytomegalovirus (CMV) promoter which is constitutively active. Finally the pOG44 plasmid contains the FLP recombinase enzyme under the control of the CMV promoter. pcDNA5/FRT and pOG44 are co-transfected into the cells leading to a recombination event between the FRT sites and inserts the pcDNA5/FRT plasmid, leading to the inactivation of the lacZ-Zeocin fusion gene, introduction of a hygromycin resistance gene and tetracycline-inducible expression of the gene of interest.

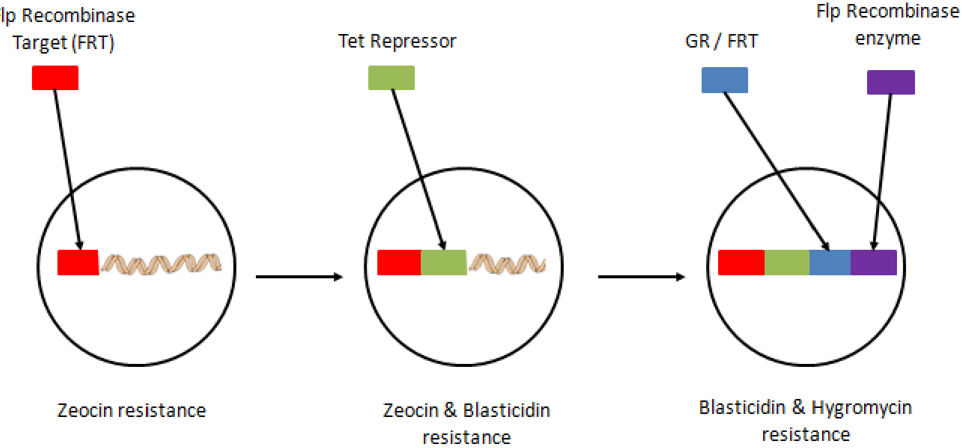


Figure 2.01: The Flp-In TRex system of expression relies on stable transfection of cells with 4 plasmids to introduce tetracycline-inducible expression of genes into cells. The host cell lines are first transfected with a plasmid containing a Flp-recombinase target (FRT) site and selected for using zeocin resistance. Stably transfected cells are then transfected with a plasmid containing a tetracycline (Tet) repressor which controls expression of the gene of interest and selected for using zeocin and blasticidin resistance. Finally the gene of interest and a second FRT site are co-transfected into cells along with a plasmid containing the Flp recombinase enzyme and selected for using blasticidin and hygromycin resistance.

2.7.1. Preparation of pcDNA5/FRT/TO and GR plasmids

NEB5α *E. coli* cells were transformed with the pcDNA5/FRT/TO plasmid and spread onto ampicillin selection plates (100µg/ml). The plates were incubated at 37°C, 5% CO_2 for 16 hours. Colonies were selected and suspended in LB Broth with 100µg/ml ampicillin and the cultures were agitated at 37°C, 5% CO_2 for 16 hours. Plasmid DNA was isolated using QIAgen Miniprep column (QIAgen) and the total DNA concentration of samples measured by NanoDrop measurement at 260nm absorbance.

The isolated plasmid DNA was then digested with restriction enzymes Xho1 and BamH1 for 3 hours at 37°C. The samples were mixed with loading dye and separated by electrophoresis on a 1% agarose gel. Bands were visualised using universal hood II (Bio-Rad, Hertfordshire, UK). Bands were excised from the gel, total sample DNA was isolated using MinElute columns (QIAgen) and DNA concentration measured using NanoDrop measurement at 260nm absorbance. Following excision of GR DNA from the pcDNA3.1 plasmid, pcDNA5/FRT/TO and GR DNA were transformed into NEB5α *E. coli* cells using a ratio of 3:1 pcDNA5/FRT/TO DNA: GR DNA. Transformed NEB5α cells were spread onto LB agar ampicillin selection plates (100µg/ml) and incubated at 37°C, 5% CO_2 for 16 hours.

2.7.2. Initial screening

DNA from samples confirmed to contain pcDNA5/FRT/TO/GR DNA was isolated from excised bands and transformed into DH5α *E. coli* cells. Cells were mixed with 5µl pcDNA5/FRT/TO/GR DNA and stored on ice before heat shocking at 42°C for 30 seconds. Cells were then suspended in LB broth and agitated at 37°C for 60 minutes before being spread onto ampicillin selection plates (100µg/ml) and incubated at 37°C, 5% CO_2 for 16 hours. Plates were then stored at 4°C.

2.7.3. Ethanol precipitation and sequencing

pcDNA5/FRT/TO/GR plasmid DNA was isolated from transformed DH5α *E. coli* colonies using Miniprep columns (QIAgen). Total DNA volume was made up to 100µl using TE buffer (10ml 1M Tris-Cl pH = 7.5, 2ml 500mM EDTA pH 8). 10µl of 3M sodium acetate buffer (3 M sodium acetate in ddH_2O, pH adjusted to 5.2 using glacial acetic acid) was added to equilibrate ion concentration and 250µl 100% ethanol. Samples were stored at - 20°C for 1 hour.

Samples were centrifuged at 14,000rpm for 30 minutes and the supernatant was discarded. Pellets were re-suspended in 250µl 70% ethanol and centrifuged at 14,000rpm for 20 minutes. The resulting supernatant was discarded and the remaining ethanol allowed to evaporate. Samples were then sent for DNA sequencing (Eurofins MWG Operons, London, UK).

2.8. Transfection of mammalian cells

Transfection of pcDNA5/FRT/TO and pOG44 plasmids into HeLa lacZeo/TO cells

HeLa lacZeo/TO cells were seeded at $1x10^5$/ ml and grown for 3 days. Cells were transfected using 3 µg, 6 µg or 9 µg of plasmid DNA, with pOG44 and pcDNA5/FRT/TO plasmids in a ratio of 9:1 and FuGENE HD reagent (Roche, Lewes, UK) in a 3:1 or 3:2 ratio of FuGENE: DNA. 24 hours after transfection, cells were treated with zeocin and blasticidin to provide selection pressure for successfully transfected cells.

2.8.1. Transfection of pFRT/lacZeo into DMS-79 cells

DMS-79 cells were seeded at $1x10^5$/ ml and incubated at 37°C, 5% CO_2 for 5 days. Cells were pipetted to break up aggregates 24 hours before transfection. DMS-79 cells were transfected using 6µg, 9µg or 12µg and FuGENE HD reagent in a 3:2 ratio of FuGENE:DNA. Cells were

treated with zeocin 48 hours after transfection to provide selection pressure for successfully transfected cells.

2.9. Statistics

Densitometry of western blots is presented as mean ± standard error of the mean (S.E.M) of n=3 independent experiments. Quantification of qPCR results is presented as mean ± SEM of 3 replicate readings of n=3 independent experiments.

Two sample T-tests were used to determine whether differences between two differently treated cell samples were significant. One way analysis of variance (ANOVA) with a post-hoc Tukey adjustment was used to determine whether differences between 3 or more differently treated cell samples were significant.

All statistical analyses were carried out using Minitab statistical and process management software, version 15. Statistical significance was indicated by $p \leq 0.05$ represented by *, $p \leq 0.01$ represented by ** and $p \leq 0.001$ represented by ***.

Chapter 3: Results 1

Regulation of GR in SCLC cells

Introduction

Small cell lung cancer is an extremely aggressive malignancy which typically metastasises early and although initially sensitive to treatment, tumours quickly develop resistance. Some SCLC tumours secrete hormones such as ACTH or more commonly its pro-hormone pro-opiomelanocortin (POMC), leading to ectopic ACTH syndrome. In pituitary cells this POMC secretion is under the control of a negative feedback loop, however the secretion of POMC in these SCLC cell lines is resistant to glucocorticoids.

GR protein levels in SCLC cells have been shown to be very low, allowing these cells to evade glucocorticoid-induced apoptosis; however the regulation of GR protein levels in these cells is not fully understood (Kay *et al*, 2011; Sommer *et al*, 2007). Investigation into the regulation of GR protein levels in SCLC cells may provide insight into mechanisms through which GR can be upregulated in these cells, conferring sensitivity to glucocorticoid-induced apoptosis.

3.1. GR protein levels in nSCLC and SCLC cell lines

Our group has previously shown that GR protein levels are significantly lower in SCLC cells than nSCLC cells (Kay *et al*, 2011). In order to investigate this further, I compared GR protein levels in a number of different nSCLC and SCLC cell lines (Fig. 3.01).

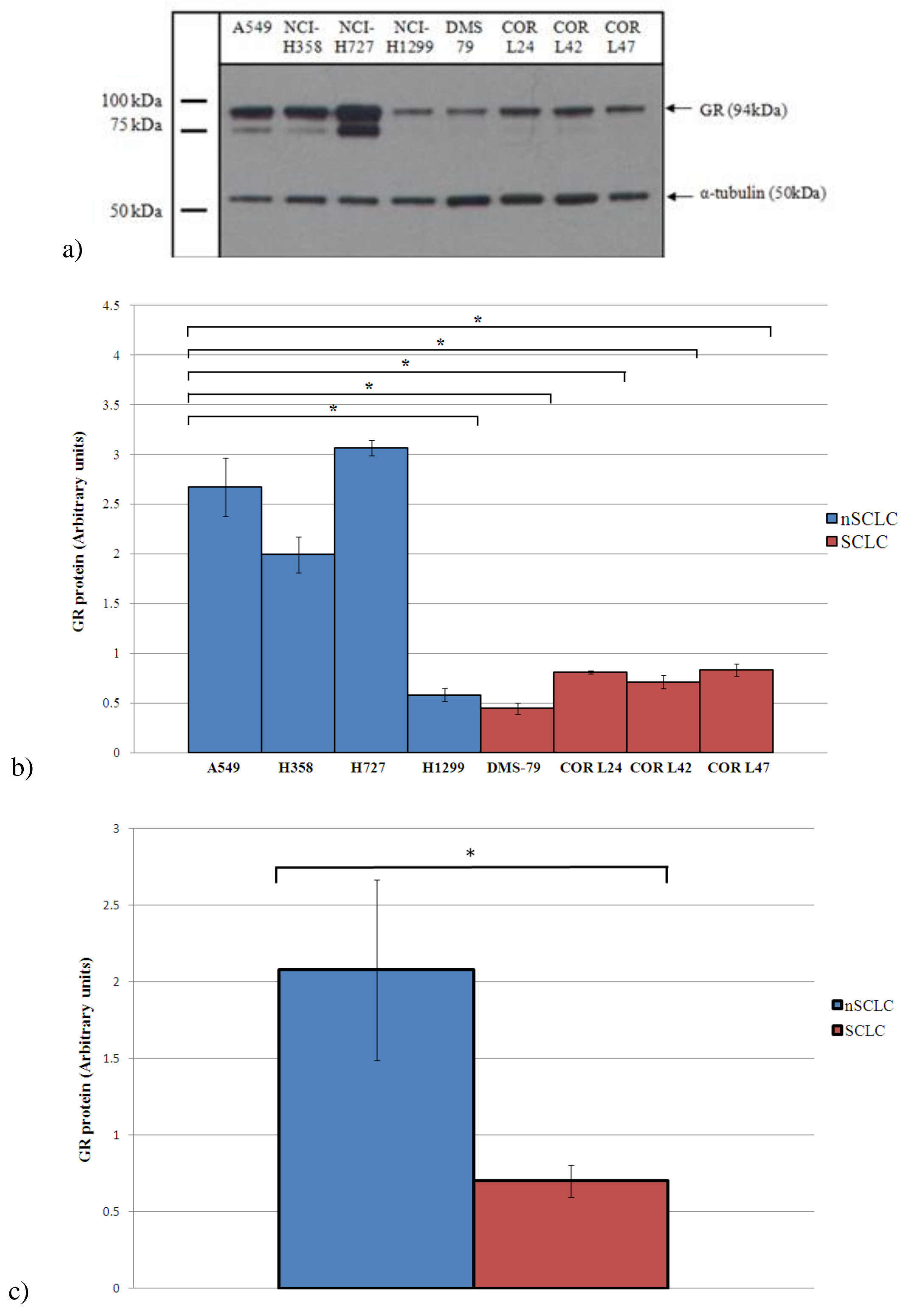


Figure 3.01: GR protein levels in nSCLC and SCLC cell lines. nSCLC cell lines (A549, NCI-H358, NCI-H727 and NCI-H1299) and SCLC cell lines (DMS-79, COR L24, COR L42 and COR L47) were seeded at 1×10^5 cells /ml and grown to 90% confluency. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) and c) Densitometry was used to quantify GR protein relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) n=3. Significance was calculated using a Student's t test comparing to A549 cells, * indicates $p \leq 0.05$.

Significantly higher levels of GR protein was detected in nSCLC cells compared to SCLC cell lines (Fig. 3.01). GR protein levels varied greatly between the nSCLC cell lines tested; NCI-H727 cells showed the highest levels of GR protein of all nSCLC cells tested while NCI-H1299 cells showed much lower levels of GR protein. In contrast, consistently low levels of GR protein were detected in SCLC cells.

Two bands were detected for GR in the nSCLC A549, NCI-H358 and NCI-H727 cell samples. The more prominent, heavier band is GRα (94kDa) and the lighter band is likely to be an alternative GR isoform. The detection of multiple bands is due to the antigen of the GR antibody used, which binds to amino acids 176-289 of the protein and is therefore able to detect various GR isoforms.

3.2. <u>Effect of glucocorticoid treatment on GR protein and GR-regulated genes in SCLC cells</u>

SCLC cells have previously been shown to be resistant to glucocorticoids (Ray *et al*, 1996). In order to investigate this further, DMS-79 cells were treated with dexamethasone to investigate the effect of glucocorticoid treatment on GR protein levels. Glucocorticoid-induced changes in GR and GR-regulated gene expression were also investigated.

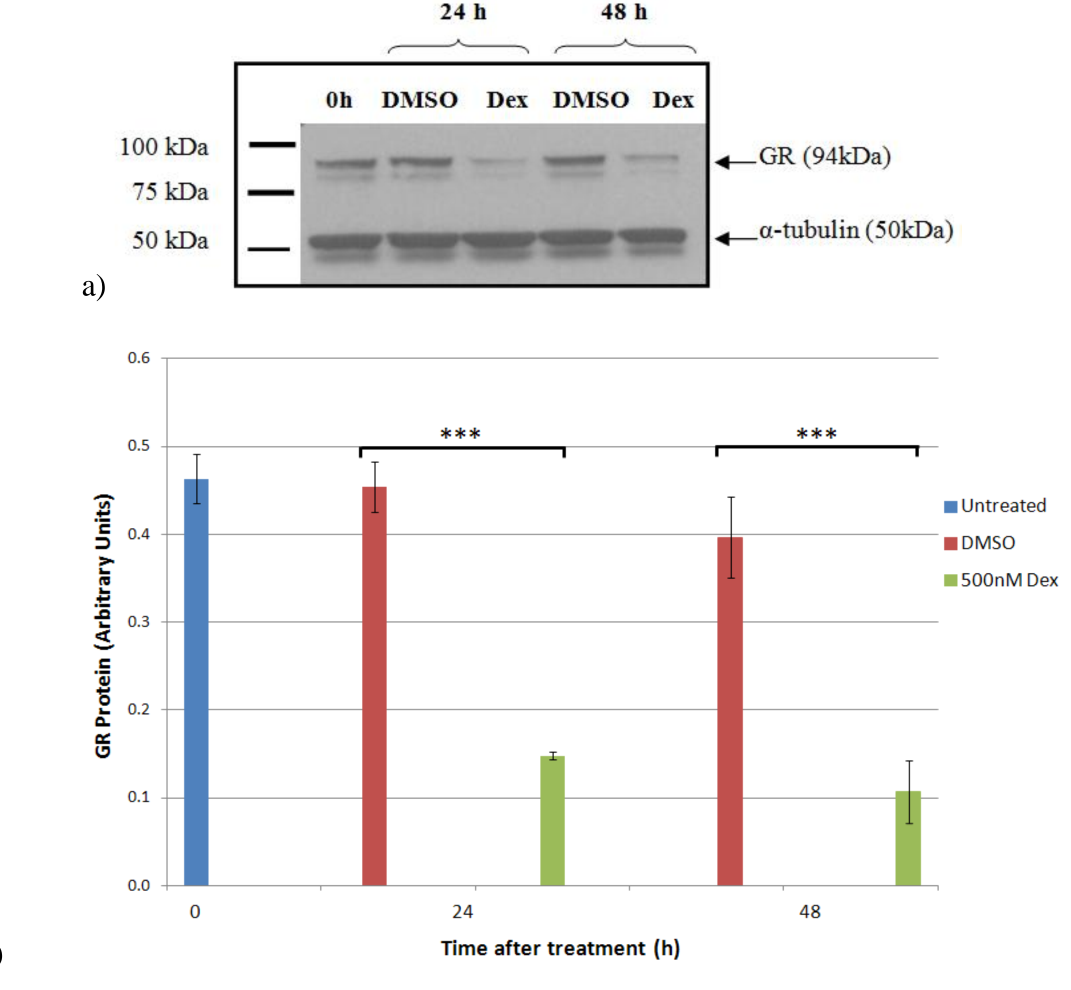


Figure 3.02: Effect of dexamethasone on GR protein levels in DMS-79 cells. DMS-79 cells were seeded at $1x10^5$ cells /ml and treated with 500nM dexamethasone and samples taken at 24 and 48 hours after treatment. a) Samples were analysed by western blot for GR and α-tubulin. b) Densitometry was used to quantify GR expression relative to α-tubulin loading. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. *** where $p \leq 0.001$.

Figure 3.02 shows a significant reduction in GR protein levels in DMS-79 cells following treatment with 500nM dexamethasone, compared to cells treated with DMSO ($p \leq 0.001$). This effect was seen following 24 and 48 hour dexamethasone treatment. Two bands for GR were detected in all samples; the heavier band is GRα (94kDa) and the lighter band is likely an alternative GR isoform such as GRδ (74kDa), a truncated form of the protein identified within DMS-79 cells.

qPCR was performed to quantify changes in GR gene expression following dexamethasone treatment of DMS-79 cells. Glucocorticoid-induced changes in gene expression of GR-

regulated genes pro-opiomelanocortin (POMC, downregulated by GR in glucocorticoid-sensitive cells) and glucocorticoid-induced leucine zipper (GILZ, upregulated by GR in glucocorticoid-sensitive cells) was also investigated.

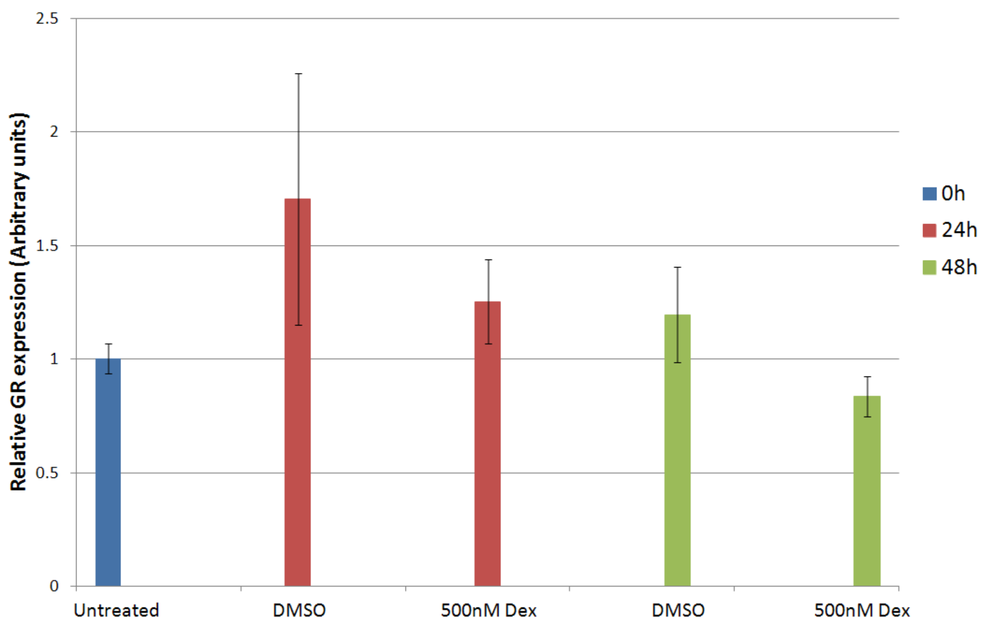


Figure 3.03: Effect of dexamethasone on GR gene expression in DMS-79 cells. DMS-79 cells were treated with 500nM dexamethasone and samples taken at 24 and 48 hours after treatment. cDNA was analysed by quantitative PCR for GR and GAPDH as an endogenous control gene. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

No significant changes in GR gene expression were seen following treatment of cells with 500nM dexamethasone (Fig. 3.03). This suggests that the decrease in GR protein level observed following dexamethasone treatment of DMS-79 cells is not due to a reduction in GR gene expression within these cells.

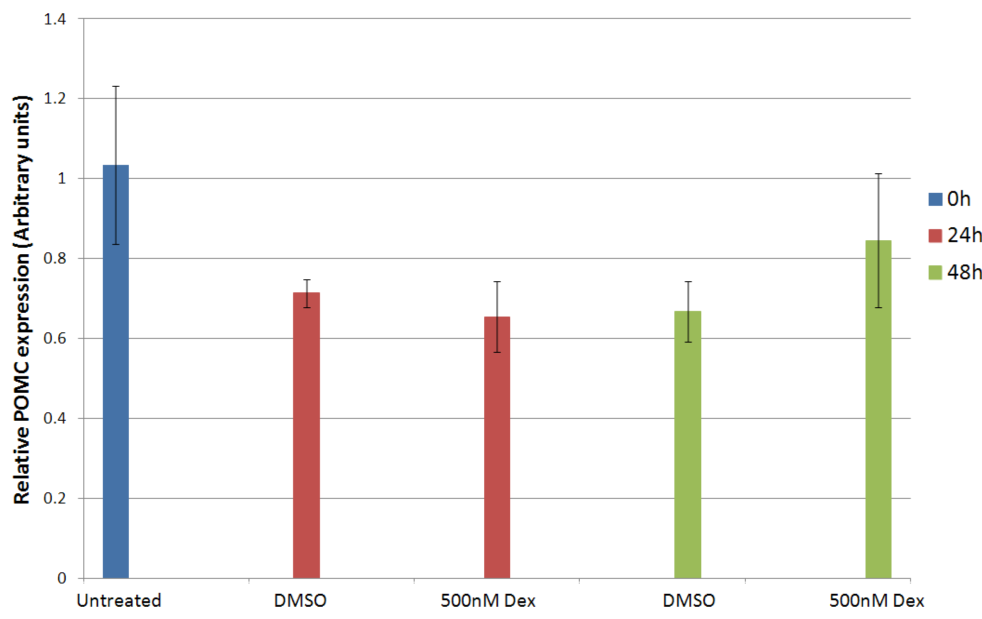


Figure 3.04: Effect of dexamethasone on POMC gene expression in DMS-79 cells. DMS-79 cells were treated with 500nM dexamethasone and samples taken at 24 and 48 hours after treatment. cDNA was analysed by quantitative PCR for POMC and GAPDH as an endogenous control gene. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

Figure 3.04 shows no significant changes in POMC gene expression following treatment of DMS-79 cells with 500nM dexamethasone over 48 hours compared to cells treated with DMSO.

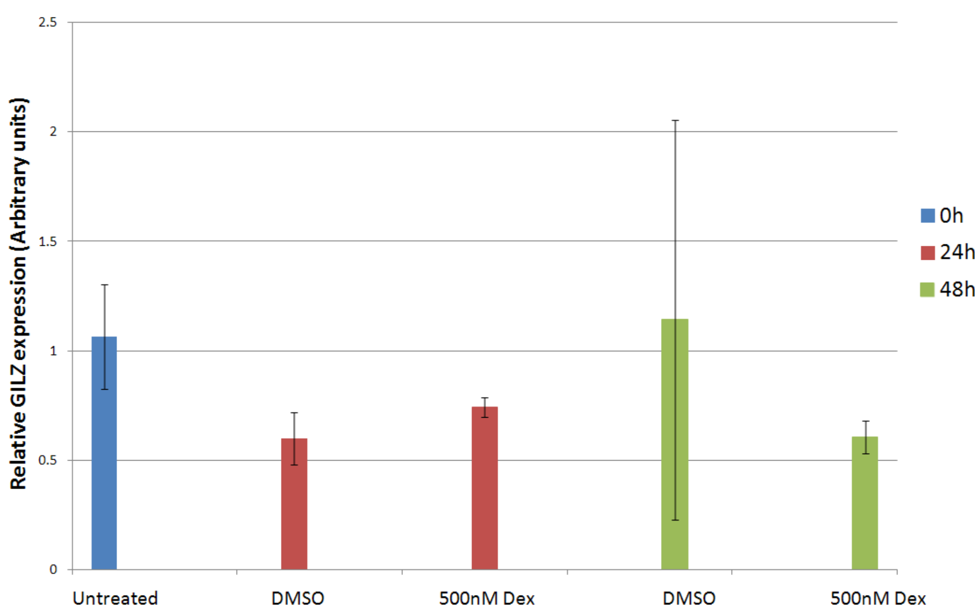


Figure 3.05: Effect of dexamethasone on GILZ expression in DMS-79 cells. DMS-79 cells were treated with 500nM dexamethasone and samples taken at 24 and 48 hours after treatment. cDNA was analysed by quantitative PCR for GILZ and GAPDH as an endogenous control gene. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

No significant changes in GILZ gene expression were seen following treatment of DMS-79 cells with 500nM dexamethasone over 48 hours compared to cells treated with DMSO (Fig. 3.05).

3.3. Effect of time in culture on GR

While glucocorticoid treatment of DMS-79 cells was seen to significantly decrease GR protein levels (Fig. 3.02), GR gene expression in these cells was not affected (Fig. 3.03). Therefore I wanted to further investigate the regulation of GR protein levels in these cells, firstly the effect of time in culture on GR protein levels in DMS-79 cells. Here, nSCLC cell line A549 cells were used as a control.

DMS-79 cells

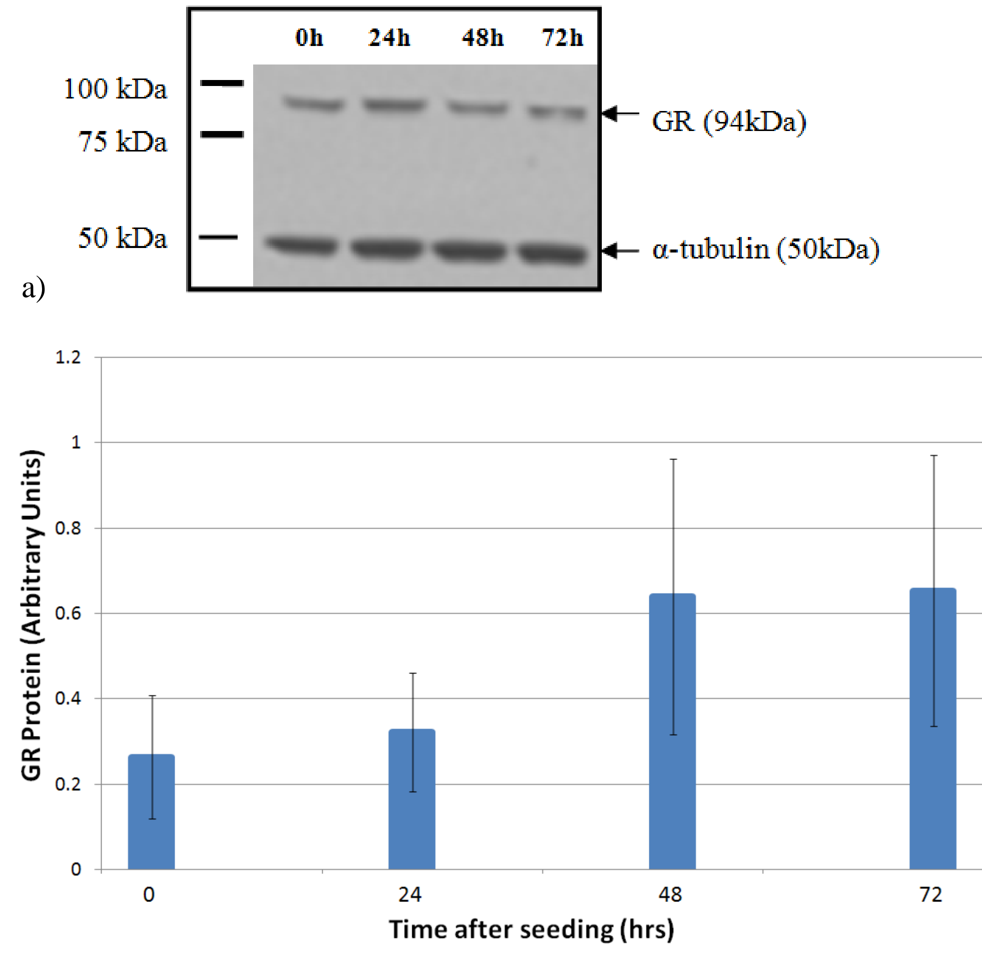


Figure 3.06: GR protein in DMS-79 cells over 72 hours in culture. DMS-79 cells were seeded at $1x10^5$/ml and samples taken at 0, 24, 48 and 72 hours after seeding. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

A549 cells

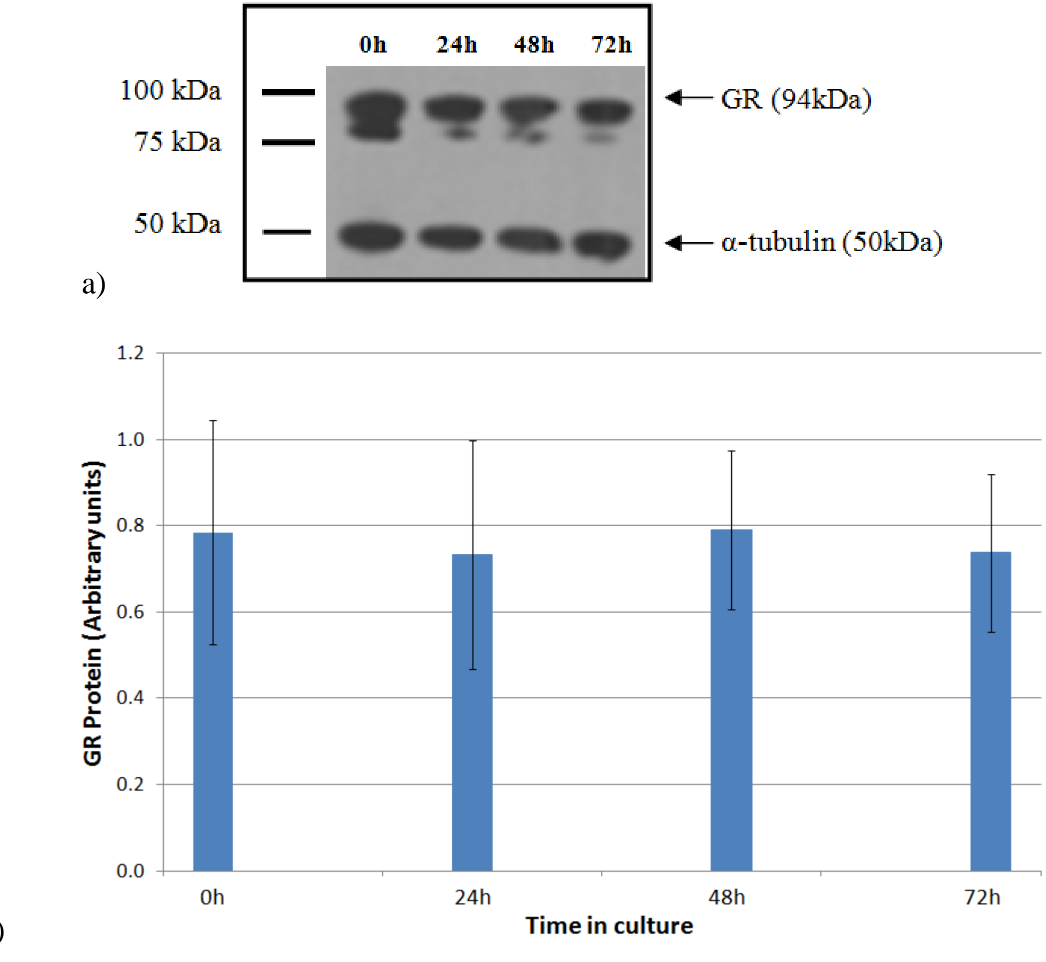


Figure 3.07: GR protein in A549 cells over 72 hours in culture. A549 cells were seeded at $1x10^5$/ml and samples taken at 0, 24, 48 and 72 hours after seeding. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. * where $p \leq 0.05$.

Figures 3.06 and 3.07 showed no significant difference in GR protein levels in SCLC cell line DMS-79 cells or nSCLC cell line A549 cells over 72 hours in culture.

3.4. <u>Effect of varying glucocorticoid concentration on GR</u>

In most cell lines, GR is ubiquitinated following ligand binding and targeted for degradation by the proteasome. Steady state GR expression is therefore regulated by glucocorticoid availability. In order to investigate the sensitivity of SCLC cell lines to glucocorticoids, DMS-79 cells were treated with a range of concentrations of dexamethasone or hydrocortisone and samples taken 48 hours after treatment. A549 cells were again used as a control cell line.

3.4.1. Effect of dexamethasone on GR protein levels in DMS-79 cells

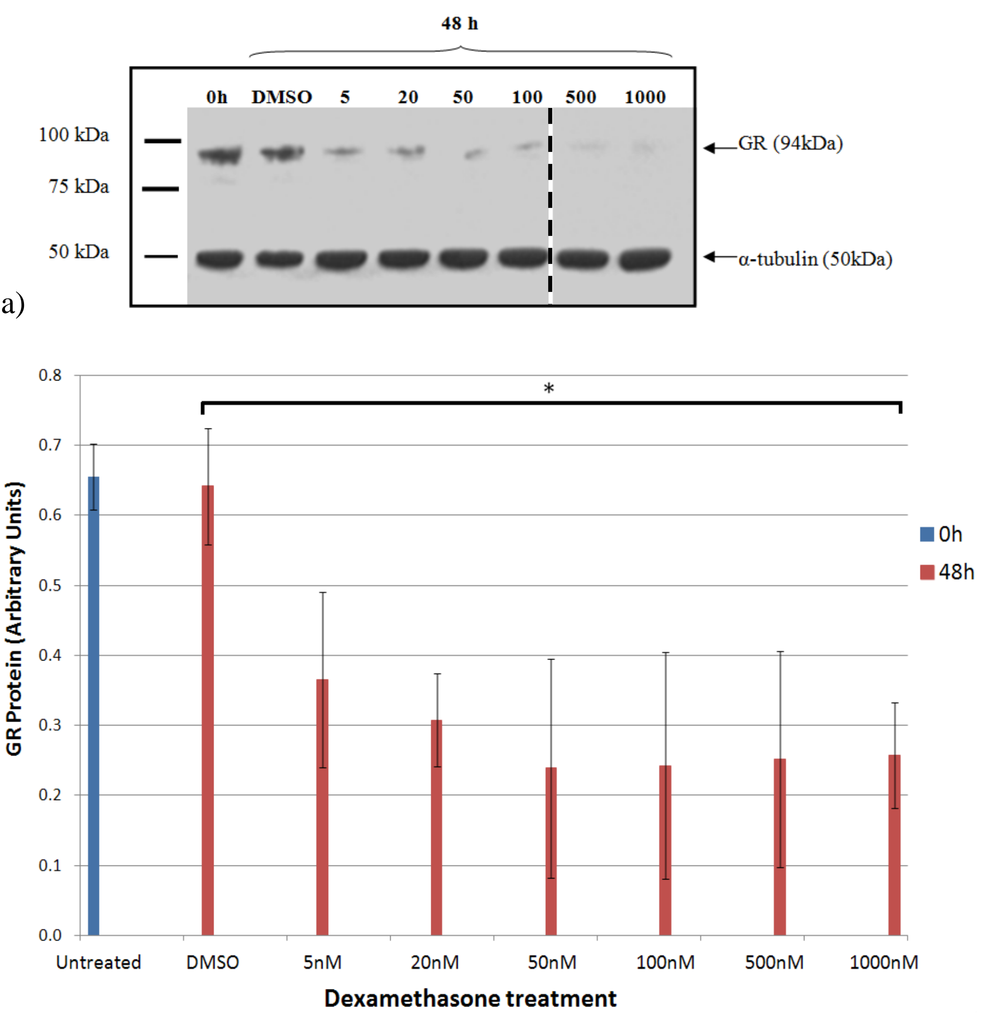


Figure 3.08: Effect of 5-1000nM dexamethasone treatment on GR in DMS-79 cells over 48 hours. DMS-79 cells were treated with dexamethasone (5-1000nM) for 48 hours. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. Data was analysed using ANOVA with a post-hoc TUKEY adjustment, * where $p \leq 0.05$. Dotted line indicates exclusion of some data from the figure.

Dexamethasone treatment for 48 hours showed a trend to decrease GR protein expression in DMS-79 cells (Fig. 3.08). A significant decrease in GR protein levels was observed following treatment of cells with 1000nM dexamethasone.

3.4.2. Effect of dexamethasone on GR protein levels in A549 cells

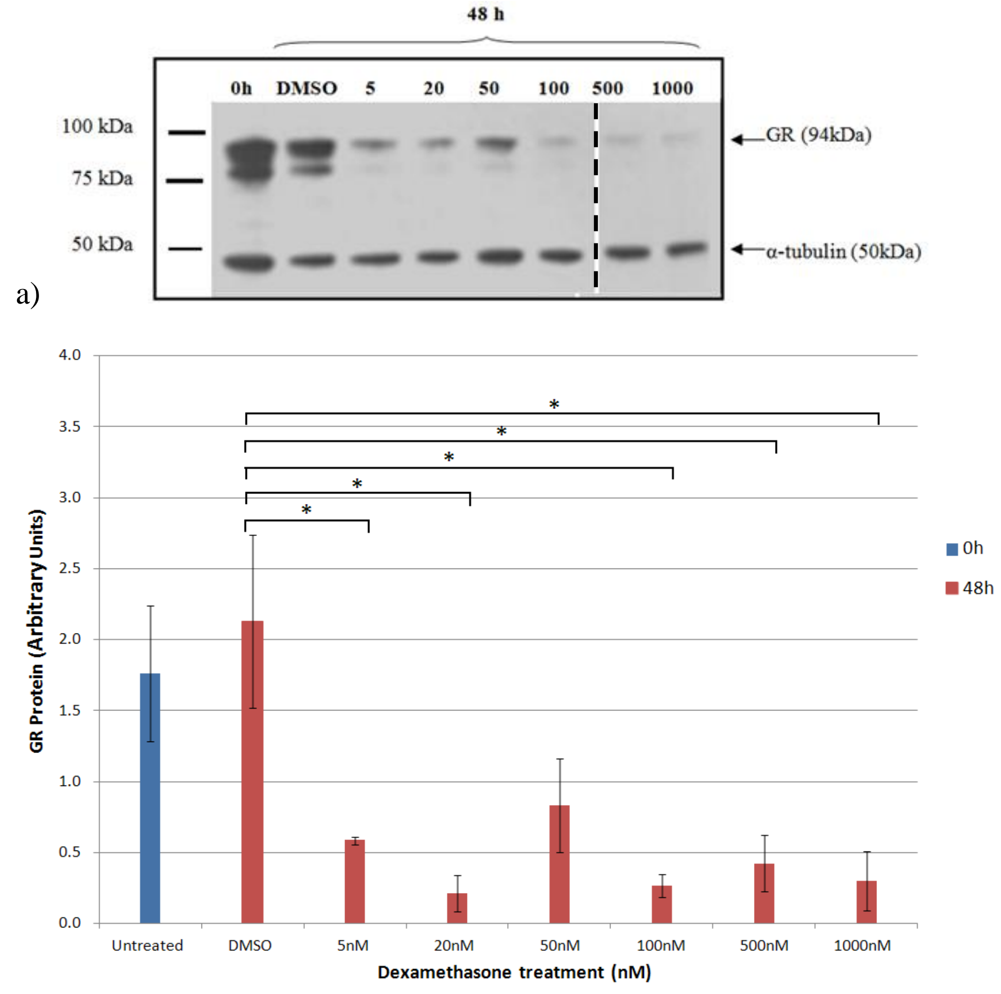


Figure 3.09: Effect of 5-1000nM dexamethasone treatment on GR in A549 cells over 48 hours. A549 cells were treated with dexamethasone (5-1000nM) for 48 hours. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. Data was analysed using ANOVA with a post-hoc Tukey adjustment, * where $p \leq 0.05$. Dotted line indicates exclusion of some data from the figure.

A significant decrease in GR protein levels was observed following treatment of A549 cells with 5nM dexamethasone for 48 hours (Fig. 3.09). All dexamethasone concentrations tested caused a significant decrease in GR protein, with the exception of 50nM dexamethasone. This was mostly likely due to variation between replicates.

3.4.3. Effect of hydrocortisone on GR protein levels in DMS-79 cells

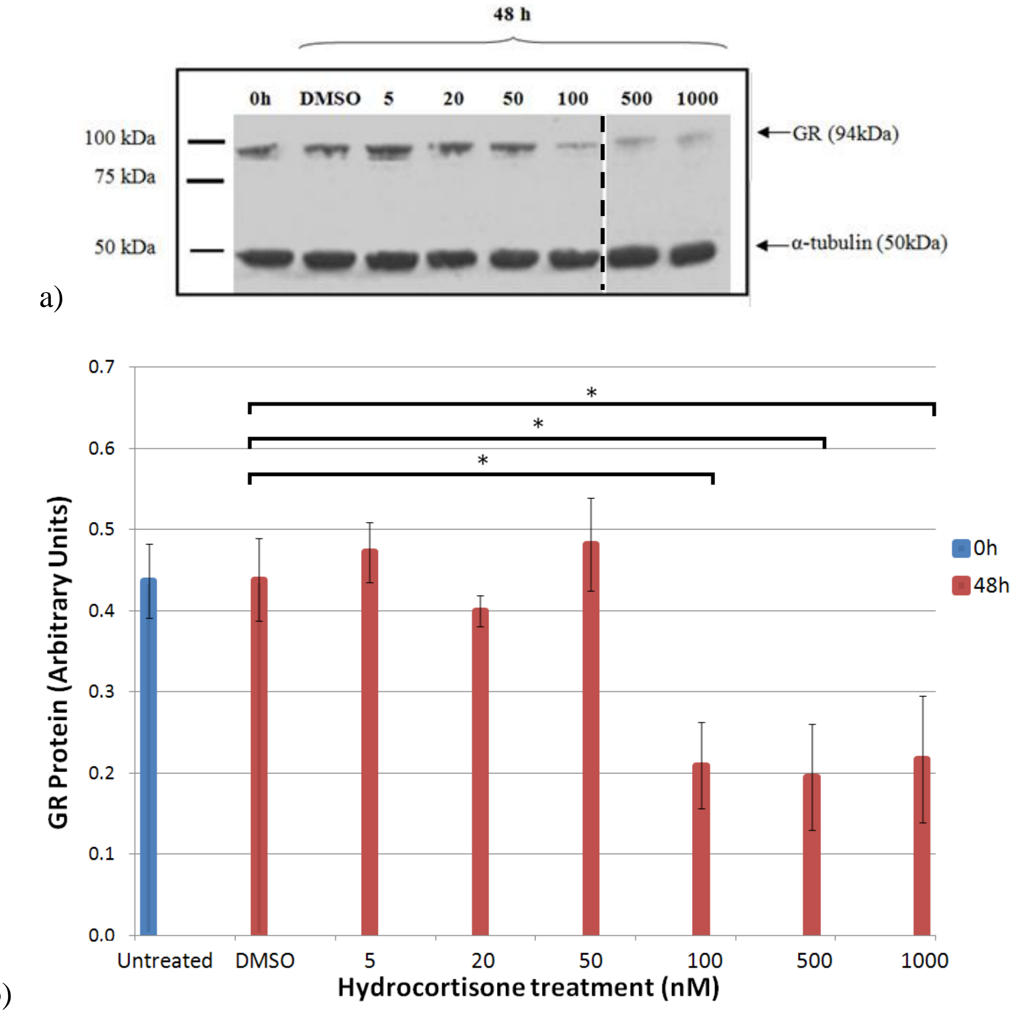


Figure 3.10: Effect of 5-1000nM hydrocortisone treatment on GR in DMS-79 cells over 48 hours. DMS-79 cells were treated with hydrocortisone (5-1000nM) for 48 hours. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. Data was analysed using ANOVA with a post-hoc TUKEY adjustment, * where $p \leq 0.05$. Dotted line indicates exclusion of some data from the figure.

Figure 3.10 shows that treatment of DMS-79 cells with 100nM, 500nM or 1000nM hydrocortisone for 48 hours leads to a significant decrease in GR protein levels. Lower concentrations of hydrocortisone (0-50nM) had no significant effect on GR protein levels in these cells. This may be due to overexposure of this blot, which can conceal differences in loading and make accurate comparisons between samples difficult, however this was necessary to detect extremely low levels of GR protein.

3.4.4. Effect of hydrocortisone on GR protein levels in A549 cells

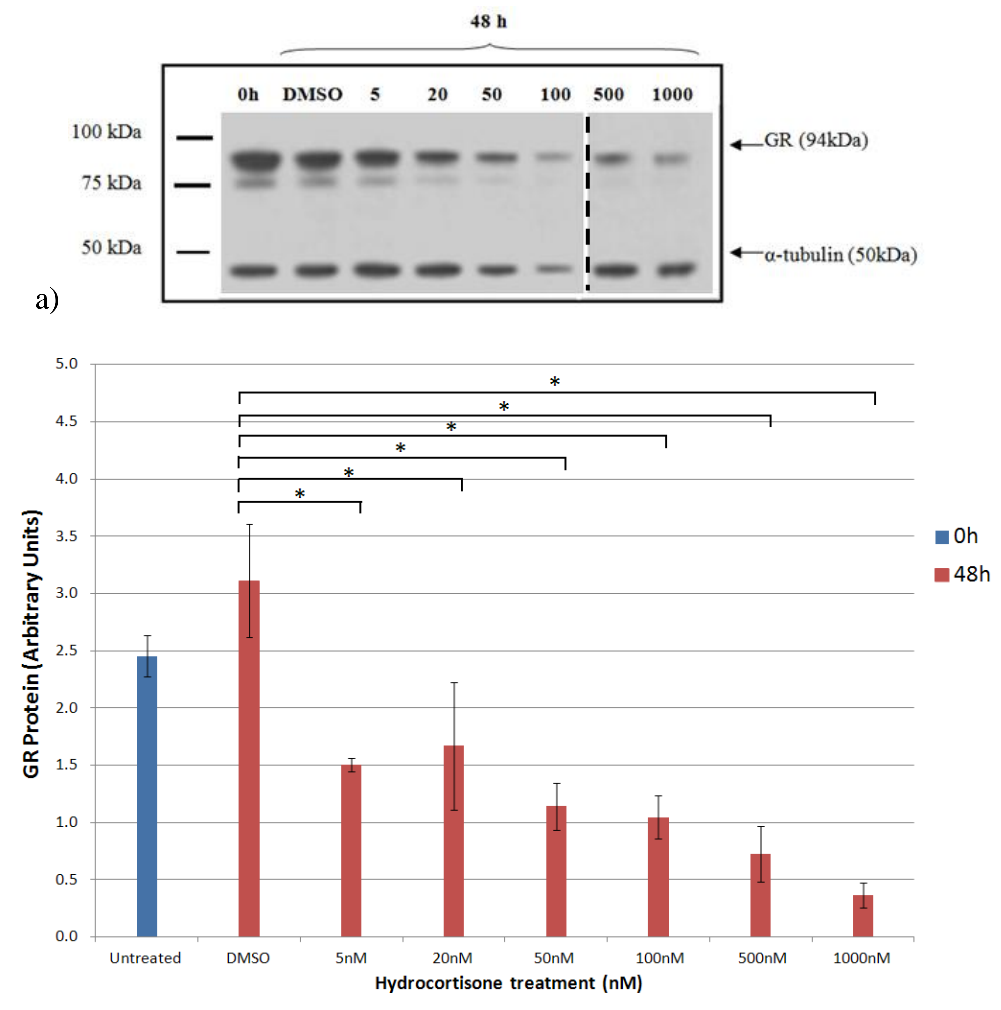


Figure 3.11: Effect of 5-1000nM hydrocortisone treatment on GR in A549 cells over 48 hours. A549 cells were treated with hydrocortisone (5-1000nM) for 48 hours. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. Data was analysed using ANOVA with a post-hoc TUKEY adjustment, * where $p \leq 0.05$. Dotted line indicates exclusion of some data from the figure.

A significant decrease in GR protein levels was seen in A549 cells following 48 hour treatment with all hydrocortisone concentrations tested (Fig. 3.11).

3.5. Recovery of GR protein after removal of glucocorticoid treatment

I then wanted to investigate the longevity of the glucocorticoid-induced decrease in GR protein levels observed in DMS-79 cells and determine whether and how quickly GR protein levels recovered to that of untreated cells. In order to do this, DMS-79 cells were treated with dexamethasone for 48 hours then washed to remove glucocorticoids.

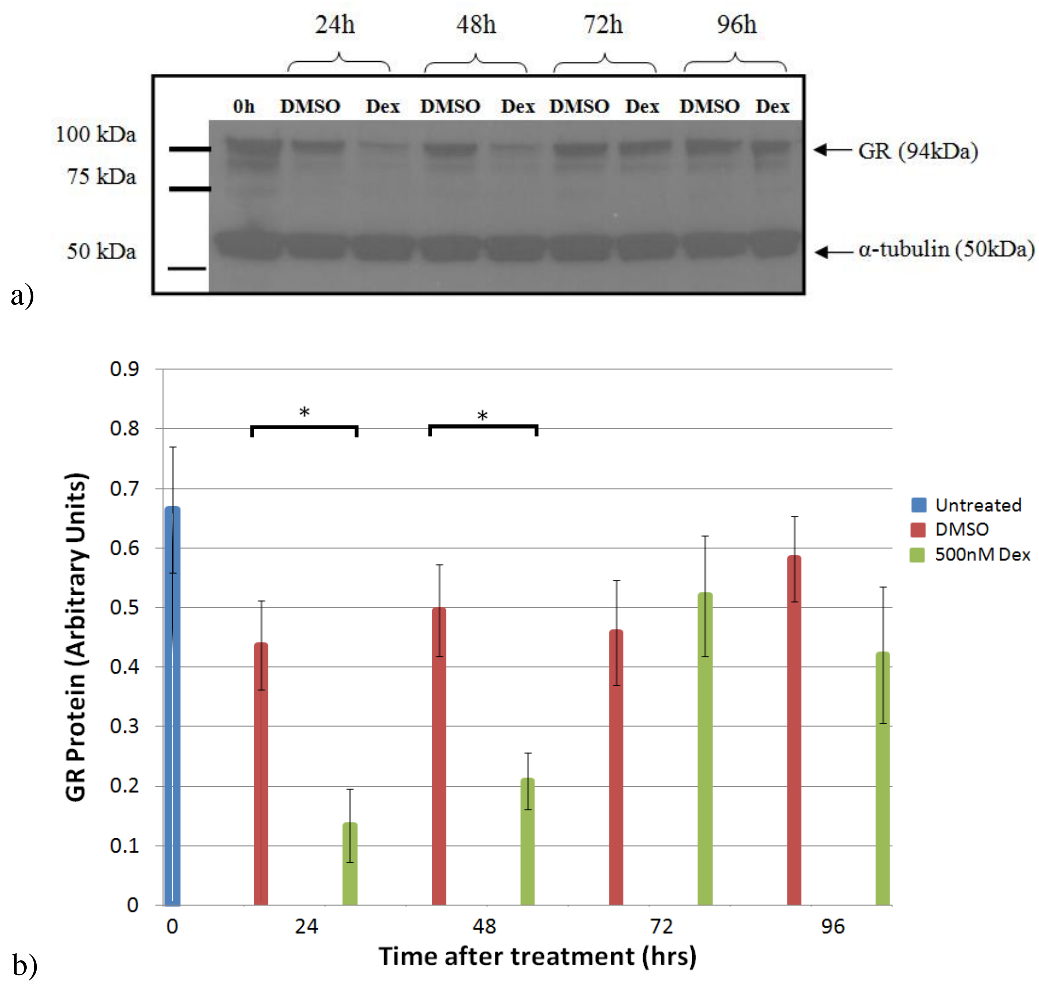


Figure 3.12: GR protein in DMS-79 cells treated with 500nM dexamethasone and washed after 48 hours. DMS-79 cells were treated with either DMSO or 500nM dexamethasone and samples were taken 24 and 48 hours after treatment. Cells were then pelleted and washed twice with serum free media before re-suspension of cells to the original volume. Samples were taken 72 and 96 hours after glucocorticoid treatment. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. * where $p \leq 0.05$.

Figure 3.12 shows a significant decrease in the level of GR protein in DMS-79 cells treated with 500nM dexamethasone for 24 and 48 hours. Following removal of dexamethasone, the level of GR protein detected in DMS-79 cells was not significantly different to that of cells

treated with DMSO. This suggests that although GR protein levels are reduced by dexamethasone treatment, this decrease is not permanent and upon removal of dexamethasone GR protein levels recover to that of untreated cells within 24 hours.

Chapter 4: Results 2

GR protein expression in SCLC cell xenografts

Introduction

SCLC tumours have been shown to be heterogeneous and cells within each tumour vary in morphology, phenotypic characteristics and rate of proliferation (Stovold et al, 2013). The function of this heterogeneity is not fully understood, however cells of a particular phenotype may be more likely to metastasise or be resistant to various therapy options.

I have shown that GR protein is tightly regulated at low levels in SCLC cells *in vitro*. In order to further investigate GR protein levels in these cells, I wanted to use an *in vivo* model. Subcutaneous DMS-79 xenografts were sectioned and stained for GR with a view to understanding the significance of GR in the context of tumour heterogeneity and the effect of irradiation on GR protein expression in these cells.

4.1. GR protein expression

A549 cell pellets and DMS-79 tumour sections were stained for GR to investigate whether GR can be observed in xenografts by this method and whether the protein is expressed homogeneously or heterogeneously in tumours. A549 nSCLC cell pellets were used as a GR positive control and to assess the sensitivity of the antibody. DMS-79 xenograft tumours stained using a non-specific IgG antibody at the same concentration as the GR antibody were used as a negative control.

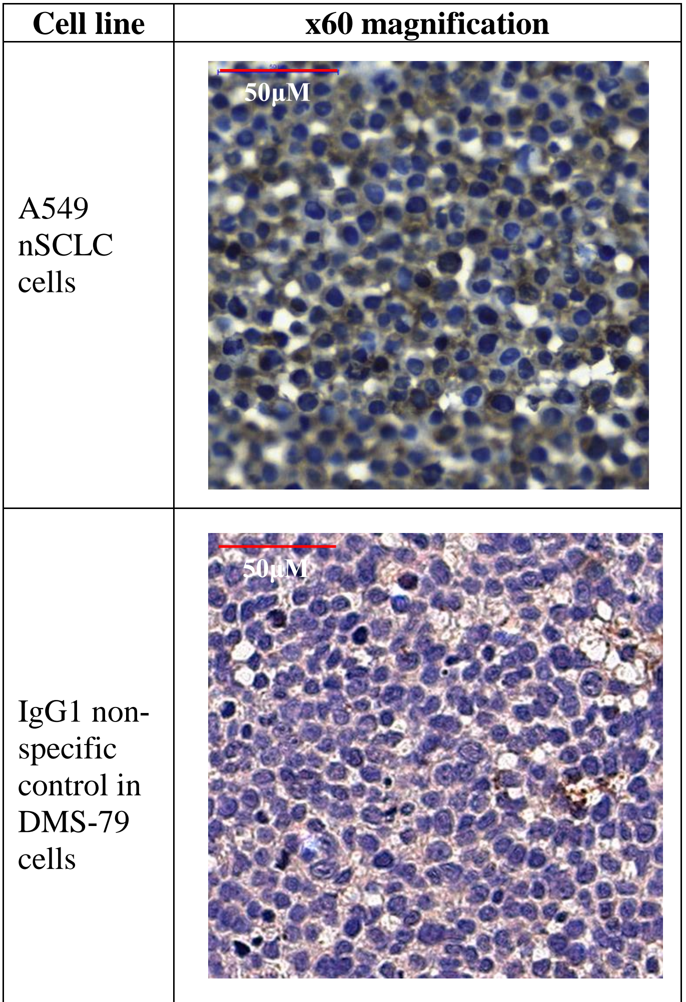


Figure 4.01: GR expression in A549 nSCLC cells and IgG1 non-specific control in DMS-79 tumours. A549 and DMS-79 cells were pelleted and embedded in paraffin wax. Cell pellets were sliced into 5µm sections and stained for GR. Nucleii were counterstained with haematoxylin.

GR protein was strongly detected in all A549 nSCLC cells, as indicated by the dark brown staining (Fig. 4.01). DMS-79 tumours when stained using a non-specific IgG antibody as a negative control showed very weak background staining.

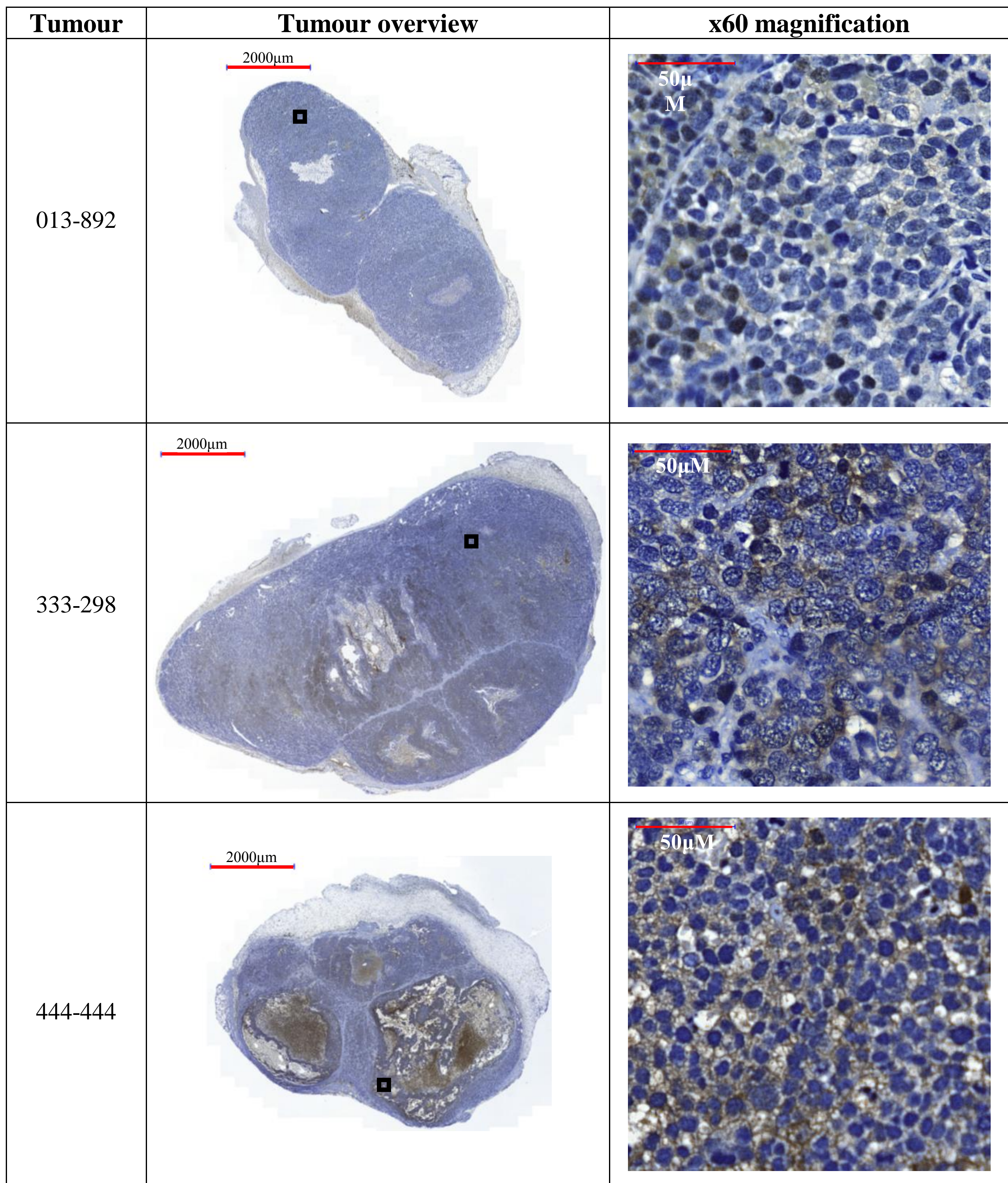


Figure 4.02: GR expression in DMS-79 xenografts. Tumour xenografts were sliced into 5µm sections and stained for GR. Nucleii were counterstained with haematoxylin.

All DMS-79 xenografts tested showed weak positive GR staining and GR protein was detected in virtually all cells. These cells have a mutation in one allele of the GR gene which generates an in frame termination codon within exon 7. This gives rise to a truncated GR protein which lacks the ligand-binding domain, known as GRδ or GR-P (Gaitan *et al*, 1995). The positive staining observed in DMS-79 xenograft cells reflects the presence of GR and GRδ which is unable to bind to glucocorticoids. Some variation in staining intensity was seen between cells within each xenograft, suggesting that some cells may express higher levels of GR than others. However, the GR staining observed in the DMS-79 tumours was very similar to the background levels observed using a non-specific IgG control. This makes it difficult to determine whether this method accurately indicates GR positive expression in the SCLC tumours. Further optimisation with other antibodies to GR would be required to prove whether there is staining above the non-specific seen with IgG.

4.2. Effect of xenograft irradiation on GR protein expression

Currently a combination of chemotherapy and radiotherapy is used to treat patients with SCLC. Radiotherapy is effective since exposure to radiation damages DNA and slows the rate of tumour growth. SCLC tumours are very aggressive and although initially sensitive to therapy, quickly become resistant. Since SCLC tumours have been shown to have a heterogeneous phenotype, some cells may be more sensitive to irradiation than others, leaving a small number of irradiation-resistant cells to continue to grow (Stovold *et al*, 2013). Therefore despite GR expression being difficult to see in the untreated tumours, I wanted to investigate whether irradiation of DMS-79 cell xenografts would increase GR protein expression in these cells and thereby increase sensitivity to glucocorticoid-induced apoptosis.

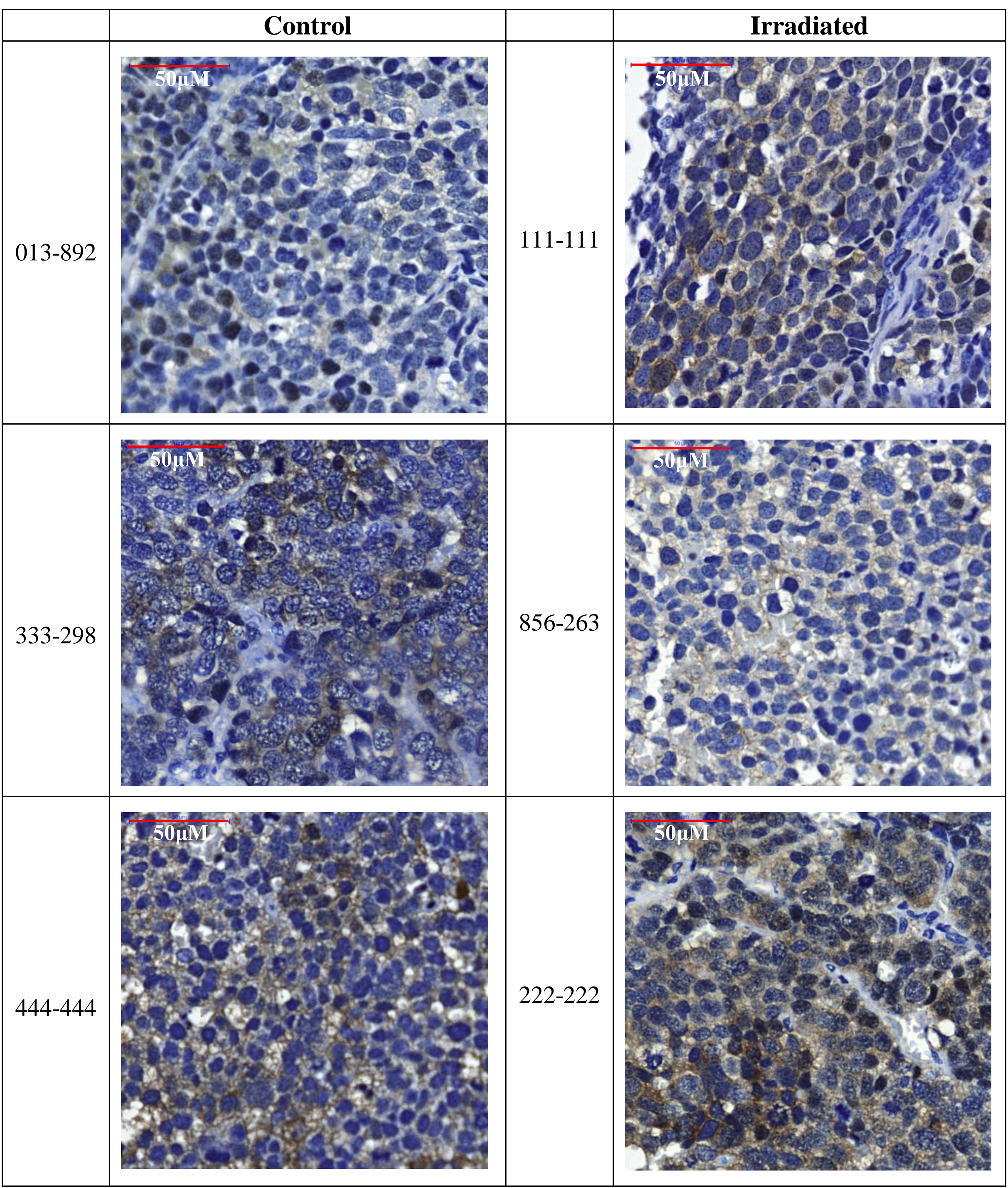


Figure 4.03: GR expression in irradiated DMS-79 xenografts. Control and irradiated DMS-79 tumour xenografts were sliced into 5μm sections and stained for GR. Nucleii were counterstained with haematoxylin.

Generally, weak GR staining was observed in both control and irradiated DMS-79 tumour xenografts. Some variation in GR protein levels was observed within xenografts, with some tumour areas showing darker positive staining than others (figure 4.03, 111-111 and 222-222 particularly), but this again is difficult to quantify using this method of detection. Over all the

tumour sections, the level of GR protein detected within irradiated tumours appeared to be similar to that of control tumours. This suggests GR positive cells are not significantly affected by irradiation treatment.

Chapter 5: Results 3

GR auto-upregulation

Introduction

SCLC cells have been shown to express low levels of GR protein and this is known to decrease the propensity of these cells to undergo glucocorticoid-induced apoptosis. Over-expression of GR in SCLC cells has been shown to restore glucocorticoid sensitivity to these cells (Sommer *et al*, 2007; Sommer *et al*, 2010). The mechanisms underlying glucocorticoid-induced apoptosis are not fully understood, specifically the immediate downstream targets of GR which lead to initiation of apoptotic pathways.

GR is known to regulate expression of Bcl-2 family proteins, which play an important role in apoptosis (Kay *et* al, 2011). Transfection of cells to overexpress GR prevents investigation into the immediate downstream targets of GR, since it is difficult to differentiate between the genetic changes induced by increased GR expression and those induced by the aggressive transfection process itself. Therefore in order to investigate the specific pathways, I looked at alternative mechanisms of upregulating GR protein levels in SCLC cells.

In acute lymphoblastic leukaemia (ALL) cells, GR gene expression arising from promoter 1A is regulated by binding of transcription factors c-Myb and c-Ets (Geng & Vedeckis, 2005). Binding of GR and c-Myb to promoter 1A triggers auto-upregulation of GR expression while binding of GR and c-Ets represses GR auto-upregulation. I wanted to investigate whether this mechanism could be induced in SCLC cells.

5.1. GR promoter usage in DMS-79 cells

The GR gene contains 9 alternative promoters which control expression of 9 alternative unexpressed first exons and GR expression arising from these is known to be differentially regulated in different cell lines (Breslin *et al*, 2001; Turner *et al*, 2005; Presul *et al*, 2007). In order to investigate which GR promoters contribute to total GR gene expression in DMS-79 cells, primers were designed to amplify mRNA spanning each exon 1: exon 2 boundary. PCR was then performed to detect expression of GR mRNA containing each alternative exon 1 sequence.

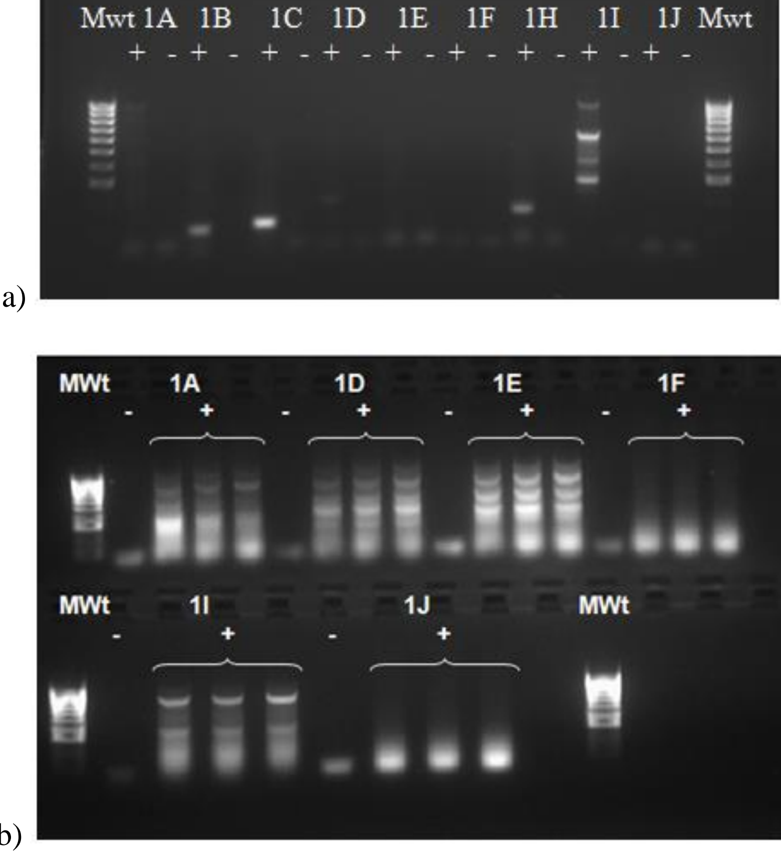


Figure 5.01: Testing of GR exon 1 primers in pooled cDNA samples. RNA was extracted from a number of different cell lines and pooled (SCLC cell lines; DMS-79, COR L24 and COR L47 cells, nSCLC cell line A549 cells, HeLa cells and HEK-293 cells). RNA was reverse transcribed and PCR was performed to detect the presence of GR mRNA containing each alternative exon 1 sequence. a) PCR samples run on a 1% gel. b) Temperature gradient PCR to optimise primer binding, samples run on a 1% gel.

Primers designed to amplify GR exons 1F and 1J could not detect a PCR product, while multiple PCR products were detected using primers designed to amplify GR exons 1A, 1D, 1E and 1I. However, primers designed to amplify GR exons 1B, 1C and 1H were able to detect a single, specific PCR product. Therefore these were tested for efficiency and subsequently used to investigate GR promoter usage in DMS-79 cells.

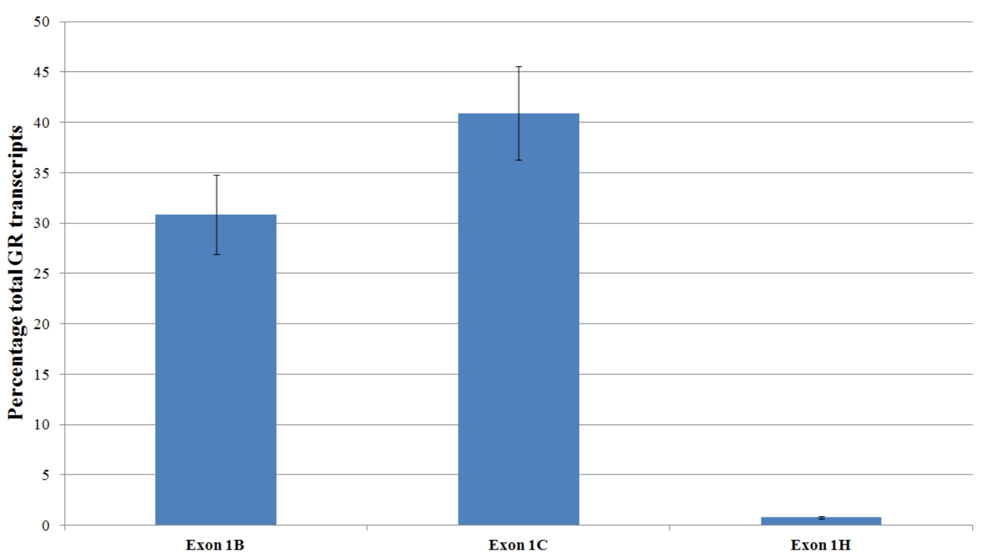


Figure 5.02: Percentage of total GR transcripts containing exons 1B, 1C and 1H in DMS-79 cells. DMS-79 cells were seeded at $1x10^5$/ml and grown for 5 days. RNA was extracted from samples and reverse transcribed to cDNA. Quantitative PCR was carried out to detect total GR and GR mRNA transcripts containing exons 1B, 1C and 1H. Data is displayed as percentage of total GR gene expression arising from each promoter. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

GR promoters contribute differentially to total GR gene expression in DMS-79 cells. On average, over 40% of total GR gene expression arises from promoter 1C and over 30% arises from promoter 1B. Transcription arising from GR promoter 1H is extremely low compared to the other GR promoters tested, with only 1% of total GR mRNA transcripts containing exon 1H.

5.2. GR protein levels in DMS-79 and CEM C7 cells

In the ALL cell line CEM C7, upon addition of ligand, GR and c-Myb bind to the GR gene and trigger auto-upregulation of GR gene expression from promoter 1A. I first wanted to compare the levels of GR protein in DMS-79 and CEM C7 cells and investigate the effect of glucocorticoid treatment on these cells. Although α-tubulin was used as a loading control in previous experiments, the masses of c-Ets and α-tubulin are very similar (50kDa) meaning the bands would be too close together to accurately distinguish. Therefore β-actin (36kDa) was used as a loading control in this experiment.

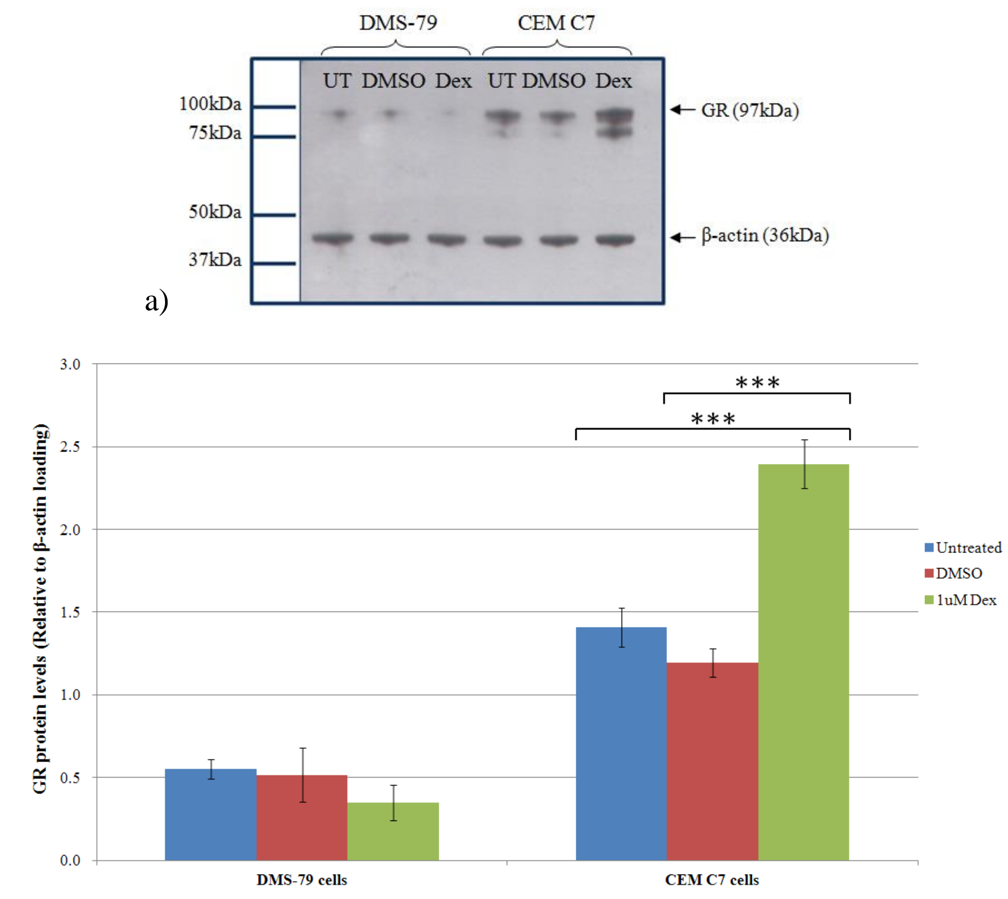


Figure 5.03: GR protein levels in DMS-79 and CEM C7 cells following 24 hours glucocorticoid treatment. DMS-79 and CEM C7 cells were treated with either DMSO or 500nM dexamethasone and samples were taken 24 hours after treatment. a) Samples were analysed by western blot for GR and β-actin as a loading control. b) Densitometry was used to quantify GR expression relative to β-actin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. *** where $p \leq 0.005$.

DMS-79 cells express significantly lower levels of GR than CEM C7 cells (Fig. 5.03). This was expected since SCLC cell lines are known to express low levels of GR protein. Following glucocorticoid treatment, a decrease in GR protein levels is seen in DMS-79 cells. Again, this was expected and supports the findings of the previous chapters. GR gene expression is upregulated in CEM C7 cells following glucocorticoid treatment and a significant increase in GR protein levels was observed.

5.3. c-Myb protein levels in DMS-79 and CEM C7 cells

Regulation of GR gene expression in ALL cells relies on the binding of c-Myb or c-Ets to the GR gene. I therefore wanted to compare the levels of these transcription factors in DMS-79 and CEM C7 cells.

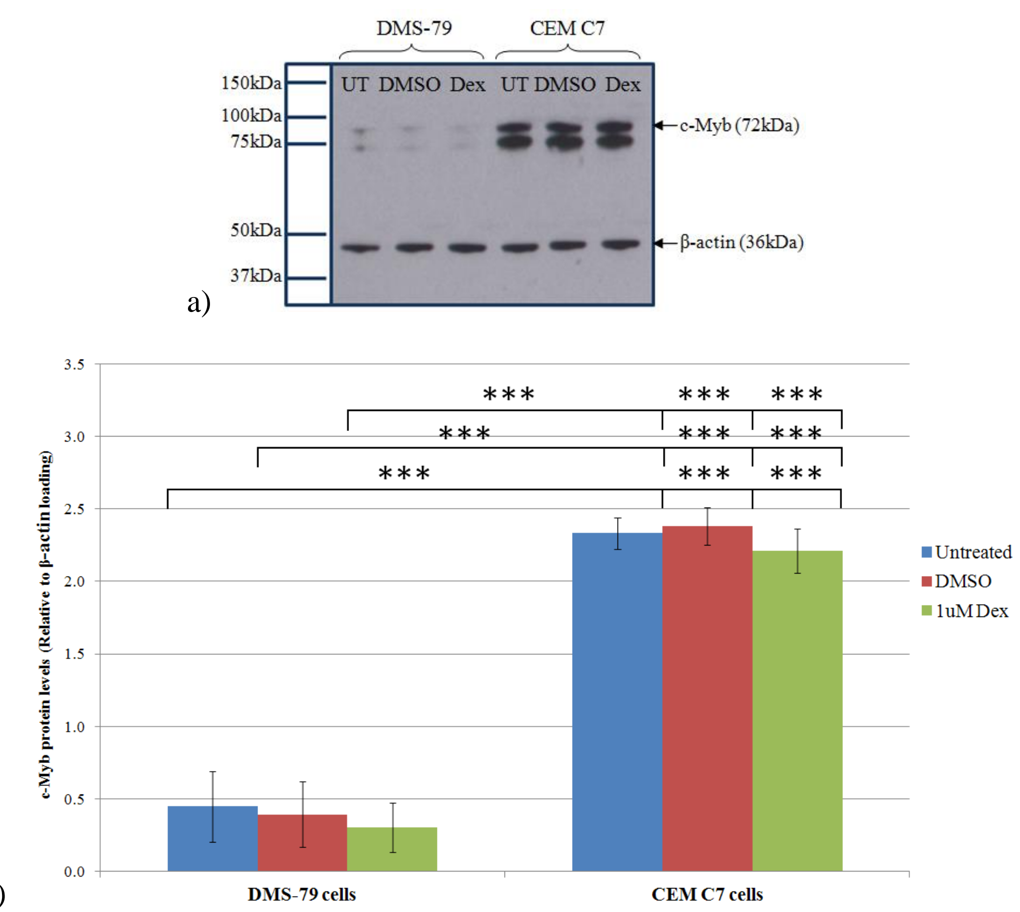


Figure 5.04: c-Myb protein levels in DMS-79 and CEM C7 cells following 24 hours glucocorticoid treatment. DMS-79 and CEM C7 cells were treated with either DMSO or 500nM dexamethasone and samples were taken 24 hours after treatment. a) Samples were analysed by western blot for c-Myb and β-actin as a loading control. b) Densitometry was used to quantify c-Myb expression relative to β-actin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments. *** where $p \leq 0.005$.

5.3.1. c-Ets protein levels in DMS-79 and CEM C7 cells

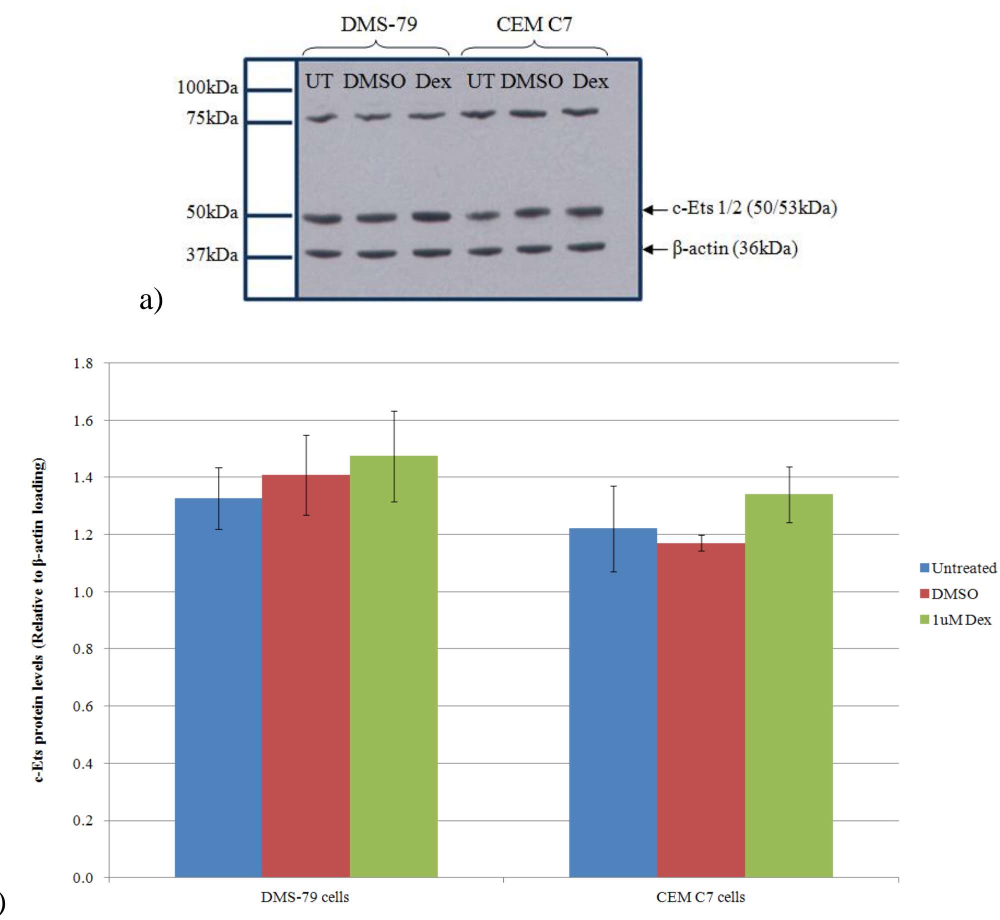


Figure 5.05: c-Ets protein levels in DMS-79 and CEM C7 cells following 24 hours glucocorticoid treatment. DMS-79 and CEM C7 cells were treated with either DMSO or 500nM dexamethasone and samples were taken 24 hours after treatment. a) Samples were analysed by western blot for c-Ets and β-actin as a loading control. b) Densitometry was used to quantify c-Ets expression relative to β-actin. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

Figure 5.04 shows that c-Myb protein levels were significantly higher in CEM C7 cells than in DMS-79 cells. Since availability of c-Myb protein is an important factor in GR auto-upregulation, this was an encouraging observation. Furthermore treatment of cells with dexamethasone treatment did not significantly alter the level of c-Myb protein.

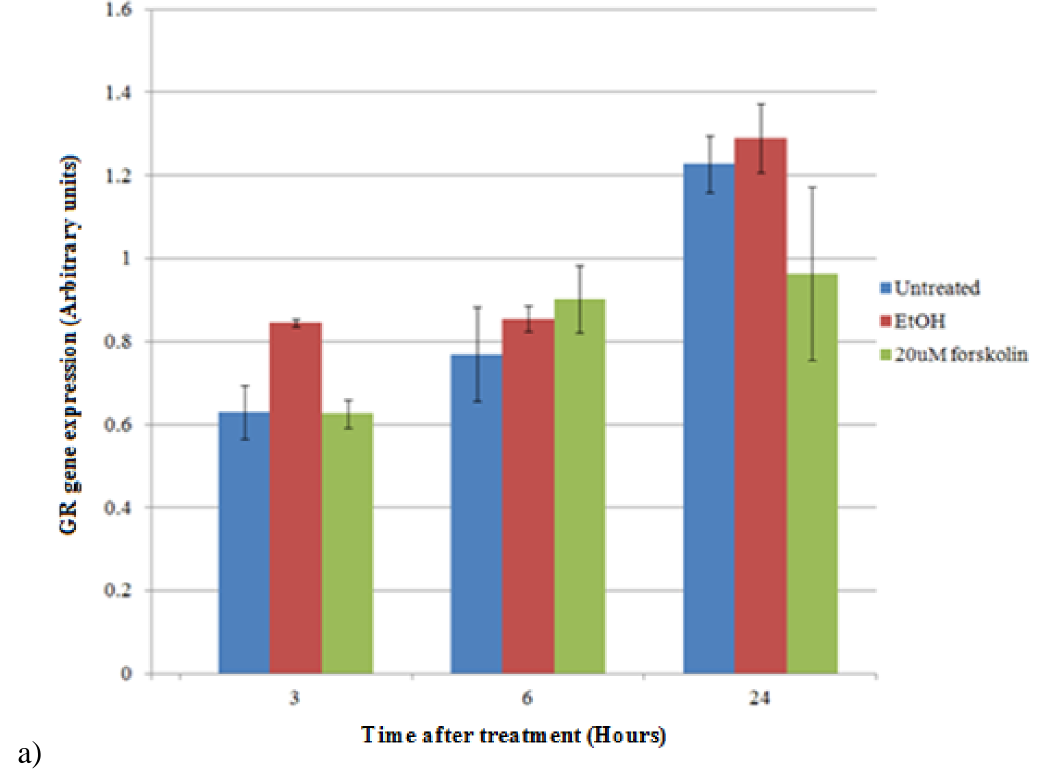
The level of c-Ets protein was comparable between DMS-79 and CEM C7 cells and no significant difference was seen (Fig. 5.05). Treatment of cells with dexamethasone had no effect on the level of c-Ets protein detected in either cell line.

5.4. Upregulation of c-Myb in DMS-79 cells

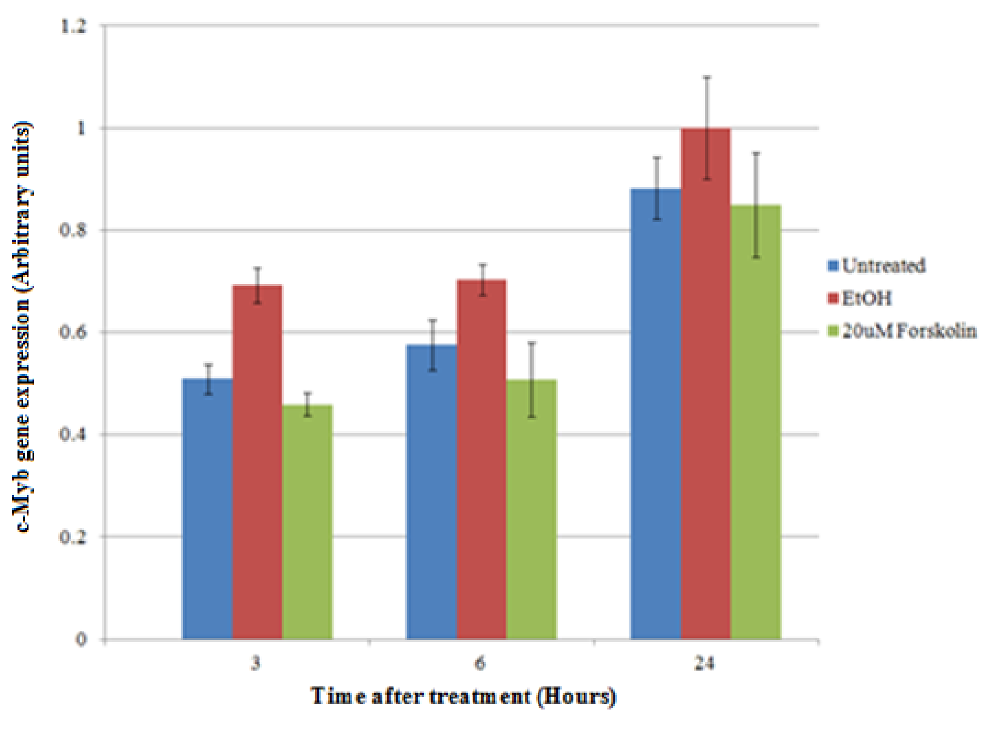
DMS-79 cells showed significantly lower levels of c-Myb protein compared to CEM C7 cells. Since c-Myb is known to play an important role in GR auto-upregulation, I wanted to investigate whether this mechanism could be induced in DMS-79 cells through an increase in c-Myb protein levels.

Treatment of human erythromyeloblastoid leukaemia cell line, K562 cells with forskolin, an adenylate cyclase activator, has been shown to increase c-Myb expression (Kuroyanagi *et al*, 2006). Therefore I wanted to investigate the effect of forskolin treatment on c-Myb gene expression in DMS-79 cells, with a view to inducing GR auto-upregulation in these cells.

5.4.1. Effect of forskolin on GR (a) and c-Myb (b) gene expression in DMS-79 cells



a)



b)

Figure 5.06: GR and c-Myb gene expression in DMS-79 cells following forskolin treatment. DMS-79 cells were treated with either ethanol or 20nM forskolin and samples were taken 3, 6 and 24 hours after treatment. Samples were analysed by qPCR for GR, c-Myb and GAPDH as an endogenous control gene. Values depict mean ± standard error of the mean (SEM) of 3 independent experiments.

No change in GR gene expression was observed following treatment of DMS-79 cells with forskolin (Fig. 5.06a). Figure 5.06b shows that treatment of DMS-79 cells with forskolin did not significantly alter expression of c-Myb in these cells. This suggests that activation of adenylate cyclase does not induce c-Myb gene expression in these cells.

Chapter 6: Results 4

Over-expression of GR

Introduction

Since treatment of cells with forskolin did not alter expression of GR or c-Myb in DMS-79 cells, alternative mechanisms of increasing GR protein levels were investigated. Transient GR over-expression is not suitable for investigating the immediate downstream targets of GR which trigger glucocorticoid-induced apoptosis. In order to differentiate changes in gene expression caused by glucocorticoid treatment and those caused by the transfection protocol, cells must be allowed to recover before samples are taken.

I wanted to investigate whether DMS-79 cells could be stably transfected with a tetracycline-inducible GR expression vector. This would allow cells to be transfected and recover from the transfection protocol while expressing endogenous levels of GR protein. Treatment of cells with tetracycline induces over-expression of GR in stably transfected cells and therefore the immediate downstream targets of GR can be investigated.

HeLa cells were used as a control cell line in this investigation since they are available already stably transfected with two of the four plasmids required for tetracycline-induction of gene expression.

6.1. Generation of the pcDNA5/FRT/TO/GR plasmid

The plasmid provided with the tetracycline-inducible system of expression lacked the gene of interest therefore the GR gene was isolated and ligated into the pcDNA5/FRT/TO plasmid prior to transfection of DMS-79 cells.

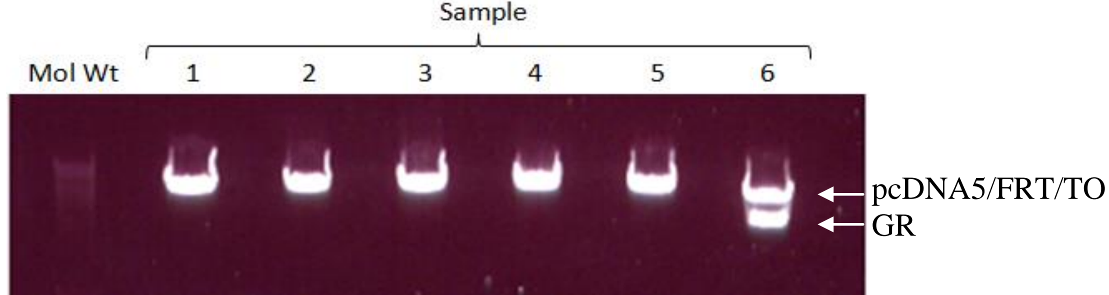


Figure 6.01: GR cDNA in pcDNA5/FRT/TO plasmid. The pcDNA5/FRT/TO plasmid was digested with restriction endonucleases Xho1 and BamH1. GR cDNA was cut from the pcDNA3-GR plasmid using Xho1 and BamH1, run on a 1% agarose gel and extracted. GR was then ligated into the pcDNA5/FRT/TO plasmid using an E3 ligase and transformed into NEB 5α *E. coli*. Colonies were selected, resuspended in LB broth and grown overnight at 37°C. DNA was extracted from these colonies, digested with Xho1 and BamH1 restriction endonucleases and run on a 1% agarose gel.

Colonies 1-5 did not contain the GR cDNA, as indicated by the single band which denotes the linear pcDNA5/FRT/TO plasmid backbone (Fig. 6.01). GR was successfully ligated into pcDNA5/FRT/TO within colony 6 since both the linear pcDNA/FRT/TO plasmid backbone and another band were detected. This second band was extracted from the gel and subsequently confirmed to be GR cDNA through sequencing.

6.2. Hygromycin Sensitivity in HeLa lacZeo/TO Cells

The pcDNA5/FRT/TO plasmid contains a hygromycin resistance gene. Following transfection, cells must be treated with hygromycin in order to select for successfully transfected cells. This ongoing treatment also maintains a selection pressure on the cells which ensures they remain stably transfected. The concentration of hygromycin required to induce 90% cell fatality varies between cell lines therefore I wanted to investigate the optimum hygromycin concentration to select for successfully transfected HeLa cells.

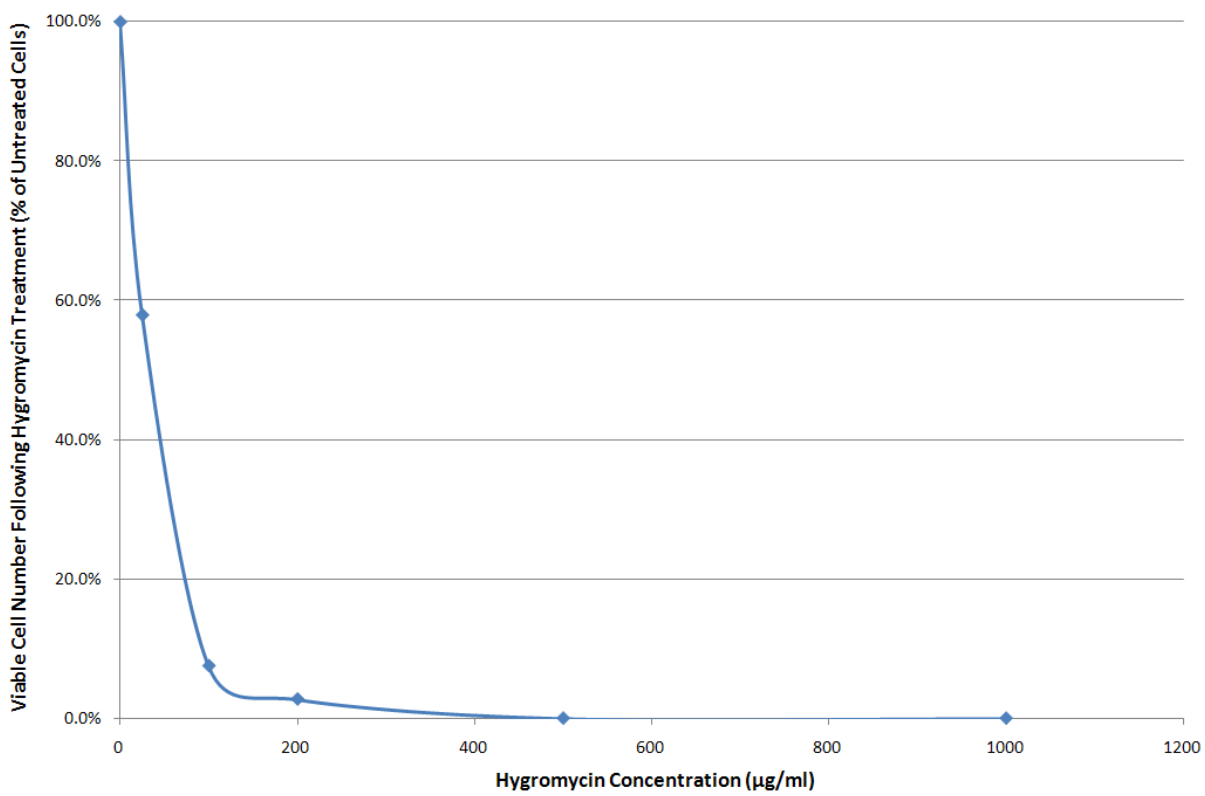


Figure 6.02: Hygromycin kill curves in HeLa lacZeo/TO cells. HeLa lacZeo/TO cells were treated with 0-1000µg/ml hygromycin. After 5 days, cells were counted using trypan blue exclusion. Graph depicts the % cell viability compared to HeLa lacZeo/TO cells treated with DMSO.

Figure 6.02 shows approximately 90% cell fatality in HeLa lacZeo/TO cells following treatment with 100µg/ml hygromycin. Therefore 100µg/ml hygromycin was used to select for HeLa cells successfully transfected with the pcDNA5/FRT/TO plasmid.

6.3. <u>Tetracycline-induction of GR expression in HeLa cells</u>

Following transfection of HeLa lacZeo/TO cells with the pcDNA/FRT/TO/GR plasmid, cells were treated with tetracycline to confirm they had been stably transfected with a tetracycline-inducible system of GR gene expression.

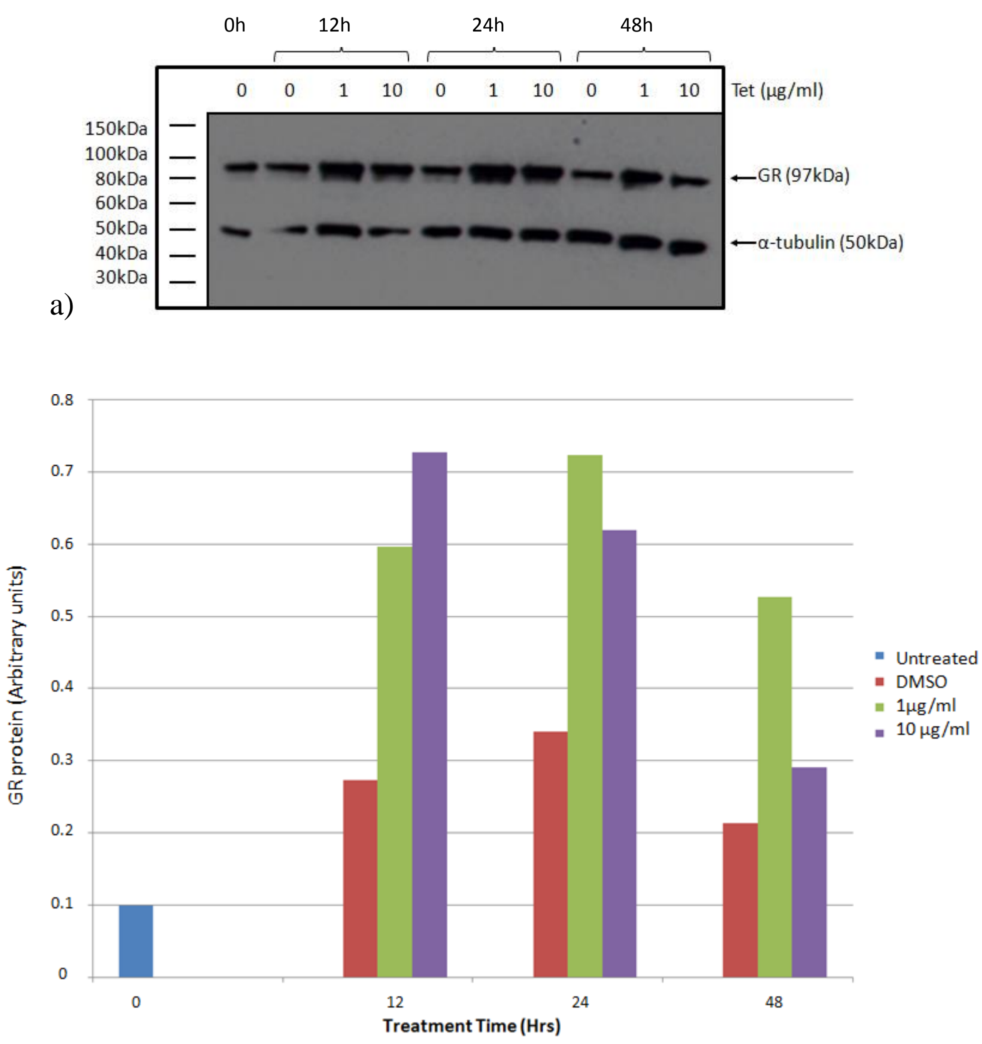


Figure 6.03: Tetracycline-induction of GR expression in HeLa/GR cells. HeLa/GR cells were treated with 0, 1 or 10µg/ml tetracycline and samples taken at 3, 6 and 24 hours after treatment. a) Samples were analysed by western blot for GR and α-tubulin as a loading control. b) Densitometry was used to quantify GR expression relative to α-tubulin. Values depict the mean of 2 independent experiments.

Treatment of HeLa GR cells with tetracycline induces an increase in the level of GR protein detected in HeLa/GR cells (Fig. 6.03). This indicates that a tetracycline-inducible system of GR expression has been established in these cells. GR protein expression levels were comparable in cells treated with 1µg/ml and 10µg/ml tetracycline.

Chapter 7: Discussion

7.1. GR gene expression in DMS-79 cells is resistant to glucocorticoids

SCLC is an aggressive neuroendocrine cancer with a particularly poor prognosis. Despite extensive research into new treatments, SCLC tumours quickly metastasise and develop resistance to therapy. SCLC patients may develop ectopic ACTH syndrome which is characterised by the secretion of pro-hormone POMC or hormone ACTH by the tumour. In pituitary cells, a negative feedback loop inhibits secretion of POMC however SCLC cells are resistant to glucocorticoids and this is used as a diagnostic test for ectopic ACTH syndrome (Oliver *et al*, 2003).

DMS-79 cells express low levels of GR protein to evade apoptosis. The mechanisms underlying glucocorticoid-induced apoptosis are not fully understood; however over-expression of GR in DMS-79 cells has been shown to upregulate expression of pro-apoptotic genes Bad and Bax *in vitro* (Sommer *et al*, 2007). Therefore understanding the regulation of GR protein levels and the mechanisms underlying glucocorticoid-induced apoptosis may lead to the discovery of new therapeutic strategies.

In order to understand the process of glucocorticoid-induced apoptosis in SCLC cells, I first wanted to investigate the effect of glucocorticoids on the regulation of GR gene expression and the expression of downstream GRE-containing target genes in these cells. DMS-79 cells showed no change in GR gene expression following 24 and 48 hour treatment with 500nM dexamethasone. This suggests that GR gene expression in these cells is resistant to treatment with high concentrations of glucocorticoids. My findings support those of previous publications which suggest SCLC cells are resistant to glucocorticoids (Ray *et al*, 1994). This has previously been shown to be due, at least in part, to low levels of GR protein in SCLC cells compared to nSCLC cells (Kay *et al*, 2011). In DMS-79 cells, this low level of GR is

caused by a mutation within one allele of the GR gene, leading to the formation of a truncated GR protein lacking the ligand-binding domain (Gaitan *et al*, 1995). GR expression in DMS-79 cells is further reduced by methylation of the ubiquitous GR promoter 1C (Kay *et al*, 2011).

Expression of pro-hormone pro-opiomelanocortin (POMC), a downstream, negatively regulated GRE-containing gene, was also investigated to determine the effect of glucocorticoids on GR regulated genes in DMS-79 cells. POMC gene expression was unaltered following treatment with 500nM dexamethasone for 24 and 48 hours. The effect of glucocorticoid treatment on a downstream, positively regulated GRE-containing gene; glucocorticoid-induced leucine zipper (GILZ) was also investigated in DMS-79 cells. GILZ gene expression was unaltered by treatment of DMS-79 cells with dexamethasone at 24 and 48 hours. These results support those of previous publications indicating that DMS-79 cells express low levels of GR and are resistant to glucocorticoids (Kay *et al*, 2011; Ray *et al*, 1994). Taken together, these findings suggest that at a transcriptional level, DMS-79 cells are resistant to glucocorticoids.

7.2. DMS-79 cells tightly regulate GR protein expression

I have shown that DMS-79 cells are resistant to treatment with high concentrations of glucocorticoids at the mRNA level. Therefore I wanted to investigate the effect of glucocorticoids on GR protein expression in these cells with a view to understanding the underlying regulatory mechanisms and the ways in which these might be manipulated to increase GR protein levels and glucocorticoid sensitivity. nSCLC cell line A549 cells were used as a control in these experiments since these cells are sensitive to glucocorticoids (Waters *et al*, 2004). GR was detected in both DMS-79 and A549 cells however the level of GR detected in DMS-79 cells was significantly lower than that of A549 cells. These findings support those of previous publications which suggest that GR protein levels are tightly

regulated to maintain a low level in SCLC cells since decreased GR expression has been shown to provide a survival advantage to these cells (Kay *et al*, 2011; Sommer *et al*, 2007).
I wanted to investigate the effect of increasing cell turnover and cell density on GR protein in DMS-79 cells. GR protein expression was seen to remain constant over 72 hours in culture, suggesting that DMS-79 cells maintain low levels of GR protein expression as cells approach confluency to maintain resistance to glucocorticoid-induced apoptosis (Sommer *et al*, 2007). This constant level of GR protein expression was also seen in nSCLC cell line A549 cells.

In order to further understand the regulation of GR levels within these cells, I wanted to investigate whether GR protein levels are affected by glucocorticoid treatment in DMS-79 cells. Treatment of DMS-79 cells with high concentrations of dexamethasone or hydrocortisone for 48 hours significantly decreased the level of GR protein detected in these cells. DMS-79 cells have previously been shown to be resistant to glucocorticoid treatment, as demonstrated by failure to inhibit POMC secretion in these cells (Ray *et al*, 1994). GR protein levels were significantly decreased following treatment with lower concentrations of hydrocortisone (100nM) than dexamethasone (1000nM).

Previous studies in glucocorticoid-resistant acute lymphoblastic leukemia (ALL) cells suggest that a threshold level of GR protein expression is required to convey glucocorticoid sensitivity (Helmberg *et al*, 1995; Geley *et al*, 1996). I have demonstrated that GR protein expression in DMS-79 cells is extremely low and these cells showed no decrease in GR gene expression following 48 hour treatment with 500nM dexamethasone. Taken together, these findings suggest that the observed decrease in GR protein levels is due to an increase in proteasomal degradation of GR following glucocorticoid binding, rather than downregulation of GR protein expression. This could be investigated by measuring GR protein levels following

treatment of DMS-79 cells with both dexamethasone and a proteasomal inhibitor such as MG-132.

Over-expression of GR in SCLC cells has been shown to increase the rate of apoptosis, both *in vitro* (Sommer *et al*, 2007) and *in vivo* (Sommer *et al*, 2010). My findings indicate that glucocorticoid treatment over 48 hours in SCLC cells leads to a decrease in GR protein levels *in vitro*. Therefore investigation into the mechanisms underlying this loss of GR protein is important since low levels of GR inhibit glucocorticoid-induced apoptosis in these cells. A decrease in GR protein levels was observed following treatment of DMS-79 cells with glucocorticoids dexamethasone (1000nM) and hydrocortisone (100nM) over 48 hours however it was not clear whether this decrease in GR protein was maintained over time. Therefore I wanted to investigate whether removal of glucocorticoids would restore GR protein levels to that of untreated cells.

Again GR protein levels were seen to decrease following treatment of DMS-79 cells with dexamethasone for 24 and 48 hours. These results support those of my earlier experiments in which a decrease in GR protein levels in DMS-79 cells was observed following glucocorticoid treatment. Following the removal of glucocorticoids, GR protein levels were seen to increase to that of vehicle-treated cells within 24 hours. This supports my hypothesis that the decrease in GR protein levels observed in DMS-79 cells following dexamethasone treatment is due to proteasomal degradation, since removal of glucocorticoids prevents ligand binding and hyperphosphorylation of GR which triggers degradation of the protein.

Taken together, these findings suggest that DMS-79 cells consistently express low levels of GR protein and that the observed decrease in GR protein levels following glucocorticoid treatment is reversed following removal of glucocorticoids.

7.3. GR protein expression in DMS-79 cell xenografts

My results show that DMS-79 cells *in vitro* maintain low levels of GR protein in order to evade glucocorticoid-induced apoptosis. I wanted to further investigate the significance of this observed low level of GR protein using DMS-79 cell xenografts.

DMS-79 cells within xenografts were seen to have a similar morphology to that of DMS-79 cells *in vitro*, displaying large nuclei and scant cytoplasm. This finding is important since transfer of cells from *in vitro* to *in vivo* can introduce a wide range of selection factors such as blood supply and availability of growth factors and space, which can in turn alter cell morphology. This suggests that injection of DMS-79 cells subcutaneously into the flank of nude (nu/nu) female mice an does not significantly alter cell morphology.

DMS-79 cells stained using a non-specific IgG were used as a negative control while nSCLC cell line A549 cells were used as a positive control since both my results and previous publications show that these cells express high levels of GR (Waters *et al*, 2004).

DMS-79 xenograft sections tested showed extremely weak, positive staining for GR and this staining was seen to be largely cytoplasmic. Some areas were seen to stain slightly darker for GR than others, suggesting that GR protein levels may vary between cells across the tumour xenografts. This finding is supported by that of previous publications, which show that SCLC tumours have a heterogeneous phenotype and cells within these tumours express different biomarkers (Calbo *et al*, 2011).

The heterogeneity observed within SCLC tumours is often decreased following treatment, since radiotherapy is thought to eradicate a number of different cell types. The tumour is then

thought to regrow from the remaining small number of therapy-resistant cells, generating a more homogenous tumour phenotype consisting of similar cells. Therefore I wanted to investigate the effect of tumour irradiation of GR protein expression in this DMS-79 cell xenograft model. GR staining was seen to be very weakly positive across all xenograft sections tested compared to the IgG1 negative control and no obvious difference in GR staining intensity was observed following irradiation of tumour xenografts. Some non-specific staining was observed, likely due to the high concentration of GR antibody required to detect the extremely low concentrations of GR in these cells.

Taken together, these findings support those of my previous experiments suggesting that GR protein is expressed at low levels in DMS-79 cells. The low level of positive staining makes more definitive analysis very difficult, therefore this technique may not be suitable for use in detecting changes in GR protein levels in DMS-79 cells.

7.4. Increasing GR expression in DMS-79 cells

The GR gene is known to contain 9 alternative promoters which direct expression of GR mRNA transcripts containing alternative exon 1 sequences. These are cleaved during post-transcriptional modification. Expression of GR arising from each of the alternative promoters is known to vary between cell lines (Turner & Muller, 2005; Russcher *et al*, 2007). Therefore I wanted to investigate which of the GR promoters contribute to total GR expression in DMS-79 cells with a view to understanding the transcriptional regulation of GR in these cells and ways in which this may be manipulated to increase GR expression.

DMS-79 cells were seen to express GR from promoters 1B, 1C and 1H (30.8%, 40.9% and 0.8% of total GR gene expression respectively). Promoter 1C has been shown to be highly

methylated in DMS-79 cells, decreasing GR gene expression arising from this promoter (Kay *et al*, 2011). Together these 3 promoters account for approximately 73% of total GR gene expression in DMS-79 cells; the remaining 27% could not be attributed to a specific promoter since the primers designed against promoters 1A, 1D, 1E, 1F, 1I and 1J were unable to detect a specific PCR product. Although the same reverse primer was used to detect all GR exon 1 sequences, the forward primers were different and therefore bound with different efficiencies to the cDNA. As a result, this may not be an accurate representation of proportion of GR gene expression arising from each promoter. Repetition of this experiment using primers able to efficiently detect all of the alternative GR exon 1 sequences in DMS-79 would give a more accurate measure of the contribution of each promoter to total GR gene expression in these cells. A number of transcription factors are known to bind to these promoters including Yin Yang 1 and Sp1 (Breslin & Vedeckis, 1998), regulating gene expression arising from each.

It has been shown that over-expression of an exogenous GR gene in DMS-79 cells increases their sensitivity to glucocorticoids *in vitro* (Sommer *et al*, 2007), suggesting that the mechanisms underlying glucocorticoid-induced apoptosis are functional within DMS-79 cells however low levels of GR protein in these cells prevents these pathways from being activated, allowing the cells to evade glucocorticoid-induced apoptosis. Of the GR promoters identified, only promoter 1A has been shown to contain a GRE-like element which suggests that expression of GR arising from this promoter could be auto-regulated (Pedersen *et al*, 2004).

Transcription factors c-Myb and c-Ets have been shown to play a role in GR auto-upregulation from promoter 1A in ALL cell line CEM C7 cells (Geng & Vedeckis, 2005). Therefore I wanted to investigate the levels of c-Myb and c-Ets protein expression in DMS-79 cells with a view to understanding whether this auto-upregulation mechanism could be induced in these

cells to increase total GR expression and thereby sensitivity to glucocorticoid-induced apoptosis.

GR protein levels were found to be significantly higher in CEM C7 cells than DMS-79 cells. DMS-79 cells have been shown to express low levels of GR, partly due to methylation of GR promoter 1C (Kay *et al*, 2011). GR gene expression levels in DMS-79 cells were not significantly altered by 24 hour treatment with 500nM dexamethasone, contradicting the results of my previous experiments. This disparity is likely due to the high level of GR protein detected in CEM C7 cells, meaning that the exposure time of the Western blot had to be limited to prevent over exposure. Treatment with dexamethasone significantly increased GR protein levels in CEM C7 cells. This is compatible with previous work which has shown that, GR expression is auto-upregulated from promoter 1A in these cells following glucocorticoid treatment (Geng & Turner, 2005).

CEM C7 cells were seen to express significantly higher levels of c-Myb protein than DMS-79 cells ($p \leq 0.005$). This result is interesting since binding of c-Myb to footprint 12 within GR promoter 1A has been shown to promote auto-upregulation of GR expression in CEM C7 cells (Geng & Vedeckis, 2005). Therefore it is possible that an increase in c-Myb protein levels in DMS-79 cells may drive binding of c-Myb to GR promoter 1A leading to auto-upregulation of GR expression. Treatment of cells with dexamethasone did not alter levels of c-Myb in DMS-79 or CEM C7 cells compared to untreated controls.

DMS-79 and CEM-C7 cells were seen to have comparable levels of c-Ets protein. Binding of c-Ets to footprint 12 within GR promoter 1A has been shown to inhibit GR auto-upregulation (Geng & Vedeckis, 2005). These results suggest that the failure of DMS-79 cells to auto-upregulate GR following glucocorticoid treatment is not due to high levels of c-Ets expression

inhibiting c-Myb binding to footprint 12 within GR promoter 1A. Treatment of cells with dexamethasone did not alter levels of c-Ets in DMS-79 or CEM C7 cells compared to untreated controls. No GRE has been identified within the c-Ets gene.

DMS-79 cells express significantly lower levels c-Myb than CEM C7 cells, therefore increasing c-Myb expression in DMS-79 cells could potentially induce auto-upregulation from GR promoter 1A in these cells. K562 cells have been shown to increase c-Myb expression following treatment with forskolin, an adenylate cyclase activator (Kuroyanagi *et al*, 2006), therefore the effect of forskolin on c-Myb protein expression in DMS-79 cells was investigated. However DMS-79 cells showed no significant change in c-Myb protein levels following treatment of cells with forskolin. This suggests that expression of c-Myb in DMS-79 cells is not increased by treatment with this concentration of forskolin. Further investigation is required to determine whether an alternative concentration of forskolin may increase c-Myb protein expression in DMS-79 cells. Alternatively, DMS-79 cells could be transfected with a c-Myb expression plasmid to allow GR auto-upregulation to be investigated in these cells. Taken together, these results suggest that DMS-79 cells do not auto-upregulate GR expression from promoter 1A.

Over-expression of GR in DMS-79 cells has been shown to increase the potential of these cells to undergo glucocorticoid-induced apoptosis, both *in vitro* (Sommer *et al*, 2007) and *in vivo* (Sommer *et al*, 2010). These investigations have relied on the infection of SCLC cells with either a retrovirus or adenovirus containing a GR-eYFP transgene, however this technique is unable to regulate the time and level of expression of the transgene. Therefore I wanted to investigate the use of a tetracycline-inducible system in DMS-79 cells to allow the expression of the exogenous GR gene to be regulated This would alleviate the effect of changes in cell morphology and gene expression caused by transfection of cells on the results.

Furthermore, once stably transfected the SCLC cell line overexpressing GR could be used in ongoing investigations without the need for repeated transient transfection. HeLa cells expressing the tetracycline repressor from pcDNA6/TR plasmid were used as a control since these cells were available stably transfected with the TRex system of expression.

Following insertion of the GR gene into the pcDNA5/FRT/TO plasmid, HeLa pcDNA6/TR cells were co-transfected with the pcDNA5/FRT/TO/GR and pOG44 plasmids. Treatment of these cells with tetracycline showed an obvious trend to increase the level of GR protein detected compared to vehicle-treated cells, however statistical analysis of these results showed this increase was not significant. This is likely due to the high level of variation between replicates, rather than the failure of the pcDNA5/FRT/TO/GR vector to successfully integrate into these cells.

Many attempts were made to stably insert the pFRT/lacZeo plasmid into DMS-79 cells, using various cell densities, transfection methods, concentrations of plasmid and transfection reagents, however these were unsuccessful. Further work is required to determine whether insertion of the TRex system of GR expression into DMS-79 cells is feasible.

7.5. Conclusion

SCLC tumours are very aggressive and can quickly develop resistance to treatment. Some SCLC tumours secrete hormones such as ACTH or its pro-hormone POMC leading to the development of ectopic ACTH syndrome. This hormone secretion has been shown to be resistant to glucocorticoid inhibition in SCLC tumours. This thesis has demonstrated that GR in DMS-79 cells is resistant to glucocorticoids; this supports findings of previous publications. Quantitative PCR data showed that glucocorticoid treatment does not alter expression of GR

or downstream targets POMC and GILZ in DMS-79 cells. Extremely low levels of GR protein were detected in DMS-79 cells and GR was seen to be downregulated following treatment of cells with high concentrations of glucocorticoids.

The low level of GR protein in SCLC cells reduces the propensity of these cells to undergo glucocorticoid-induced apoptosis. Over-expression of GR in these cells has shown that the pathways controlling glucocorticoid-induced apoptosis are functional within SCLC cells and it has been hypothesised that a threshold level of GR protein is required to confer glucocorticoid sensitivity to these cells. Understanding the mechanisms underlying regulation of GR protein levels in SCLC cells and the pathways through which these cells undergo glucocorticoid-induced apoptosis may provide insight into novel therapeutic approaches for SCLC patients.

7.6 Future work

My results showed that the level of GR protein in DMS-79 cells is very low and treatment of these cells with high concentrations of glucocorticoids triggers down-regulation of GR protein. Treatment of alternative SCLC cell lines (COR L24, COR L47 and COR L103 cells) with glucocorticoids could demonstrate whether the effect of glucocorticoids on GR protein is comparable to that seen in DMS-79 cells. Glucocorticoid response curves in these cell lines would demonstrate the extent to which glucocorticoid resistance varies between SCLC cell lines. Furthermore, the observed decrease in GR protein levels following treatment of DMS-79 cells with glucocorticoids could not be explained by a decrease in GR gene expression. Treatment of DMS-79 cells with the proteasomal inhibitor MG-132 and glucocorticoids would demonstrate whether downregulation of GR protein is due to proteasomal degradation.

In DMS-79 cells, GR gene expression is controlled by 9 alternative promoters and the contribution of each to total GR expression is known to vary between cell lines (Turner & Muller, 2005; Russcher *et al*, 2007). My results showed that GR gene expression in DMS-79 cells arises from promoters 1B, 1C and 1H however the remaining exon 1 sequences could not be detected for technical reasons. The primers designed against these sequences could be redesigned to accurately determine the contributions of each promoter to total GR expression in these cells.

GR auto-upregulation from promoter 1A has been demonstrated in acute lymphoblastic leukemia (ALL) cell line CEM C7 cells following treatment with glucocorticoids. This mechanism has been shown to rely on binding of GR and c-Myb to footprints 11 and 12 in GR promoter 1A. My results showed that c-Myb protein levels in DMS-79 cells were significantly lower than that of CEM-C7 cells and treatment with adenylate cyclase activator forskolin failed to increase c-Myb protein levels in these cells. Infection of DMS-79 cells with a retroviral construct containing the c-Myb gene would determine whether the mechanism underlying auto-upregulation of GR from promoter 1A is functional in DMS-79 cells.

The low level of GR detected in DMS-79 cells has been shown to reduce the propensity of these cells to undergo glucocorticoid-induced apoptosis both *in vitro* (Sommer *et al*, 2007) and *in vivo* (Sommer *et al*, 2010). The mechanisms underlying glucocorticoid-induced apoptosis are not fully understood. Previous investigations have relied on infection of SCLC cells with adenoviral or retroviral constructs however the resulting GR gene expression is brief and the immediate downstream targets of GR are difficult to identify due to the time taken for cells to recover from infection. Stable transfection of DMS-79 cells with GR under the control of a tetracycline-inducible promoter would overcome these problems. My results showed that transfection of HeLa cells with the GR gene under the control of a tetracycline-inducible

promoter led to an increase in GR protein levels however attempts to repeat this transfection in DMS-79 cells were unsuccessful. Electroporation of DMS-79 cells may improve the efficiency of transfection and DMS-79 cells expressing GR under the control of a tetracycline-inducible promoter could be used to investigate the mechanism underlying glucocorticoid-induced apoptosis in these cells.

It has recently been demonstrated that SCLC cells express high levels of Bcl-2 and this has been linked to the observed low levels of GR protein in these cells (Schlossmacher *et al*, in press). Further investigation into this association and the mechanisms underlying this may lead to a deeper understanding of the way in which increased GR protein levels in these cells leads to glucocorticoid sensitivity and the pathways through which these cells undergo glucocorticoid-induced apoptosis.

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