Novel Modulators of Glucocorticoid Sensitivity

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Maryam Jangani

School of Translational Medicine
Faculty of Medical and Human Sciences
University of Manchester
Oxford Road
Manchester
M13 9PT
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List of publications arising from this thesis


List of presentations arising from this thesis

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– Oral presentation at the EMBO Nuclear Receptors, From Molecular Mechanism to Health and Disease conference in Barcelona, Spain. *Metastasis-related methyltransferase-1 (Merm1/WBSCR22) is a novel modulator of GC sensitivity*

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September 2010

– Presented two research posters at the Cold Spring Harbor Nuclear receptor from Molecular Mechanism to Health and Disease conference in New York, USA. 1- *William Beuren syndrome chromosome region 22 (WBSCR22) is a novel modulator of GR function*. 2- *Novel pharmacokinetic approach for the use of GCs in treatment of inflammatory diseases.*

July 2009
– Oral presentation at the University of Manchester Musculoskeletal Research group. *Novel pharmacokinetic approach for the use of GCs in treatment of inflammatory diseases.*

**August 2008**

– Oral presentation at the University of Manchester Endocrinology Seminar series. *Novel pharmacokinetic approach for the use of GCs in treatment of inflammatory diseases.*
List of abbreviations

ACTH: Adrenocorticotrophin hormone
AF1: Activation function 1
AF2: Activation function 2
ALL: Acute lymphoblastic leukaemia
AP-1: Activator protein 1
AR: Androgen receptor
ASPH: Aspartyl/asparaginyl beta-hydroxylase
5-Aza: 5 Aza-deoxycytidine
BAF: BRG1-associated factors
BCI-2: B-cell CLL/lymphoma2
BRG1: Brahma related gene 1
BRM: Brahma
BSA: Bovine serum albumin
CARM1: Coactivator arginine methyltransferase 1
C terminus: Carboxylic acid end of protein
cDNA: complementary deoxyribonucleic acid
C/EBPβ: CCAAT/ehnacer binding protein beta
CBP: CREB binding protein
CCL2: Chemokine (C-C motif) ligand 2
ChIP: Chromatin immunoprecipitation
ChIP-seq: Chromatin immunoprecipitation sequencing
CMV: Cytomegalovirus
CoIP: Co-immunoprecipitation
CORT: Corticosterone
CpG: Cytosine-phosphate-guanine
CREB: cyclic AMP reponse element binding protein
CRH: Corticotropin releasing hormone
DBD: DNA binding domain
ddH2O: Double distilled H2O
Dex: Dexamethasone
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DNMT: DNA methyltransferase
Dot1L: Dot1-like
DUSP1: Dual specificity phosphatase 1
ER: Estrogen receptor
FACs: Flourescence activated cell sorting
FBS: Foetal bovine serum
FKBP5: FK506 binding protein 51
FoxA1: Forkhead boxA1
GC: Glucocorticoid
GH: Growth hormone
GILZ: Glucocorticoid-induced leucine zipper
GLUL: Glutamate ammonia Ligase
GR: Glucocorticoid receptor
GRdm: GR dimer mutants
GR-LBD: GR ligand bining domain
GRE: Glucocorticoid response elements
GRIP1: Glucocorticoid receptor interacting protein 1
H3: Histone 3
H3K4: Histone lysine 4
H3K4me3: Histone3 lysine4 trimethylated
H3K9me: Histone3 lysine 9 methylation
H3K79me2: Histone3 lysine 79 dimethylated
HAT: Histone acetyltransferase
Hc: Hydrocortisone
HDAC: Histone deacetylase
HEPES: N-2-hydroxyethyl-piperazine-N’-2-ethanesulphonic acid
HMTase: Histone methyltransferase
HMM: Hidden Markov Model
HPA: hypothalamic pituitary axis
HSP: Heat shock protein
IFI16: Interferon-inducible protein 16
IFNγ: Interferon γ
IGF: Insulin-like growth factor
IgG: Immunoglobulin G
IκB: Inhibitor of kappa B
IL-6: interleukin 6
IL6ST: Interleukin-6 signal transducer
iNOS: inducible nitric oxide synthase
KDa: Kilodalton
MAPK: Mitogen activated protein kinase
Merm1: Metastasis-related methyltransferase 1
ΔMethT: Merm1 methyltransferase deletion domain
MKP-1: MAPK phosphatase 1
MMTV: Mouse mammary tumour virus
MR: Mineralocorticoid receptor
MR pred: Modified-release prednisolone
mRNA: messenger ribonucleic acid
MT1X: Metallothionine 1X
MTase: Methyltransferase
Mw: Molecular weight
MYO1B: Myosin 1B
N terminus: Amino group end of protein
NF-κB: Nuclear factor kappa B
NL: Nuclear localisation
NR: Nuclear receptor
PBS: Phosphate buffered saline
PBMC: Primary blood mononuclear cell
PCR: Polymerase chain reaction
PG: Prostaglandins
Pol II: Polymaerase II
PR: Progesterone receptor
PVN: Para ventricular nucleus
RA: Rheumatoid arthritis
RNAi: RNA interference
RPM: Revolutions per minute
PTH: Parathormone
SAM: S-adenosyl-L-methionine
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M: Standard error of the mean
shRNA: Short interfering ribose nucleic acid
siRNA: Small interfering ribose nucleic acid
SLE: Systemic lopus erythmatosus
SRC: Steroid receptor co-activator
SWI/SNF: SWItch/Sucrose NonFermentable
TAT3: Tyrosine amino transferase 3
TGF-β: Transforming growth factor- β
TIF2: Transcriptional intermediary factor 2
TF: Transcription factor
TNF-α: Tumour necrosis factor α
WBS: William Bueren syndrome
WBSCR22: Williams Beuren syndrome chromosome region 22
Abstract

Glucocorticoids (GCs) exert diverse effects on multiple cell types and tissues. The variability in GC sensitivity can give rise to disease states hence the importance of GC sensitivity modulators. GCs act through the glucocorticoid receptor (GR), a ligand-activated nuclear hormone receptor (NR), which interacts with the DNA to regulate gene transcription depending on the chromatin structure. GR itself modulates chromatin through epigenetic modification of histone residues. In the present study, novel modulators of GC sensitivity, altering GR-mediated gene expression through dynamic or epigenetic regulatory mechanisms, are identified and explored.

Metastasis-related methyltransferase1 (Merm1/WBSCR22), is a histone methyltransferase, previously shown to methylate histone H3 Lysine 9 (H3K9), a repressive methyl mark, to inhibit target gene transcription. Our GR reporter transient transfections assays showed that Merm1 potentiated GR transactivation through its methyltransferase and SAM domains. Merm1 knockdown significantly impaired both GR transactivation, and transrepression of endogenous genes, including GILZ. The ChIP assay analysis confirmed that both GR and Merm1 bound the GILZ promoter and Merm1 regulated ligand-induced GR recruitment.

Merm1 regulated tri-methylation of H3K4 (H3K4me3) and di-methylation of H3K79 (H3K79me2). At the GILZ locus, GR induced H3K4me3 and inhibited H3K79me2. Merm1 regulated both of these and also maintained basal H3K79me2. The GR-induced H3K4me3 followed by loss of H3K79me2 showed that these events were driven by H3K4 methylation. In conclusion, Merm1 regulates chromatin structure to affect GR recruitment, and mediates GR actions of transcription by histone methylation.

In the second part of the thesis, the biological consequence of temporal dynamics of GC delivery to target cell gene expression and apoptosis has been investigated. For this purpose a flow-through culture system was designed and modified for pulsatile and continuous delivery of GC to HeLa cells and primary T cells. Pulsatile cortisol caused a significant reduction in cell survival compared to continuous exposure of the same cumulative dose in HeLa population. This was due to increased apoptosis. Transcription factor (TF) binding site analysis of the microarray data identified CCAAT-displacement protein (CDP) as a common TF binding site in the differentially regulated target genes. Mouse mammary tumour virus (MMTV) gene is regulated by CDP and is also GC responsive. MMTV-Luc was also differentially regulated between pulsatile and continuous cortisol. In primary T cells, GILZ and FKBP5 genes were more highly induced with continuous than with the same equivalent concentration given in pulses. In conclusion, cortisol oscillations exert important effects on target cell gene expression, and phenotype.

In summary, GC sensitivity is modulated via different mechanisms. Our data illustrate a novel regulatory mechanism whereby GR activity is altered through histone modifications and chromatin remodelling. In addition, GC oscillations provide frequency modulation to GR-mediate gene expression with a resulting differential pattern of gene transcription and cellular response.
Declaration

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The author

The author graduated from the University of Manchester in 2007, with a BSc Honours in Pharmacology. She then took up an MRes degree course in Biomedical Sciences, in the University of Liverpool where she graduated with a 1st in 2008.

She undertook her PhD from 2008 under the supervision of Professor David Ray and Dr Rachelle Donn.

She is now pursuing a career in academic research.
Chapter 1: Introduction
Chapter 1: Introduction

1- Introduction

1.1- Overview

Glucocorticoids (GCs) belong to the group of steroids secreted by the adrenal cortex that exert a wide range of metabolic, endocrine and immunological effects on virtually all tissues and organs. They were named for their hyperglycaemic effects as low blood glucose levels were seen in adrenalectomised animals (Cohn et al. 1952). GCs are the most potent anti-inflammatory and immunosuppressive drugs used for the management of a multiple acute and chronic inflammatory conditions such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and cancer. Therapeutic use of GCs is, however, largely restricted by the broad spectrum of their metabolic side effects (den Uyl et al. 2011; Strohmayer et al. 2011).

The glucocorticoid receptor (GR) is a ubiquitous intracellular receptor that mediates the actions of GCs and is responsible for modulation of GC sensitivity in different tissues. GR plays an important role in the regulation of GC target genes through its interactions with a variety of different co-factors (Feng et al. 2006; O'Brien et al. 2010) and chromatin remodelling complexes which influence the subsequent recruitment of transcriptional machinery (John et al. 2008; Trotter et al. 2004b).

GC secretion is under the control of the hypothalamic-pituitary-adrenal (HPA) axis and occurs in pulses (Young et al. 2004). Evidence is growing to support the relevance of GCs
oscillations in consequences on down-stream gene expression (Conway-Campbell et al. 2010; McMaster et al. 2011).

In the following chapter I will discuss the role of GCs in metabolism and inflammation and explain the role of GC sensitivity co-modulators on GR-mediated gene expression. Furthermore, I will discuss the significance of pulsatile GC release on the cellular response with a focus on the molecular pathways driving these events.

1.2- **Physiological effects of glucocorticoids (GCs):**

Physiological effects of GC are mediated by the GC receptor (GR) which is ubiquitously expressed in the body. The endogenous GC hormone in man, cortisol, is involved in maintaining homeostasis during disturbed physiological conditions such as infection, anxiety and chronic disease like RA. The effects of GCs on various organs in the body are evident in conditions such as Cushing’s syndrome or osteoporosis arising through excessive GC drug treatment (Longui 2007; Schacke et al. 2002). Of the wide range of GC actions, metabolic and immunomodulatory effects are most frequently studied.

1.2-1. **GC effects on metabolism:**

As the name glucocorticoid suggests, the primary function of GCs is to regulate the blood glucose levels. During fasting, the level of circulating cortisol is elevated in order to increase serum glucose levels to allow rapid energy supply to the brain. This is brought about by less glucose uptake in the muscle and adipose tissue and more glucose absorption in neural tissue (McMahon et al. 1988). Anabolic processes in the liver and catabolic processes in peripheral organs result in higher blood glucose levels through
increased gluconeogenesis, increased protein degradation and lipolysis. GCs stimulate the synthesis of glucose from other non-hexose organic molecules such as pyruvate, lactate, glycerol and amino acids (Pilkis et al. 1992). The fatty acids released by lipolysis are used for energy production in muscle and the released by product, glycerol, is used as a substrate in gluconeogenesis. Furthermore, GCs prevent leptin secretion, a vital hormone involved in body weight and reproductive function (Zakrzewska et al. 1997). Therefore, one can envisage that these catabolic processes can contribute to some of the side effects of prolonged GC treatment, for instance via increased tendency to hyperglycaemia and diabetes or by redistributions of fat.

1.2-2. Immune and anti-inflammatory actions of GCs:

The body’s defense response to inflammation involves the activation of the different components of the inflammatory system as well as coagulation, tissue repair and activation of the HPA axis to initiate more GC production. GCs exhibit potent anti-inflammatory and immunomodulatory effects in the body. Adrenalectomized rodents, lacking the required adrenal gland for corticosterone production, showed difficulty in surviving even mild septic shock induced by injection of lipopolysaccharide (LPS) (Yeager et al. 2004).

1.2-3. Immunomodulatory effects on cells:

GCs suppress the influx and trafficking of leukocytes to the site of inflammation through a reduced leukocyte-endothelial cell interaction and hence less adhesion and migration to the site of inflammation. This is thought to be partially mediated by the actions of GCs on
activity of membrane glycoprotein CD18 as was demonstrated by investigation of Farsky et al (Farsky et al. 1995) on rats’ white blood cells. In addition, GCs reduce proliferation and activity of endothelial cells (Akkoyn et al. 2007) and cytokine secreting T cells (Abe et al. 2011). GCs also inhibit the emergence of macrophages from blood vessels. Other important effects of GCs on primary and secondary immune cells are summarised in Table 1.

Table 1. Effects of glucocorticoids on primary and secondary immune cells.

<table>
<thead>
<tr>
<th>Monocytes/macrophage</th>
<th>↓ number of circulating cells (↓ myelopoiesis, ↓ release)</th>
<th>(Buttgereit et al. 2005a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↓ expression of MHC class II molecules and Fc receptors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ synthesis of pro-inflammatory cytokines (e.g. IL-2, IL-6, TNF-a) and prostaglandins</td>
<td></td>
</tr>
<tr>
<td>T Cells</td>
<td>↓ number of circulating cells (redistribution effects)</td>
<td>(Hawrylowicz 2005)</td>
</tr>
<tr>
<td></td>
<td>↓ production and action of cytokine secreting T cells</td>
<td></td>
</tr>
<tr>
<td>Granulocytes</td>
<td>↓ number of eosinophil and basophil granulocytes</td>
<td>(Schramm et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>↑ number of circulating neutrophils</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>↓ vessel permeability</td>
<td>(Nehme et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>↓ expression of adhesion molecules</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ production of IL-1 and prostaglandins</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>↓ proliferation</td>
<td>(He et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>↓ production of fibronectin and prostaglandins</td>
<td></td>
</tr>
</tbody>
</table>
1.2-4. Effects on cytokines, chemokines and inflammatory enzymes:

Inflammatory processes are usually characterised by unregulated synthesis of pro-inflammatory mediators such as cytokines (e.g. interleukin-1 (IL-1), IL-6, tumour necrosis factor alpha (TNFα)), prostaglandin E₂ (PGE₂), chemokines (e.g. IL-8, monocytes chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, etc) and inflammatory enzymes (e.g. inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2). Furthermore, GCs block the endothelial cell adhesion molecules such as vascular adhesion molecule 1 (VCAM-1), ICAM-1 and E-selectin in order to inhibit lymphocyte adhesion and migration during inflammation (Chen et al. 2002b; Cronstein et al. 1992; Wang et al. 2008). GCs act to suppress the pro-inflammatory cytokines by induction of decoy receptors that lack the intracellular signalling properties of cytokine receptors but can instead compete with the cytokine receptor and ‘trap’ these cytokines, induce the synthesis of anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-10, and finally by anti-inflammatory cytokine receptors (e.g. (TGF)-βR and IL-10R (Almawi et al. 1996; Galon et al. 2002). Chemokines can be suppressed by GCs through transcriptional inhibition of their target gene or ‘transrepression’. COX-2, the inducible COX is induced by inflammatory and mitogenic stimuli and is involved in metabolism of arachidonic acid, AA, an important mediator of inflammatory response produced by phospholipase A₂ (PLA₂). GCs inhibit the expression and synthesis of cytosolic PLA₂ and COX-2 via genomic (Newton et al. 1997) and also non-genomic mechanisms (Croxtall et al. 2000). Finally, GCs have been shown to inhibit iNOS involved in vasodilatation of site of inflammation. *In vitro* studies on vascular smooth muscle cells confirmed that
dexamethasone suppressed the transcription of iNOS gene by inhibiting nuclear factor-κB (NF-κB) (Matsumura et al. 2001). Treatment of vascular smooth muscle cells (VSMCs) with lipopolysaccharide (LPS) plus interferon-gamma (IFN-γ) (LPS/IFN-γ) caused activation of NF-κB and iNOS promoter. LPS/IFN-γ induced mRNA and nitric oxide (NO) synthesis was then markedly reduced following Dex treatment. The mechanism of NF-κB inhibition by dexamethasone (Dex) is thought to be mediated through Dex-induced transcription of IκB-α, selective NF-κB inhibitor, and also by direct GC-induced transcriptional repression of NF-κB (Nissen et al. 2000). Another potential mechanism of GC-mediated inhibition of NF-κB is through activation of the MAPK phosphatase (MKP)-1, also known as dual specificity phosphatase 1 (DUSP1) (Abraham et al. 2006; Bhattacharyya et al. 2007). Dexamethasone inhibited p38 mitogen-activated kinase (MAPK), an upstream regulator of NF-κB, in mouse macrophages which was associated with an induction of DUSP1. The anti-inflammatory effects of dexamethasone was impaired in DUSP1 knock out macrophages (DUSP1-/-). Dexamethasone significantly inhibited COX-2, IFN-γ, IL-1α, and IL-1β in DUSP1+/+ macrophages whereas this inhibition was severely impaired in DUSP1-/- macrophages (Abraham et al. 2006).

1.3- GCs and inflammatory diseases

1.3-1. Overview of RA

Rheumatoid arthritis (RA) is a systemic disorder characterized by the chronic inflammation of synovial lining of mainly peripheral joints. It affects about 1% of the adult population worldwide and is a significant cause of disability (Scott et al. 1987) and
mortality (Pincus et al. 1986) as RA is also linked with a higher risk of cardiovascular disease development (Solomon et al. 2006). The medical cost of RA averages $5,919 per case per year in the United States (Yelin et al. 1999) and approximately £2,600 per case per year in the United Kingdom (McIntosh 1996). Women are three times more likely than men to be affected by RA. In RA, the synovial membrane is infiltrated by inflammatory cells, primarily CD4+ T cells, which play a pivotal role in cell-mediated immune responses. CD4+T cells then stimulate monocytes, macrophages and synovial fibroblasts to produce cytokines IL-1, IL-6 and TNF-α and to secrete matrix metalloproteinases (MMPs) (Fig 1.1). Formation of MMPs in turn stimulates the migration of polymorphonuclear cells from the surrounding tissue into joint fluid making the surface layer of lining cells more thickened and hyperplastic. The activated lymphocytes, macrophages and fibroblasts also induce angiogenesis leading to increased vascularity in the synovium. Extensive production of autoantibodies such as rheumatoid factors (RFs) and anti-citrullinated protein/peptide antibodies (ACPAs) by plasma cells in synovium also exacerbates the chronic inflammation. RFs are autoantibodies that have the Fc portion (fragment of antibody without antigen-binding site) of the immunoglobulin G (IgG) as their antigen. The nature of the antigen means that they aggregate into immune complexes and stimulate inflammation, causing chronic synovitis (inflammation of synovial lining of joints). Although transient production of RFs is necessary for body’s normal mechanism of removing immune complexes, in RA, there is a persistent production of RFs in the joints and they show much higher affinity (Halldorsdottir et al. 2000). Local formation of immune complexes together with increased permeability of blood vessels leads to joint
effusions containing lymphocytes and dying polymorphonuclear cells. The inflamed synovium damages the cartilage by blocking its normal route for nutrition causing swelling around the joints and also bone erosion (Sweeney et al. 2004).
Chapter 1: Introduction

The major cell types and cytokine pathways involved in joint destruction mediated by TNF-α and interleukin-1 are shown. T cells activated by cytokines and cellular contact with the extracellular matrix activate the production of cytokines, prostaglandins (PGs) and matrix metalloproteinases (MMPs). A combination of these pro-inflammatory responses leads to migration of polymorphonuclear cells and initiation of bone and cartilage erosion. Adapted from McInnes et al (McInnes et al. 2000).
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1.3-2. GC administration in RA

According to the guidelines published in 2009 by the National Institute for Health and Clinical excellence (NICE) for management of RA short-term GC therapy should be offered to alleviate the flares in people with recent onset of RA, or in established disease, to rapidly decrease the inflammation. Long-term treatments with GCs are only considered when, a) the long-term complications of long-term therapy have been fully discussed and b) all other treatment options (including biological agents) have been offered. This is because of potential side effects of long-term GC treatment such as hypertension, hyperglycaemia, osteoporosis, gastric ulceration and glaucoma. However, in the U.S. it is estimated that 44% to 75% of RA patients have GC therapy (Pincus et al. 1992) while in UK up to 30% of RA patients currently take oral steroids (Panoulas et al. 2007). A study by Hindmarsh et al (Hindmarsh et al. 2005) has suggested that parenteral GC administration results in more than 50% reduction in the total GC daily dosage with resultant sparing of hepatic GC action. The resultant bioavailability of the subcutaneously administered GC was determined to be 100% compared to only 75% when given orally. A Medtronic pump facilitated subcutaneous administration which mimicked the normal daily (=circadian) rhythm of cortisol. This supports the necessity for more investigation into the route of GC delivery particularly in patients requiring high doses of oral GCs and in those with abnormal GC pharmacokinetics (Bryan et al. 2009).
1.3-3. GCs-induced adverse effects

Despite potential short-term benefits of GCs, there is a high prevalence of long-term metabolic side effects. Examples of these side effects include effects on bone and calcium homeostasis e.g., osteoporosis, cardiovascular effects such as hypertension, atherosclerosis, and endocrine-metabolic system like suppression of HPA axis, growth failure, diabetes, and Cushingoid features (central obesity, moon face, etc). More detailed list of known GC-mediated side effects are indicated in Table 2.

1.3-3.1. Osteoporosis

Post menopausal women and also elderly men on long-term GC therapy for RA have a greater risk of developing secondary osteoporosis. This increases the fracture risk at the hip and spine due to significant bone loss in the trabecular bone (Kirwan et al. 2007). GCs stimulate osteoclast-mediated bone resorption and reduce osteoblast-mediated bone formation. It is estimated that osteoporosis will occur in 50% of patients treated with GCs for more than 6 months (Mitra 2011; Weinstein 2011). Although some studies have revealed that doses of 7.5 mg of prednisone a day or less are relatively safe. Some data has suggested that doses as little as 6.0mg prednisone per day for 6 months may cause significant bone loss (Pearce et al. 1998). Also doses as small as 5.0 mg have been shown to suppress bone formation as measured by serum and urine biomarkers (Ton et al. 2005). In inflammatory diseases like RA, osteoporosis is multifactorial and depends on level of disease activity and duration of the disease. While evidence has shown that RA itself plays a role in regional generalised bone loss, GCs have independent
suppressory effect on osteoclasts (bone resorption) and osteoblasts (bone formation) (Schacke et al. 2002) (Fig 1.2).

**Figure 1.2. Mechanism of GC-induced bone loss.**

GC can affect the osteoblasts and osteoclasts directly or indirectly leading to bone loss and osteoporosis. GH, growth hormone; IGF, insulin-like growth factor; PTH, parathormone adapted from (Schacke et al. 2002).
1.3-3.2. **Cardiovascular disease**

Prolonged high dose GC treatment plays a role in dyslipidemia, hypertension and ultimately increased mortality rate due to cardiovascular disease (Boers 2004; Choy et al. 2009; Pieringer et al. 2011). The link between GC exposure and hypertension (HT) is not very well understood but increased levels of renin substrate (angiotensinogen) and higher vascular sensitivity to adrenergic agonists are possible mechanisms of actions (Panoulas et al. 2007). On the other hand, investigations have shown that inflammatory diseases like RA are themselves a significant factor in endothelial damage and hypercoagulability as a result of chronic inflammation (Townsend et al. 2004). Data from cohorts of patients have demonstrated that while high level of RA disease activity induces dyslipidemia, treatment with GC can reverse these changes (Boers et al. 2003).
Table 2. Adverse effects of Glucocorticoids and affected organs

<table>
<thead>
<tr>
<th>Affected system</th>
<th>Undesirable effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>• Arterial hypertension</td>
</tr>
<tr>
<td></td>
<td>• Congestive heart failure</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>• Esophagitis,</td>
</tr>
<tr>
<td></td>
<td>• gastritis, peptic ulcer</td>
</tr>
<tr>
<td></td>
<td>• Digestive haemorrhage</td>
</tr>
<tr>
<td>Neuropsychiatric</td>
<td>• Psychiatric disorders in general</td>
</tr>
<tr>
<td></td>
<td>• Intracranial hypertension</td>
</tr>
<tr>
<td></td>
<td>• Ophthalmic Glaucoma</td>
</tr>
<tr>
<td></td>
<td>• Cataracts</td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>• Osteoporosis (Schacke et al. 2002); (Townsend et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Aseptic bone necrosis</td>
</tr>
<tr>
<td></td>
<td>• Myopathies</td>
</tr>
<tr>
<td>Endocrine/metabolic</td>
<td>• Truncal obesity,</td>
</tr>
<tr>
<td></td>
<td>• Supraclavicular and posterior cervical fat deposition</td>
</tr>
<tr>
<td></td>
<td>• Hirsutism, masculinization, menstrual disorders</td>
</tr>
<tr>
<td></td>
<td>• Growth failure in children and adolescents</td>
</tr>
<tr>
<td></td>
<td>• Hyperglycaemia, dyslipidemia</td>
</tr>
<tr>
<td></td>
<td>• Negative nitrogen, potassium and calcium balance</td>
</tr>
<tr>
<td></td>
<td>• Sodium retention</td>
</tr>
<tr>
<td></td>
<td>• Hypokalemia and metabolic alkalosis (Longui 2007)</td>
</tr>
<tr>
<td>Immune</td>
<td>• Decrease in inflammatory response</td>
</tr>
<tr>
<td></td>
<td>• Higher susceptibility to infections</td>
</tr>
<tr>
<td></td>
<td>• Cutaneous Striae and acne, delayed wound healing</td>
</tr>
<tr>
<td>Vascular</td>
<td>• Vasculitide</td>
</tr>
<tr>
<td></td>
<td>• Thromboembolism</td>
</tr>
<tr>
<td></td>
<td>• Arteriosclerosis (Townsend et al. 2004)</td>
</tr>
</tbody>
</table>

Adapted from Longui et al (Longui 2007).
1.4- The glucocorticoid receptor (GR):

GR belongs to the family of nuclear receptors which are ligand-activated transcription factors. Other members of the family include mineralocorticoid, thyroid hormone, retinoic acid and vitamin D₃ receptors (Hard et al. 1990). GR is encoded by NR3C1 gene and was first cloned in 1987 (Evans 1988). The human GR (hGR) is located on chromosome 5 and encodes the GRα and also GRβ. Several new isoforms of the GR proteins have been identified recently which all diverge from the original gene by alternative translation start sites and also alternative splicing of the primary GR transcript (Lu et al. 2007) GRα is expressed in all human cells and regulates the anti-inflammatory actions of GCs (Hagendorf et al. 2005; Pujols et al. 2002). GRβ is also expressed in almost all cells and tissues (epithelial cells and neutrophils are exceptions) to a much lower extent (~400 times) (Fruchter et al. 2005); (Pujols et al. 2002) and is thought to have dominant negative actions on the transactivation effects induced by GRα (Oakley et al. 1996; Oakley et al. 1997) although this remains controversial (Brogan et al. 1999). The physiological importance of the GRβ is still unknown but some studies have shown GRβ overexpression in some inflammatory diseases like asthma (Sousa et al. 2000) and chronic lymphocytic leukaemia (Shahidi et al. 1999).

1.4-1. GR structure:

The functional domains of the GR include variable N terminal domains which harbors transactivation functions especially within the so called τ1 region; a DNA-binding domain which consists of two zinc fingers important in receptor dimerisation (Luisi et al. 1991),
nuclear translocation, binding to glucocorticoid response elements (GREs) and gene transactivation and also a C terminal ligand binding domain (LBD). The LBD consists of 12α helices involved in the formation of the hydrophobic ligand binding pocket by a three layer helical sandwich (Wikström 2003). The inactivated GR resides in the cytoplasm as a multi-protein complex bound to several heat shock proteins (Hsp) including Hsp90, Hsp70, Hsp56 and Hsp40. In addition, GR also interacts with immunophilins, p23, and several kinases of the MAPK signalling pathway like Src (Kassi et al. 2011). The main purpose of these (co)chaperones is to stabilize the GR structure at the intermediate stage for high affinity ligand binding (McLaughlin et al. 2002) leading to conformational change, nuclear translocation and binding of the GR to the promoter region of target genes (Stavreva et al. 2004) for transcriptional regulation.

1.4-2. Post-translational modification of GR:

The GR is the subject of various post-translational modifications (PTMs), including phosphorylation, ubiquitination, SUMOylation and acetylation (Fig 1.3). These PTMs serve as additional means to modulate the functionality of the GR, by affecting transcriptional activity, subcellular localization, protein-protein interactions and rate of degradation.
Figure 1.3. Site of post-translational modifications of the human GR.

a) The known sites of phosphorylation in the hGR, all of which are contained within the NTD. b) reported sites of sumoylation, ubiquitination and acetylation. LBD, ligand binding domain, HR, hinge region, DBD, DNA binding domain, NTD, N-terminal domain. Adapted from Beck et al (Beck et al. 2009).
1.4-2.1. Phosphorylation

Phosphorylation is the kinase-catalysed attachment of a phospho group to a protein. This covalent addition can be reversed through the action of dephosphorylating phosphatases. The GR is a phospho-protein that is constitutively phosphorylated and upon ligand-binding the phosphorylation status of the receptor is enhanced. The level of GR phosphorylation has regulatory impacts of the proteins transcription activity, by regulating subcellular distribution, ligand- and GRE-binding affinity and protein stability.

Within the human GR six serine residues, the majority of which are located in AF-1 of the N-terminal domain, NTD, have been identified as phosphorylation sites. These residues include Serine 113 (S113), S141, S203, S211, S226 and the recently identified S404, which are conserved within the rat and the mouse. The phosphorylation properties of the individual residues have been shown to differ. It was observed that the phosphorylation of S211 is predominantly ligand-dependent, whereas S203 is basally phosphorylated at low levels, which is enhanced in the presence of hormone. There appears to be a strong association between the phosphorylation status of S211 and the transactivation potential of the GR. Indeed, the transcriptional activity of the GR has been shown to be at its maximum when the level of phosphorylation at S211 is greater than S226. Residues T8, S45, S134, S234 and S267 have also been identified as potential sites of phosphorylation during cell division (Fig 1.3). Webster et al assessed the effect of mutations at the phosphorylation sites of the murine GR (Webster et al. 1997). Here they showed that phosphorylation site mutations had a significant effect on the transactivation of a minimal
promoter. Receptor mRNA and protein levels were also abrogated in the mutant groups. However, mutations had minimal effects on the transcriptional activity of the GR from a complex murine mammary tumour virus (MMTV) promoter showing that the effects are promoter specific.

1.4-2.2. **Ubiquitination**

Following GC binding, the highly conserved ubiquitin protein is covalently attached to the GR, marking the protein for degradation via the proteasome. The involvement of this pathway in the ligand-induced down-regulation the GR was first suggested by Wallace *et al.* (Wallace *et al.* 2001). Dexamethasone-mediated degradation of the GR was abolished in COS-1 cells treated with the proteasome inhibitor, MG-132. Furthermore, immunoprecipitation studies in proteasome deficient cells revealed a higher molecular weight ubiquitinated GR. The ubiquitin-proteasome pathway enables the rapid turnover of the GR, thus regulating its transcriptional activity (Duma *et al.* 2006; Nicolaides *et al.* 2010). Ligand binding induces the hyperphosphorylation of the GR (Beck *et al.* 2009; Webster *et al.* 1997), and it is believed that the phosphorylation status of the protein is the signal that confers the enzymes of the ubiquitination process to recognise the receptor. The poly-ubiquitination of the receptor facilitates its recognition and ultimate degradation by the proteasome (Wall *et al.* 2003). Eukaryotic proteins that are rapidly degraded have long been linked with regions rich in proline, glutamic acid, serine and threonine, also referred to as PEST regions (Rogers *et al.* 1986). These regions have been shown to be flanked by a lysine residue (Fig 1.3), which has been identified as a candidate
site for substrate recognition by the E2/E3 enzymes (Rogers et al. 1986). Indeed, the mutation of such a lysine residue to an alanine in the mouse GR abolished Gc-mediated down-regulation of the receptor (Wallace et al. 2001).

1.4-2.3. SUMOylation

The covalent attachment of the small ubiquitin-related modifier-1 (SUMO-1) protein to the GR receptor occurs at 3 specific lysine residues, Lys227, Lys293 and Lys703 (Tian et al. 2002) (Fig 1.3). These lysine residues are contained within a consensus motif, ΨKXE (where Ψ is a large hydrophobic amino acid and X is any amino acid), of which there are 2 within the NTD, a region that serves as the primary SUMOylation site in the human GR, and one in the LBD (Tian et al. 2002). SUMOylation is a multi-step reaction, much like ubiquitination, although the enzymes involved are specific to each process, and SUMOylation impacts on a wider range of biological functions, such as protein stability, subcellular localisation, transcriptional activity and protein-protein interactions (Tian et al. 2002; Zhou et al. 2005). The sumoylation pathway involves three distinct enzymes, the first is the E1 enzyme that catalyses the ATP-dependent activation of SUMO-1. Then the SUMO-1 conjugating E2 enzyme (Ubc9), facilitates the attachment of SUMO-1 to the specific lysine residue within the GR. Finally the process is completed by an E3 ligase. It is thought that SUMOylation results in a decrease in the transcriptional activity of the GR, as those receptors in which SUMOylation has been abrogated show a greater transcriptional activity at minimal promoters than their wild type conformations (Tian et al. 2002). This however contradicts with the findings of (Le Drean et al. 2002) where it was shown that
SUMO-1 overexpression caused a five fold increase in GR-mediated transactivation of GREx4-TK-Luc reporter gene in COS-7 cells (Le Drean et al. 2002). P38 Mitogen-activated kinase (MAPK)-mediated GR phosphorylation reduces its ligand binding affinity and is thought to be involved in IL-2/IL-4 mediated GC resistance in peripheral blood mononuclear cells (PBMCs) (Irusen et al. 2002).

1.4-2.4. Acetylation

The GR protein can also be acetylated. GR acetylation has also been linked to lower affinity for NF-κB. Knockdown of histone deacetylase 2 (HDAC2) in the human alveolar epithelial cell line A549s resulted in a loss of GR association with NF-kB p65 subunit as was found by co-immunoprecipitation. Overexpression of HDAC2 in alveolar macrophages from GC resistant COPD patients restored GC-mediated inhibition of IL-β induced granulocyte/macrophage colony-stimulating factor production (GM-CSF), suggesting that acetylation is an important signal targeting GR to binding partners (Ito et al. 2006).

1.5- GR effects on gene transcription

1.5-1. Genomic mechanisms of GC actions

1.5-1.1. Transactivation

The GR homodimer can directly bind to glucocorticoid response elements (GREs), giving rise to positive regulation of gene expression known as transactivation. GREs are specific sequences in promoter region of the GC target genes to which transcription factors bind and control the activation or inhibition of the associated target gene (Cornett et al. 1998).
The DNA-bound GR recruits several co-modulator proteins involved in remodelling of the chromatin leading to the opening of the chromatin structure required for gene transcription (Kleiman et al. 2007). Alternatively, GR can bind to other DNA-bound transcription factors, such as STAT5 (signal transducer and activator of transcription 5) (Schacke et al. 2007; Sommer et al. 2008) to increase transcription activity. It has been shown that GC-induced transactivation is required for regulation of metabolism and glucose homeostasis in the liver (Hudson et al. 2007) through synthesis of enzymes involved in gluconeogenesis and lipid metabolism. However, transactivation is also responsible for most of the deleterious side effects of therapeutic GCs such as inhibition of bone formation and osteoblasts apoptosis and prevention of bone formation leading to osteoporosis (Introduction section 1.3-3.1) (Kleiman et al. 2007). It is known that GR expression is a prerequisite for the transactivation mechanisms, as the GR$^{-/-}$ mice, lacking the GC receptor, died shortly after birth due to various serious abnormalities in lung maturation (Cole et al. 1995). However, mice carrying the dimerisation-deficient GR, (point mutation A458T, impairing dimerisation and hence GRE binding), overcame the lethal phenotype of the GR-knock out mice (Reichardt et al. 1998) emphasising the significance of the GRE-independent activities of the GR. However, it is now clear that GR dimer mutants (GRdm) are able to bind some GREs and in fact the DNA-binding mechanism of GR is likely to be more complex (Adams et al. 2003).
1.5-1.2. Transrepression

Transcription of genes can also be inhibited by GR through different mechanisms. GR directly interacts with negative GREs (nGREs) e.g., pro-opiomelanocortin, α-fetoprotein and prolactin gene (Sakai et al. 1988) interfering with binding of other transcription factors inhibiting the transcription of these genes (Sommer et al. 2008). It is known that GCs suppress the transcription of inflammatory cytokines like interleukin (IL-1) and IL-2 through nGREs (Falkenstein et al. 2000). Only a few genes are known to be regulated by this direct mechanism and so the overall role of the nGREs is still poorly understood (Cato et al. 2004). Alternatively, GR can displace the transcription factors from their positive GREs through direct interaction of the GR and the transcription factors (Buttgereit et al. 2004). However, the major transrepression mechanism involves direct protein-protein interaction of the GR with transcription factors involved in expression of pro-inflammatory genes. Because this mechanism is fully functional in dimerisation-deficient GR, GR\textsuperscript{dim/dim}, it is known that this pathway occurs through GR monomers (Reichardt et al. 2001). The transcription factors to which GR is tethered include, activator protein-1 (AP-1) (Chinenov et al. 2007) and nuclear factor-κβ (NF- κβ) (Nissen et al. 2000) and interferon regulatory factor 3 (IRF3) (Chinenov et al. 2007). The tethering complex inhibits the recruitment of co-activators or co-repressors while interfering with the interaction of RNA polymerase II (Nissen et al. 2000). These act to reform the structure of chromatin changing it into a transcriptionally non-permissive state. In addition to the above, GR can also compete with some transcription factors in binding to the co-activators of the target genes (Stahn et al. 2007). Furthermore, NF-κB can be suppressed by GC-mediated
induction of IκB, an inhibitor of NF-κB, which sequesters NF-κB in the nucleus and promotes the net dissociation of DNA bound NF-κB (Auphan et al. 1995), or through GC-induced leucine zipper protein (GILZ), also demonstrated to inhibit AP-1 (Mittelstadt et al. 2001). In endothelial cells, however, GR does not inhibit NF-κB through increased IκBα synthesis neither inhibit nuclear accumulation of NF-κB, or inhibit DNA binding, suggesting that GR blocks NF-κB-mediated transactivation. In fact, it is evident that in COS-7 cells of bovine aortic endothelial lineage, GR competes with the NF-κB co-activators, CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) for binding. Overexpression of CBP and SRC1 in COS7 cells relieved the GR-mediated inhibiton of NF-κB-depednet reporter gene activation and rescued NF-κB-mediated inhibiton of GR-dependent reporter gene activation (Sheppard et al. 1998).

Another potential mechanism for GR-mediated transrepression involves the MAPK phosphatases, MKP-1 in particular (also known as dual specificity phosphatase 1, DUSP1). GCs induce the expression of DUSP1 (Imasato et al. 2002; Lasa et al. 2002) which in turn inhibits the expression of mitogen activated kinases p38 (p38 MAPK) and c Jun N-terminal kinase (JNK) (Chen et al. 2002a; Zhao et al. 2005). Dexamethasone inhibited the JNK and p38 MAPK in a DUSP1 dependent manner in mouse macrophages (Abraham et al. 2006). The inhibitory effects of dexamethasone on pro-inflammatory genes such as cyclooxygenase 2 (COX-2) and IL-1β was impaired in DUSP1 knock out macrophages (DUSP1-/-) indicating a role for DUSP1 in mediating the anti-inflammatory effects of GCs. DUSP1 is a known target of c-Jun, a transcription factor for AP-1 (Breitwieser et al. 2007;
Kristiansen et al. 2010). Since, p38 MAPK and JNKs phosphorylate and therefore activate AP-1 transcription factor, c Jun (Kallunki et al. 1996; Karin 1995), and also NF-KB (Beyaert et al. 1996; Lee et al. 1997), it is possible that DUSP-1 could also target GC-mediated transrepression through inhibition of NF-kB activation although this is yet to be experimentally confirmed.

GCs, therefore, through transrepression mechanisms inhibit the functions of pro-inflammatory mediators, including cytokines (e.g. IL1, IL-2, TNF-α, IFN-γ) and prostaglandins and cell adhesion molecules and enzymes (De Bosscher et al. 2000).

1.5-2. Non-genomic mechanisms of GC actions

Apart from the classical genomic effects of the GCs, some of their very rapid effects are also mediated by non-genomic mechanisms. Genomic mechanisms require many steps, all of which need time. Translocation into the nucleus and transcription of the target gene are both time consuming and take at least 15 to 30 minutes. While the non-genomic effects are not well understood, it is thought that they prepare the cells for the forthcoming long-term effects of the genomic effects through rapid effects on second messengers, water and electrolyte balance and also glucose metabolism (Buttgereit et al. 2002). Buttgereit et al (Buttgereit et al. 2002) have claimed that non-genomic pathways act as temporal changes that fill the gap between the immediate need for changes and long-lasting, slow genomic effects. Two main categories have been specified by the Mannheim Classification for non genomic mechanisms: A) direct effects and B) indirect effects. Direct effects are further divided into non-specific (receptor independent) and
specific (receptor-dependent involving ligand binding) effects (Falkenstein et al. 2000). Based on these classifications, three different mechanisms have been proposed for non-genomic effects of glucocorticoids. These include the non-specific interaction of GCs with cellular membranes, non-genomic effects mediated by cytosolic GR (cGR) binding and specific effects mediated by the membrane-bound GR (mGR) (Fig 1.4).

1.5-2.1. GC interaction with the cellular membrane

It is believed that GCs at high concentrations can interact with membrane lipids there by changing their physiochemical properties as well as altering the activities of membrane associated proteins (Buttgereit et al. 2004). These changes result in reduced calcium and sodium movements as well as ATP use across the membranes of immune cells and hence a rapid immunosuppression and reduced subsequent inflammation (Buttgereit et al. 2002). In addition to this, GCs can also bind to mitochondrial membranes and increase proton permeability. This impairs the oxidative phosphorylation and ATP production (Buttgereit et al. 1994). These effects are usually seen in high doses of GCs and may contribute to clinically relevant outcomes of GCs as ATP is vital for housekeeping activities of immune cells together with their specific function such as cytokine synthesis, migration, antigen processing and phagocytosis.
Figure 1.4 Mechanisms of actions of GCs.

Lipophilic GCs pass easily through the plasma membrane and bind to cGR. This is followed by either the GR-mediated genomic effects (I) or by GR-mediated non-genomic effects (II). GCs can also interact with cellular membranes through mGR (III), or via non-specific interactions with cell membranes (IV) m=mechanism. Adapted from Bartholome et al. (Bartholome et al. 2004).

1.5-2.2. Non-genomic mechanisms regulated by cGR

The ligand-free GR is bound to several proteins such as (Hsps and src (Hache et al. 1999; Limbourg et al. 2003; Pratt et al. 1997)). Ligand-bound GR dissociates from these proteins facilitating its translocation into the nucleus. There is evidence that the released proteins like SRC, HSP-70 and HSP-90 are all involved in mediating the non-genomic effects of GCs
Another observation by Croxtall et al (Croxtall et al. 2000) includes the release of arachidonic acid (AA) from the membrane –associated phospholipids regulated by different GC-mediated proteins (such as growth factors, adaptor proteins, MAPK and lipocortin 1). AA plays a key role in controlling cell growth, metabolic and inflammatory processes in the cells and is subject to inhibition by glucocorticoids via a cGR-dependent manner i.e sensitive to RU486 (GR antagonist), and is transcription-independent i.e. insensitive to actinomycin D (inhibitor of transcription) (Kayahara et al. 2005).

**1.5-2.3. Specific non-genomic effects mediated by membrane-bound GR**

Recent studies have detected the presence of a membrane-bound GR (mGR) in amphibian and neuronal membranes and in lymphoma cells (Gametchu et al. 1999). mGR has also been identified in human peripheral blood mononuclear cells using high-sensitive immunofluorescent staining (Bartholome et al. 2004). The antibody used for the deletion also recognised both cGR and mGR. However, overexpression of the cGR did not have any effect on the expression of mGR on the cell surface membrane. Therefore, it was concluded that mGR is not a form of cGR transported to the membrane but rather a variant of cGR produced by differential splicing or post-translational modification (Bartholome et al. 2004). Stahn et al (Stahn et al. 2007) have reported an increase in the percentage of mGR positive monocytes by immunostimulation using lipopolysaccharide (LPS). An increased number of mGR in B-lymphocyte cells has also been shown in patients with active RA (Buttgereit et al. 2005a; Buttgereit et al. 2005b). In patients with systemic
lupus erythematosus the expression of mGR was reported to be up-regulated, in some cases even higher that those with RA (Spies et al. 2006). Together these findings suggest that mGR plays a role in pathogenesis of inflammatory diseases. Because of its role in mediating the GC-induced apoptosis of immune cells (Gametchu et al. 1999), higher expression levels in chronic diseases is suggestive of a protective role for mGR. Recent studies in T cells have revealed that some of the immunosuppressive effects of GCs are mediated non-genomically through a membrane-bound GR. Lck and Fyn are members of the Src family of tyrosine kinase family and are involved in mediating the T-cell receptor (TCR) signalling pathway (Palacios et al. 2004). Experiments involving HSP90 siRNA constructs have revealed that GR is part of the TCR signalling complex involving HSP90, Lck and Fyn. HSP90 siRNA-transfected cells were not able to assemble this TCR-associated multiprotein complex, and accordingly HSP90 siRNA treatment mimics GC effects on LCK/FYN activities. These observations support a model for non-genomic GC-induced immunosuppression on the basis of dissolution of membrane-bound GC-receptor multiprotein complexes after GC-receptor ligation (Lowenberg et al. 2006).

1.6- Epigenetic modulation and transcriptional regulation

Eukaryotic DNA is packaged into a chromatin structure which consists of repeating nucleosomes formed by wrapping 147 base pairs of DNA around an octamer of four core histones (H2A, H2B, H3 and H4). The temporal and spatial extraction of information from the genome is essential in normal growth and development. Epigenetics from the Greek “epi” for “above” genetics were first defined by Waddington et al as “casual interactions
between genes and their products which bring phenotype into being” (Waddington 1959). Epigenetics now refers to stably maintained mitotically and potentially meiotically heritable patterns of gene expression occurring without changes in the DNA sequence (Gallou-Kabani et al. 2007; Waddington 1959). The epigenome includes DNA methylation and histone modifications together with RNA interference (Lippman et al. 2004).

1.6-1. DNA methylation

In higher eukaryotes including mammals, DNA methylation occurs more commonly at C5 positions of the cytosine residues and can be inherited through multiple cellular divisions. This is predominantly seen in the short canonical sequence 5’-CG-3’. Small regions of genome in which CG sequences are enriched are known as CpG islands and are frequently found near the 5’ end of the genes (Cross et al. 1994; Illingworth et al. 2010). DNA methylation involves addition of a methyl group to the cytosine residue through specific enzymes called DNA methyltransferases (MTases) (Hermann et al. 2004). All DNA MTases use the universal methyl donor, S-adenosyl-L-methionine (Ado-Met), also known as SAM to transfer the methyl group onto the DNA bases. Three main DNMTs have been identified i.e. DNMT1, DNMT3a and DNMT3b (Hermann et al. 2004). DNA methylation is associated with a structural change of chromatin into a repressed state and inhibition of gene expression or gene silencing. Methylation induced inhibition of gene expression is mediated via two basic mechanisms. In the first, methylation can directly repress transcription by blocking transcriptional activators and transcription factors from binding to DNA sequence (Watt et al. 1988). In the second, gene silencing can be mediated by
methyl-CpG binding proteins (MBPs) (e.g. MeCP2 (Jones et al. 1998), MBD1-4 (Hendrich et al. 1998)) which recognize methylated DNA and recruit co-repressors (e.g. NuRD (Zhang et al. 1999) and N-CoR (Yoon et al. 2003)) and histone deacetylases (HDACs) to silence gene expression (Bogdanovic et al. 2009).

1.6-2. Histone modulation

Histones are the chief protein components of chromatin, acting as spools around which DNA winds. Histones, especially at their amino terminal tails, are subject to a wide range of post translational modifications including, acetylation, methylation, ubiquitination, phosphorylation, glycosylation, ribosylation and carbonylation which all happen in a dynamic manner by the histone modifying enzyme complexes (Aravind et al. 2011; Ho et al. 2010; Khorasanizadeh 2004). There are two basic mechanisms by which histone modification can affect transcriptional output. First, it is believed that histone modifications alter the electrostatic charge on histones resulting in a structural change of histone and its binding to DNA. The second mechanism proposes that histone modifications are sites of recognition by proteins containing bromodomains and chromodomains that recognize acetylated or methylated lysines, respectively. The histone code hypothesis was first stated by Strahl et al and states that distinct covalent modifications on histones influences chromatin structure and leads to different patterns of gene expression (Strahl et al. 2000). Histone acetyltransferases (HATs) are important factors as they acetylate histone tails and give rise to chromatin decondensation providing the platform for recruitment of non-histone transcriptional regulatory proteins.
and gene activation, while histone methyltransferases (HMTs) methylate histone N-terminal tails and promote a tighter binding of histones to DNA and inhibition of transcription.

1.6-3. Histone acetylation

Lysine residues on histone proteins, especially on histone H3, are subject to acetylation and methylation by a large number of HATs and HMTs and have been most extensively studied. Acetylation of lysine9 on histone3 (H3K9) is a mark of activation of gene transcription. In a study by Astrand et al, Xenopus oocytes were injected with GR mRNA and treated for 4hrs with corticosterone. The results showed a significant increase in H3K9 and H3K14 acetylation, a mark of activation of gene transcription (Astrand et al. 2009). This was associated with a significant rise in GR-driven transcription of mouse mammary tumour virus, MMTV, which was reconstituted into the oocytes. In the presence of HDAC inhibitor, TSA, a known histone deacetylase inhibitor, hyperacetylation of H3K9 and H3K14 was observed which caused pleiotropic effects on chromatin structure and a significant loss of hormone induced MMTV transcription. Acetylation of histone lysine residues has also been associated with cell proliferation, cancer and inflammation. A study by Bai et al, showed hyperacetylated H3 and H4 in hepatic stellate cells (HSCs) (Bai et al. 2008). HSCs play an important role in the pathogenesis of hepatocellular carcinoma (HCC). Myocyte enhancer factor 2, MEF2, which is a key transcription factor known to cause over expression and recruitment of HATs and class II HDACs, was identified to be the key factor. MEF2 caused the considerable hyperacetylation of
histones and progression of carcinogenesis in HCC. In another study histone 3 lysine 56 (H3K56) acetylation was investigated in response to DNA damage in human HEK293T and HeLa cells (Vempati et al. 2010). It was shown that levels of H3K56 acetylation oscillated during cell cycle with peaks during S phase of the cell cycle. The acetylation of H3K56 was found to be through the HAT, p300. It was also observed that levels of H3K56 increased significantly in response to DNA damage stimuli by γ-irradiation and hydroxyurea implicating an important role for H3K56 hyperacetylation in cancer initiation and progression.

1.6-4. Histone acetyltransferases and inflammatory diseases

Up to 30 different proteins with intrinsic HATs activity have been identified so far and are classified according to their cellular localization i.e. nuclear or cytoplasmic. Five main types of HATs have been identified according to their primary structure with three that have been extensively studied: the GNAT (GCN5-related N-acetyltransferase) family, and PCAF (p300/CBP associated factor); the p300/CBP family, including p300 and CBP (CREB-binding protein); and the MYST family, which includes Tip60 (TAT-interacting protein 60). Acetylation is an important step toward full transcriptional activation of p53 and other important cancer and inflammatory mediators such as NFkB. Phosphorylation of p53 N-terminal residues permits the interaction of p53 with CBP/p300, which acetylates p53 lysine-382 (Lys382) and with PCAF, which acetylates p53 lysine-320 (Sakaguchi et al. 1998). Puca et al demonstrated HEPK2 siRNA-dependent loss of p53 Lys382 acetylation in human colon carcinoma RKO cell lines (Puca et al. 2009). This significantly impaired p53
binding to DNA in vivo and impaired p53 induced apoptotic induction. This phenomenon was found to be due to the loss of interplay between HIPK2, p300, HDAC protein Sirt1, and p53 connecting HIPK2-dependent Ser46 phosphorylation with Lys382 acetylation/deacetylation in tumor cells subjected to genotoxic stress.

The prototypical NF-κB complex is a RelA/p50 heterodimer, which is important for NF-κB-mediated antiapoptotic effects (Karin et al. 2002). It has been previously shown that CBP/p300 acetyltransferase acetylates RelA which is important for the duration of NF-κB activity since RelA acetylation mediates the nuclear translocation of RelA and downstream induction and release of pro-inflammatory cytokines (Chen et al. 2001; Chen et al. 2004). A study in human A2058 melanoma and DU145 prostate cancer cell lines, showed that the nuclear accumulation and activity of NF-κB/p-RelA was significantly higher in cancer cell lines compared to control which was mediated through signal transducer and activator of transcription 3 (STAT3), a transcription factor commonly activated in cancer, (Lo et al. 2005; Wang et al. 2004b). This increase in NF-κB activity was found to be due to increased acetylation of RelA by CBP/p300 (Lee et al. 2009). As was demonstrated by immunoprecipitation, the proteasomal degradation of highly acetylated p-RelA by IκB was significantly reduced in cancer cells. This in turn was due to STAT3-mediated reduction of RelA affinity for IκB. According to the findings by (Lee et al. 2009), the constitutive activation of NF-κB involved both STAT3 and p300 and in cancer tissues all three proteins were pulled down in the same DNA binding complex. All together these data suggest that driving the equilibrium between acetylation and deacetylation of NF-κB towards a more
hyperacetylated state contributes to activation of NF-κB in tumor cells and tumor microenvironment.

1.6-5. Histone methylation

Methylation of histone lysine residues in eukaryotes has been a remarkable area of research since Rea et al found histone methyltransferase Suv39H1 to methylate histone H3 lysine 9 (H3K9) in vivo, with deregulated SUV39H1 causing abnormal mitotic divisions (Rea et al. 2000). Histone H3 lysine residues are subject to methylations by a large family of S-adenosyl methionine-dependent histone methyltransferases (HMTs). Major methylation sites on histones are located in the tail of H3K4, H3K9, H3K27, H3K36, and the nucleosome core region H3K79 (Dambacher et al. 2010).

1.6-5.1. Histone 3 lysine 4 methylation (H3K4)

Methylation of histone H3 at lysine 4 (H3K4) whether it is mono (me), di- (me2) or tri- (me3) methylation, positively correlates with transcriptional activation (Barski et al. 2007) and is catalyzed by Set7/9 and Mll (Mixed lineage leukemia) enzymes in humans (Daniel et al. 2005; Wang et al. 2001). Mll2 knock out mouse embryonic stem cells, showed decreased rate of differentiation and proliferation compared to wild type in studies of (Lubitz et al. 2007) which further indicates the significance of H3K4 methyltransferases. This was found to be due to increased apoptosis which in turn was a result of reduced Bcl-2 expression. Chromatin immunoprecipitation (ChIP) demonstrated substantial decrease in H3K4me3 on the promoter region of Bcl-2 gene because of the Mll2 deficiency.
Histone 3 Lysine 9 (H3K9) methylation

Lysine 9 methylation of histone H3 is methylated by a range of different enzymes including Suv39h, G9a, ESET and GLP/Eu-HMTase1 (Sims, III et al. 2003). Histone H3K9 methylation has been shown to inhibit H3K9 acetylation and H3K4 methylation in vitro, both of which are methyl marks for active chromatin state. This suggests that H3K9 methylation may define a silent, transcriptionally inactive chromatin (Barski et al. 2007; Daniel et al. 2005). G9a is SET domain containing histone methyltransferase with known lysine methylating capacity on H3K9 and H3K27 (Rice et al. 2003). In a recent study by Chen et al, it was shown that G9a caused a significant down regulation of the cell adhesion molecule, Ep-CAM through H3K9 dimethylation on Ep-CaM promoter (Chen et al. 2010). ShRNA-mediated knock-down of G9a in lung adenocarcinoma cell lines caused an increase in Ep-CAM expression which was associated with suppression of cell invasion in in vitro and metastasis in vivo.

H3 lysine 79 (H3K79) methylation

H3K79 dimethylation (H3K9me2) is a mark of active gene expression in mammalian cells (Im et al. 2003a), while H3K79 tri-methylation (H3K79me3) is associated with transcriptionally repressed gene in human cell lines (Barski et al. 2007). The SET domain and non-SET domain containing histone methyltransferases are two major classes of proteins that methylate H3K79. Disrupter of telomeric silencing 1 (Dot1) is the only member of the non-SET domain containing histone methyltransferases (Feng et al. 2002). Schulze et al found that H3K79 dimethylation was enriched in the promoter region of
active genes involved in M/G1 phase of the cell cycle. Deletion of cell cycle related proteins, Swi4 and Swi6, resulted in a decrease in H3K79me2 but not H3K79me3 linking H3K79 dimethylation to cell cycle. DOT1 siRNA-treated U2OS osteosarcoma cell lines showed significantly less H3k79 methylation which was associated with a reduction in the loading 53BP1 (P53 binding protein 1) to gamma irradiation-induced double strand breaks (DSB) (Schulze et al. 2009). Immunoprecipitation assays showed a direct binding of H3 to 53BP1. Further protein analysis showed that increased exposure of pre-existing methylated Lys79, rather than newly methylated Lys79, accounted for recruitment of 53BP1 to sites of DNA DSBs (Huyen et al. 2004).

1.7- GR and chromatin remodelling

Nuclear receptors, such as GR and the progesterone receptor (PR) are capable of inducing chromatin remodelling events on the response elements of their target genes to facilitate gene transcription (Beato et al. 2000; Wiench et al. 2011b). GR has been shown to induce chromatin remodelling on hormone response elements along the mouse mammary tumor virus (MMTV) long terminal repeat. In T47D breast cancer cells, GR and PR hormone-dependent activation of the MMTV promoter was dependent on GR forming a complex with the ATP-dependent Brahma-related gene (BRG1) subunit of the SW1/Snf (SWItch/Sucrose NonFermentable) chromatin remodeler (Fryer et al. 1998).

The ATP-dependent SWI/SNF family of nucleosomal remodelling complexes play important roles in gene expression by altering local chromatin structures and facilitating the binding of transcription factors to sequence specific DNA (Kadonaga 1998). Human
SWI/SNF is a large multiprotein complex that contains either Brahma (BRM) or BRG1 as the central catalytic ATPase, as well as, 10-12 BRG1-associated factors (BAFs). GR mediated MMTV transcription in SW-13 small cell carcinoma cell lines, requires the recruitment of the BRG1 chromatin-remodelling complex as shown by Trotter et al (Trotter et al. 2004a). The adrenal cortex-derived SW-13 cell lines, lacking the endogenous BRG1 and hBrm, were stably transfected with MMTV luciferase reporter and a GR expression plasmid. Luciferase analysis of these cells transfected with either wild type or mutant BRG1 expression plasmid demonstrated that Dex-induced MMTV transcription and luciferase production was only produced in the wild type group. Further RT- PCR analysis of the stable cell lines showed that BRG1 enhanced the GR-mediated gene expression. Johnson et al (Johnson et al. 2008) analysed this finding further and illustrated that BRG1 and Brm have distinct kinetic properties on the MMTV promoter. RNA fluorescence in situ hybridization (FISH) analysis, reported a hormone-dependent enrichment of signal at the MMTV array which was lost in the absence of dexamethasone. This was associated with a significant increase in the loading of RNA pol II on MMTV array. Dominant negative form of BRG and Brm both inhibited the hormone-dependent transcription although Brm was identified as the weaker effector of transcription.

John et al (John et al. 2008), showed that GR binding to the promoter regions of target genes was associated with a hypersensitive transition of the local chromatin region. Moreover, it was shown that GR loading occurs at DNase I hypersensitive sites (DHSs) that
are either hormone inducible (de novo) or constitutively present in chromatin (pre-programmed) from cells untreated with hormone. In a subset of genes studied, ATP-dependent Brg1 activation was required for hormone-dependent or independent hypersensitive transition of the genes. An interesting finding in this paper was the observation that GR interacts with pre-existing accessible sites on chromatin. Some of these sites were shown to be dependent on the Swi/Snf complex since this hypersensitive chromatin state was clearly lost in the cells expressing the mutant Brg1 variant (dnBrg1) which lacked the ATPase subunit. In a more recent study, DNase I sensitivity and GR binding to chromatin across the whole genome, was investigated pre- and post-dexamethasone treatment (John et al. 2011). In a mouse pituitary cell line, more than 95% of the GR occupancy occurred at pre-hormone treated DNase I sensitive regions of chromatin whereas, in mammary 3134 cells, no pre-hormone GR binding was observed. However, almost all (99%) of GR occupancy post –dexamethasone treatment was associated with raised DNase I sensitivity. It was also observed that only 11.4% of GR occupancy sites were shared between the two different cell types reporting of cell- type specific differences in baseline chromatin accessibility patterns. These findings are incompatible with the previous assumption of the GR as the pioneer factor for chromatin remodelling events (Fletcher et al. 2000; Fryer et al. 1998), and demonstrate that lack of remodelled chromatin provides a barrier for GR binding and transcriptional activation by GR (Biddie et al. 2009; John et al. 2008).
Biddie et al (Biddie et al. 2011) identified AP-1 as an important transcription factor regulating chromatin remodelling at the GR binding sites. Indeed inhibition of DNA binding of endogenous AP-1 using a dominant negative Fos mutant, Acidic-fos (A-fos), expressing cell line demonstrated significant attenuation of GR recruitment to GREs and GR-regulated gene transcription. At the majority of regulatory elements (>70% of all GR sites), the dominant mechanism of GR-chromatin interactions involved the utilization of AP1 to mediate interactions with GR at either composite or non-composite elements in order to facilitate the chromatin remodelling prior to hormone stimulation. Data also suggested that in a minority of sites (about 15% of total chromatin GREs) GR can act as a “pioneering factor” which will require a subset of chromatin remodelling complexes, including histone methyltransferases and DNA modifying enzymes.

To elucidate the underlying factors giving rise to the formation of DHSs and accessible chromatin, Wiench et al (Wiench et al. 2011a), measured the CpG content of the DHS regions. Interestingly, the defined DHS sites were enriched in CpG dinucleotides in comparison with the rest of the genome, exhibited a cell-type specific pattern and were hypomethylated correlating with chromatin accessibility and GR binding. Comparing between the pre-programmed and de novo (hormone-induced) DHS sites revealed that the observed increase in CpG content within GR-bound DHSs is due to CpG enrichment at pre-programmed sites only. The pre-programmed DHS sites shared between mammary epithelial 3134 and pituitary corticotroph AtT-20 were fully demethylated in both cell types. Tissue-specific DHSs were demethylated in the cell types where the sites were
accessible, but hypermethylated in the cells where they were inaccessible and lacked GR binding. The CpG enriched regulatory regions of pre-programmed DHSs were hypomethylated in GR associated regions and hyper-methylated in inaccessible chromatin regions. Inhibitors of DNA methyltransferases (Dnmts) increased the accessibility of chromatin but failed to show increased GR binding, indicating that other contributing factors are involved in chromatin remodelling. Lastly, GC hormone stimulation triggered a rapid demethylation reaction at the DHSs suggestive of an active enzymatic mechanism involving a deamination-glycosylation-base excision repair pathway (Gehring et al. 2009; Kangaspeska et al. 2008; Metivier et al. 2008). All together, the above studies demonstrate the dependence of GR on pre-existing chromatin architecture. The considerable difference in the basal chromatin accessibility between different cell types drives cell specific GR-mediated binding and gene expression.

1.8- Co-activators and co-repressors of GR

1.8-1. P160/SRC

The P160 family of co-activators were among the first co-factors found to associate with the nuclear receptors in a ligand-dependent pattern (Kurokawa et al. 1995). Three closely related SRC/P160 co-factors (SRC1/NcoA-1/p160, SRC2/Tif2/GRIP1, SRC3/pCIP/AIB-1/ACTR/RAC/TRAM-1) have been identified by expression cloning and yeast two hybrid screening assays (Anzick et al. 1997; Li et al. 1997; Onate et al. 1995). The p160 family proteins contain a conserved amino-terminal basic helix-loop-helix PAS domain involved in protein-protein interactions via LxxLL motifs known as NR boxes (Heery et al. 1997).
These motifs form amphipathic helices providing a hydrophobic surface on the helix that is able to fit into the AF-2 transactivation domain and is then held in place with a charge clamp provided by the conformation of the LBD induced by ligand binding. This has been shown as the GR binds SRC-1 when bound to dexamethasone and fails to recruit SRC-1 when bound to the partial agonist RU486 (Stevens et al. 2003). A C-terminal non-conserved motif, the NR box, appears to confer specificity for the GR, as a second charge clamp in the GR, but not other nuclear receptors, binds to this motif (Bledsoe et al. 2002). However, removal or mutation of single LXXLL motifs does not completely abolish the association with receptors which suggests that multiple interactions with nuclear receptor boxes are required. SRC2 has been reported to enhance the functions of nuclear receptor transactivation domains AF-1 and Af-2 (Ma et al. 1999). Studies with CBP antibodies illustrated that functional interaction with CBP/p300 required LXXLL-containing motifs in SRC1 and reported receptor-specific differential utilisation of LXXLL-containing motifs of the SRC-1 coactivator (Mclnerney et al. 1998).

Although some SRC family proteins have been shown to have intrinsic histone acetyltransferase activity, their role in up-regulating transcription has been shown to require the recruitment of other known HATs such as CBP/p300 and p/CAF (Chen et al. 1997). In here, they reported a novel steroid receptor coactivator, ACTR, sharing prominent region high sequence similarity with SRC1 and SRC2 that illustrated strong association with CBP/p300. This nuclear co-activator complex then binds nuclear receptors and stimulates their transcriptional activities in a hormone-dependent fashion.
Furthermore, ACTR also known in humans as p/CIP (p300/CBP interacting protein)/ SRC-3/AIB1 was found to be methylated by the coactivator arginine methyltransferase 1 (CARM1). This methylation was found to be crucial for both stability of ACTR protein and its cellular turn over and also in dissociation of CBP/p300 and ACTR in mouse embryonic fibroblasts (Naeem et al. 2007).

1.8-2. P300/CBP

The histone acetyltransferase complex cAMP response element-binding protein (CREB) binding protein (CBP) and the homologous protein p300 interact with the AF1 transactivation domain of GR and contact the AF-2 domain indirectly via binding to p160 family of co-activators (Jenkins et al. 2001; Kobayashi et al. 2000; Yao et al. 1996). CBP may also bind to general transcription factors including TBP and TFIIB (Yuan et al. 1996) and also recruit RNA polymerase II by interaction with helicase domain of the polymerase (Kee et al. 1996). Bannister et al (Bannister et al. 1996) showed that CBP not only recruits histone acetyltransferases (HATs) such as p/CAF, it also holds intrinsic HAT activity. Transient recruitment of CBP to GC-regulatory regions tracked an ultradian cortisol rhythm, resulting in ultradian changes in lysine acetylation on histone H4. Pulsatile acetylation of histone H4 was concomitant with RNA polymerase II recruitment to Period 1 gene and its rhythmic gene transcription (Conway-Campbell et al. 2011b).

1.8-3. Interferon-inducible factor (IFI16)

The interferon-inducible HIN-200 family proteins encode a class of homologous proteins that share a 200-amino acid signature motif (HIN). Four human (IFI16, AIM2, MNDA and
IFIX) and five mouse members (p202a, p202b, p203, p204, and p205) of this family have been identified. Indeed, IFI16 is widely expressed in normal human endothelial and epithelial cells in addition to hematopoietic cells (Gariglio et al. 2002; Wei et al. 2003). IFI16 overexpression in musculoskeletal tumour cells was associated with inhibition of proliferation and induced a senescent phenotype while loss of IFI16 expression has been associated with various forms of human cancers, including those of the pancreas, prostate, and breast (Trapani et al. 1992; Xin et al. 2003) demonstrating a role for IFI16 in tumour suppression. IFI16 physical binding to the C terminal region of p53 binding protein (p53BP) augmented the p53-mediated transcriptional activity (Johnstone et al. 2000). This was found to be direct binding of the HIN-A domain of IFI16 to the C terminal region of p53 while HIN-B domain binds to the core DNA binding region of p53 giving rise to the effect of full length IFI16 on p53-DNA complex formation and transcriptional activation. IFI16 has also been shown to interact directly with GR and modulate both GR-mediated transactivation and transrepression. IFI16 enhanced GR-mediated transactivation of TAT-Luciferase and restored GRmediate transrepression of NF-κB-Luc reporter gene in HeLa cells (Berry et al. 2010). It was shown that GR and IFI16 co-localised to the same nuclear regions and direct binding of IFI16 to GR ligand binding domain (LBD) was required for modulating GR functions.

1.8-4. CARM1

Co-activator associated arginine methyltransferase 1 (CARM1); also known as protein arginine methyltransferase 4 (PRMT4) is a protein arginine methyltransferase that
regulate crucial cellular functions including transcription, mRNA processing and stability and translation. It is known for its positive effects on transcription by methylating histone H3 at arginine 17 and 26 (Bedford et al. 2005; Ma et al. 2001; Schurter et al. 2001). CARM1 has been identified to associate with steroid receptor co-activators like SRC1, SRC2 and SRC3 (Chen et al. 2000; Chen et al. 1999; Ma et al. 2001). Studies with 1471.1 cell line which contains multiple copies of chloramphenicol acetyltransferase (CAT) gene, controlled by MMTV promoter, showed that GR-mediated activation of CAT gene increased significantly when CARM1 was overexpressed (Ma et al. 2001). The CAT activation was even higher when CARM1 and GRIP1 were coexpressed in these 1471.1 cells. A CARM1 mutant, lacking the methyltransferase activity failed to enhance the CAT activity. ChIP studies also indicated a dex-regulated increase in the methylation of histone H3 arginine17 on the MMTV promoter by CARM1 which was associated with higher GR and pol II recruitment. In another study, gene expression profiling of lung from CARM1 knock-out mice, revealed dysregulation of cell cycle genes and markers of differentiation. Interestingly, there was an overlap in gene expression between the CARM1 knock-out and the GR knock-out lung. In the absence of CARM1, hyper-proliferation of pulmonary epithelial cells was observed which correlated with immature alveolar type II cells and an absence of alveolar type I cells. As the gene expression profiling suggests, these phenotypes might in part be caused by attenuation of the glucocorticoid receptor-mediated signalling in the absence of CARM1 (O’Brien et al. 2010).
1.9- **Circadian and ultradian rhythm of GC release:**

In mammals the endocrine system is one of the major signalling systems to use frequency encoding. The pulsatile release of gonadotropin-releasing hormone (GnRH) is essential for luteinising hormone (LH) and follicle stimulating hormone (FSH) secretion. In patients suffering from infertility, ovulation was induced by pulsatile delivery of gonadotropin-releasing hormone (GnRH) through a battery-driven infusion pump designed to deliver boluses of luteinizing hormone releasing hormone (LHRH) every 90 mins. Cycles of ovulation was detected in all patients with ten resulting in pregnancies (Pampori *et al.* 1991). Continuous high doses (200 or 500 µg twice daily) of GnRH agonists however, suppress the pituitary gonadal axis through down-regulation of the GnRH receptor (Labrie *et al.* 2005).

In fact the pulsatile release of a number of endocrine hormones such as LH (Knobil *et al.* 1980; Minabe *et al.* 2011), prolactin (Bertram *et al.* 2010) and insulin (Knobil *et al.* 1980) is critical for their physiological functions. The hypothalamic-pituitary-adrenal (HPA) axis helps to maintain physiological and stress-related homeostasis of the central nervous system (CNS), as well as maintaining physiological metabolic, cardiovascular, and immune functions (Chrousos *et al.* 1992; Chrousos 1995). Dysregulation of the HPA axis is involved in several behavioural, circadian, endocrine/metabolic and immune disorders (Alesci *et al.* 2005; Dekkers *et al.* 2000; Harbuz *et al.* 1999). The secretion of GCs as the end product of the HPA axis shows significant temporal regulation, with both pulsatile (“ultradian” = shorter than a day) (Jasper *et al.* 1991) and “circadian” (= about one day) rhythmicity (Haus 2007; Lightman 2008; Windle *et al.* 1998a). Frequent (every 10 mins) blood-
sampling technique in female rats allowed the circadian rhythm of corticosterone to be resolved into a series of ultradian pulses. These were equally distributed (mean interval, 50.9 ± 3.7 min) throughout the 24-hr cycle, but their amplitude varied significantly, with peaks between 1800–2200 h (137 ± 9 ng/ml) and troughs between 0600–1000 hr (75 ± 17 ng/ml) (Windle et al. 1998a). The pulsatile secretory profile of endogenous GC has been reported in other species as well as humans and varies with normal physiological changes such as puberty, gender, lactation and aging. This pattern of release indicates continuous, but variable, activity of the HPA axis throughout the day (Conway-Campbell et al. 2011a).

In humans ultradian pulses of the endogenous GC hormone, cortisol, are released into the plasma every 71.8 min (Veldhuis et al. 1990a; Young et al. 2004). The magnitude of secreted endogenous GC in humans varies in a diurnal pattern with the lowest plasma hormone levels between 8 p.m. to 2 a.m. followed by a rise in the early morning reaching a maximum between 8 a.m. to 10 a.m (Lightman et al. 2008; Young et al. 2004). The most frequently reported pattern alterations in pulsatile GC release include changes in pulse frequency (Windle et al. 2001), pulse mass (Henley et al. 2009) and pulse amplitude (Deuschle et al. 1997) over the circadian cycle which occur during disease states (Windle et al. 2001), chronic (e.g., noise (Windle et al. 1998b) or fasting stress (Bergendahl et al. 2000) and sleep apnea (Henley et al. 2009). Measurements of rat hippocampal extracellular corticosterone concentrations under baseline and stress conditions revealed ultradian and circadian rhythms that were stressor-dependent (Droste et al. 2008). This implies that stress-induced rise in glucocorticoid release is ensured to exert its important
actions on several body functions like emotion, cognition, and more importantly energy metabolism.

1.9-1. SCN control and HPA axis:

SCN is a small paired nucleus in the anterior hypothalamus (Kleiman et al. 2007). SCN lesions abolished the circadian rhythm in mice (Harmar et al. 2002) (Meijer et al. 2003). In rodents these lesions lead to abnormal feeding patterns and body temperature. Although the circadian locomotor rhythms were restored after transplantation of SCN grafts into the SCN lesioned hamsters, endocrine rhythms, including those of glucocorticoids, failed to recover suggesting a requirement for axonal connections (Lehman et al. 1987). Interestingly, the SCN also exhibits ultradian rhythms of activity (Yamazaki et al. 1998). Although the significance and contributions of these rhythms to the ultradian rhythms of HPA axis and GC release has not yet been fully understood (Lowry 2002), a recent study by Nader et al (Nader et al. 2009) on HeLa cells, has demonstrated that circadian rhythm-mediated transcription factor CLOCK controls the transcriptional activity of the GR by histone acetyltransferase (HAT) activity. This suggests that the circadian rhythm and at its core the master oscillator, the SCN, indirectly influences the function of various organs and tissues modulated by the actions of GCs.

1.9-2. Glucocorticoid synthesis and release:

Corticotropin-releasing hormone (CRH) and Arginine Vasopressin (AVP) released from the para ventricular nucleus (PVN) into the hypophyseal portal blood vessels are the principal hypothalamic factors responsible for stimulating Adrenocorticotropic Hormone (ACTH)
secretion by the corticotrope cells of the anterior pituitary (Jacobson 2005). The frequency of cortisol pulses released from the adrenal cortex is tightly dependent on the episodic secretory events of ACTH from anterior pituitary (Spiga et al. 2011). This in turn is regulated by rhythmic synthesis and release of CRH from hypothalamus. Studies on unstressed rats by Watts et al (Watts et al. 2004) revealed that transcription of crh mRNA is subject to diurnal variations. CRH synthesis, in intact animals, is maintained by nocturnal episodes of crh gene transcription. Thus CRH might play a role in pulsatile secretion of ACTH as well. The cumulating concentrations of the endogenous GC, cortisol is regulated primarily by increasing or decreasing the amplitude rather than the frequency of secretory episodes (Sarnyai et al. 1995; Veldhuis et al. 1990b) and is believed to occur through modulation of ACTH release (Desir et al. 1980). Synthesis of cortisol from cholesterol depends on the ACTH receptor-mediated rise in cholesterol desmolase activity and transcription of enzyme-encoding genes required for cortisol synthesis (William GH 1998). An important aspect of the pituitary–adrenal system is the negative feedback of glucocorticoids on ACTH release. In the hypothalamus cortisol inhibits the release of CRH and ACTH respectively in a negative feedback manner. A study on adrenalectomized rats confirmed that circulating corticosterone is sufficient to completely inhibit a daily rhythm of avp gene transcription and therefore prevent its own synthesis (Watts et al. 2004). Also, acute administration of prednisolone produced a rapid significant decline in ACTH and cortisol pulsatile rhythms in adult healthy males within 60 minutes since the administration of the synthetic GC inferring rapid feedback inhibition at the anterior pituitary (Russell et al. 2010).
1.9-3. Ultradian rhythm and GR-mediated transcription

The pattern of GC hormone secretion is highly pulsatile. Since every tissue in the body expresses the glucocorticoid receptor (GR), it is important to understand how tissues respond to the pulsatile GC secretion and the molecular mechanisms involved in gene regulation by GR. Stavreva et al (Stavreva et al. 2009), demonstrated that physiological GR functions requires the pulsatile induction of the endogenous hormone. In this study they utilised the 3617 mouse cell line which contains an amplified array of GR responsive promoter structures (the MMTV array), to directly visualise the interactions of GR with the GREs in real time in live cells. Here they demonstrated that ultradian hormone induction in 3617 mouse cells stimulated discrete pulses of GFP-GR interactions with the GRE rich MMTV arrays. Unliganded GR formed a complex with the multicomponent chaperone protein. Inhibition of Hsp90 with the selective Hsp90 inhibitor geldanamycin (GA), which blocked the GR-chaperone complex formation, abolished the GR-ligand interaction and inhibited the GR-chromatin association. Thus failure to “re-ligand” the receptor prevents the receptor from re-entering the rapid exchange cycle on chromatin.

Out of the GR ligands studied only the two endogenous hormones, hydrocortisone and corticosterone, were able to produce the complete GR cyclic interactions with the MMTV promoter GREs, whereas the other synthetic GCs including dexamethasone failed to show the cyclic GR binding at the MMTV promoter array. Therefore, nuclear GR recycling is important for GR activation and gene transcription. It was also shown by chromatin immunoprecipitation (ChIP) that RNA polymerase II (Pol II) was recruited to the GREs in a cyclic pattern. The rate of Pol II exchange at the MMTV promoter was significantly lower
during maximal GR binding indicating the productive engagement of Pol II. This was also associated with pulsatile expression of some highly up-regulated GR target genes including glucocorticoid-leucine zipper protein (GILZ) whereas constant hormone stimulation produced continuous release of RNA.

Cycling of other nuclear receptors including the androgen receptor (AR) and estrogen receptor (ER) at their specific binding sites has been explained previously (Kang et al. 2002; Metivier et al. 2003). However, this cycling is known to be due to an intrinsic property of these receptors and exists in the state of constant hormone stimulation. GR cycling, as described by Stavera et al (Stavreva et al. 2009), however reflects pulsed release of glucocorticoids from the adrenal glands and is therefore driven externally and occurs through pulsatile GC induction. It is therefore of great importance to fully investigate the biological consequence of this pulsatility and explore the underlying molecular mechanisms of this phenomenon.

Conway-Campbell et al (Conway-Campbell et al. 2011b) investigated the intracellular GR signalling in response to alternative patterns of GC exposure. Using ChIP assays they were able to show cycles of GR interaction with the specific promoters of PER1, MT1, TAT and POMC in different mouse and human cell lines. This recruitment was rapidly followed by cyclic histone H4 acetylation, cyclic histone acetyltransferase P300/CBP binding and finally by RNA pol II recruitment at the specific promters. GR synthesis and nuclear-cytoplasmic transition was not required for the cycles of GR activity as shown by cyloheximide inhibition of protein synthesis and also agglutinin, which inhibits all nuclear
pore transitions. However when, HSP90 was inhibited through geldanamycin, GR ligand rebinding was impaired and this attenuated GR cyclical transcriptional activity. This strongly suggests that the chaperone activity of HSP90 is necessary for maintaining the correct GR conformation for ligand rebinding and reactivation in the nucleus and also for physiological GR functions.

The significance of ACTH rhythmicity for the maintenance of normal ultradian GC secretion and GC-mediated steroidogenesis was explored in a study by (Spiga et al. 2011). Hourly but not continuous infusions of ACTH to methylprednisolone-mediated HPA axis suppressed rats, restored pulsatile release of the endogenous GC, corticosterone, which was associated with episodic transcription of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage (P450scC) genes. This study indicates that pulsatile ACTH activation of the adrenal cortex is not only critical for the secretion of corticosterone but also induces episodic transcription of the rate-limiting enzymes necessary for physiological steroidogenic responses.

In line with these finding, McMaster et al (McMaster et al. 2011), also showed that pulsatile cortisol exerted a differential pattern of gene expression to that of continuous treatment. Pulsatile cortisol caused a significantly more apoptosis compared to continuous exposure of the same cumulative dose in HeLa cells. Microarray profiling of HeLa cells identified genes that were differentially regulated by pulsatile vs continuous cortisol. Several transcription factor binding sites were enriched in these differentially regulated target genes, including CCAAT-displacement protein (CDP) and SP-1. A CDP-
regulated reporter gene (MMTV-Luc) was, as predicted, also differentially regulated by pulsatile compared to continuous cortisol delivery. The differential pattern of expression of genes could not be explained through autoregulation of the GR, or differential post-translational modification of the GR, Ser\textsuperscript{211}, implying a post GR activation mechanism.

These preliminary findings together with other studies mentioned above, contribute to the growing observations of the importance of oscillatory signalling pathways by which cells coordinate multi-gene responses to extracellular signal. These insights provide clues into the molecular mechanisms that regulate the GR functions and can bring new approaches for synthesis of novel GR ligands. Given their widespread medical use, further studies are necessary to understand the potential of ultradian therapy for GC administration.

1.9-4. The significance of ultradian GC release in physiology

It is increasingly recognised that the pulsatile secretion of glucocorticoid signals are critical determinants of target organ responses. Findings by Windle et al (Windle et al. 1998a) reported an increase in the amplitude of corticosterone ultradian pulses in periods when the rats were exposed to white noise, as psychological stress stimulation, resulting in a rise of plasma corticosterone level. The pulsatile ultradian rhythm suggested alternate periods of secretion (rising phase) and inhibition (falling phase), which were found to have a profound effect on the corticosterone responses to acute stress. The response to the stress was not significant when the stress coincided with the falling phase
of ultradian pulses in which release of corticosterone is inhibited and HPA axis is insensitive to stress stimuli (Windle et al. 1998b).

The ultradian rhythm of GCs release is necessary for maintaining the homeostasis of a number of endocrine and metabolic activities. Dysregulation of this ultradian pattern through disease states (Arlt et al. 2003; Pariante et al. 2008) and/or long-term glucocorticoid treatment (Claahsen-van der Grinten HL et al. 2011) produces potentially life-threatening and debilitating conditions and disorders. Adrenal insufficiency is a life-threatening disease that manifests itself with early morning fatigues and an impaired health-related quality of life. Primary adrenal insufficiency, Addison’s disease, is due to the absence of adrenal tissue while the secondary form is due to impaired ACTH secretion because of pituitary failure or long term GC therapy (Arlt et al. 2003). GC replacement therapy has been suboptimal for treatment of all forms of adrenal insufficiency due to a number of side effects such as hypertension, impaired growth and cardiovascular disease indicating a need for a therapy that can replicate a more physiologically relevant rhythm of GC release. Modified release hydrocortisone which releases the drug 4hrs after ingestion was given to six healthy males, whose HPA axis were suppressed with administration of dexamethasone, at 10pm (Newell-Price et al. 2008). This allowed the cortisol to reach a peak at around 2.45 a.m. reproducing the normal daily morning rise in serum cortisol. In another study, 14 patients with congenital adrenal hyperplasia, characterised with a defect in cortisol synthesis and androgen excess, were given a modified release hydrocortisone tablet three times a day (Verma et al. 2010). The twenty-
four hour cortisol sampling showed three cortisol peaks a day with one at 6:00 hours and significantly lower cortisol levels in the afternoon and night time relative to the conventional hydrocortisone. Patients also demonstrated well-controlled adrenal androgens overnight showing that the initial early morning rise in serum GC is needed. This study indicates a requirement for physiological cortisol profiles which would likely result in improved clinical outcome of diseases with a deregulated daily profile of cortisol release.

Obstructive sleep apnea (OSA) is a common condition associated with nocturnal awakening. The significant cardiovascular and metabolic comorbidity seen in sleep apnea have been linked to marked disturbances in the ultradian secretory dynamics of cortisol and ACTH release in untreated patients (Henley et al. 2009). Previously it had been shown that sleep deprivation and continuous disruption during sleep significantly raised the plasma cortisol and made the HPA axis highly sensitive to negative feedback inhibition (Spath-Schwalbe et al. 1991).

1.9-5. The HPA axis in rheumatoid arthritis

A number of cytokines follow a diurnal pattern of secretion all of which are directly or indirectly regulated by cortisol (Petrovsky et al. 1998). Tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-12 and interferon-gamma (IFN-γ) production measured in LPS-stimulated whole blood from 13 male and female normal human volunteers demonstrated a diurnal variation which was inversely related to the pattern of cortisol release. The peak of plasma circulating T cells, in particular CD4+ T cells coincided with
the peak level of cytokines which raises the possibility that diurnal variation of cytokines is either a consequence of variation in circulating T cells or the cause of it. The early morning elevation of cortisol suppressed cytokine production. However, levels of CD4+ and CD8+ T cells did not change until two or three hours later.

Due to the importance of cytokines in immune and inflammatory reactions, their temporal relationship with symptoms of autoimmune disease such as rheumatoid arthritis (RA) has been frequently explored and reported (Arvidson et al. 1994; Cutolo et al. 2005; Cutolo et al. 2008). Patients with RA complain of joint stiffness and swelling on walking which is worst in early morning and improves as the morning progresses. Crofford et al (Crofford et al. 1997), investigated circadian variation of cortisol and IL-6 levels in newly-diagnosed patients and showed a significantly higher plasma IL-6 levels in patients with RA. In the RA group there was a temporal correlation between the elevated levels of IL-6 and ACTH/cortisol release with peak IL-6 preceding the rise in cortisol/ACTH by 1-2 hrs. In these patients elevated circulating IL-6 failed to increase the basal cortisol release. Furthermore, Perry et al (Perry et al. 2009) as well as reporting abnormalities in plasma cortisol and IL-6 concentrations and dynamics, also linked the overall rise in IL-6 to the circadian variations in symptoms. Increased production of other pro-inflammatory cytokines such as TNF-α, IL-1, IL-2 and IFNγ, have also been numerousely reported in RA patients (Cutolo et al. 2003; Cutolo et al. 2008).

To determine a defective HPA axis underlying RA increasing number of studies have investigated the diurnal variations in cortisol release between healthy volunteers and
patients. Patients with RA failed to mount an increase in serum cortisol levels relative to control despite high levels of IL-1 and IL-6 following surgery (Chikanza et al. 1992). Since CRH release showed normal results in these patients, a hypothalamic defect was blamed for the lack of response. Other studies have shown a defect at the adrenal level in RA. Serum IL-6 and TNF-α levels were measured and compared between a group of healthy volunteers and RA patients (Straub et al. 2002). Significantly elevated levels of cortisol IL-6 and TNF-α was observed in 34 patients with RA (male and female) compared to 112 healthy controls. However, the ratio of ACTH/cortisol induction to IL6 and TNF-α was significantly lower than that seen in the healthy controls. As a result, the HPA axis fails to respond to elevated systemic inflammation through increasing the levels of rhythmic cortisol release. These findings are suggestive of a defect in the HPA axis in RA. In fact a number of reports have utilised 24 hour blood sampling and have suggested that HPA axis is unable to mount an appropriately enhanced glucocorticoid response to alleviate joint inflammation (Chikanza et al. 1992; Harbuz et al. 1999; Jessop et al. 2005; Neeck et al. 1990; Zoli et al. 2002) although one study suggests the unresponsiveness of the HPA axis to be an ongoing adaptation to the disease state, with higher priority for proper regulation of core body functions over immune homeostasis (Imrich et al. 2010).

1.9-5.1. Modified-release GC intervention in treatment of RA

There are new approaches for the optimisation of glucocorticoid therapy in RA. When patients were administered with low doses of prednisolone at either 2 a.m. or 7.30 a.m. the group with early (2.00 a.m.) treatment of prednisolone showed smaller duration of
morning stiffness, joint pain and also lower serum concentration of IL-6 (Arvidson et al. 1997). Some recent approaches include the modified release (MR) GCs that adapt the timing of glucocorticoid release to the circadian rhythm of endogenous cortisol release. The MR prednisolone tablets used for the study by Buttgereit et al (Buttgereit et al. 2008) of 288 patients with RA, released the drug 4hrs after ingestion in contrast to the immediate release-tablets which immediately release the drug following ingestion (Buttgereit et al. 2008). The MR tablet was given to patients at 10 p.m. releasing the drug at around 2 a.m. allowing for the GC release to be adapted to the endogenous circadian rhythm. Both serum IL-6 concentrations and the duration of morning stiffness were significantly reduced (p= 0.045) by the MR prednisone after 3 months but were unchanged by immediate-release prednisone. However, MR prednisolone did not change the safety profile of the drug relative to the immediate release. This study provides evidence for the significance of timed glucocorticoid therapy and the need for further investigations on the long-term effects of timed drug release on basic inflammatory processes and the entire range of symptoms of rheumatoid arthritis. The effects of long-term low-dose MR prednisolone therapy on the HPA axis of 28 patients with RA was investigated in a follow-up study (Alten et al. 2010). Corticotrophin-releasing hormone (CRH) was not changed in MR vs. immediate release (IR) prednisolone, showing that no HPA axis suppression developed with the tablets. The results were the same even after 12 months of treatment with MR tablets. These studies therefore reveal a novel approach for optimising GC therapy in patients with RA.
1.10- Williams Bueren syndrome chromosome region 22 (WBSCR22/Merm1)

1.10-1. Williams Bueren syndrome

Williams Bueren syndrome (WBS), also known as Williams’ syndrome, is caused by deletion of 1.5-1.8 Mbp on chromosome 7 spanning 26-28 genes (Fig 1.5). WBS is a complex multi-developmental disorder characterized by multisystemic manifestations such as congenital vascular and heart disease, hypertension, characteristic facial features, premature aging, mild mental retardation, hypercalcemia and glucose intolerance (Morris et al. 1988; Pober et al. 2007; Pober 2010). Flourescence in situ hybridization (FISH) readily detected the most deleted segments to be between two highly homologus duplicons of 350-500 kbs in 90-99% of adults with WBS (Mila et al. 1999; Perez Jurado et al. 1998; Tassabehji et al. 1999).

Transcriptome profiling of lymphoblastoid cell lines from 6 patients with Williams’ syndrome showed that glycolysis and gluconeogenesis were the most significantly affected pathways, with multiple down-regulated genes involved in glucose catabolism to pyruvate in liver. This may lead to glucose intolerance and diabetes in WBS (Antonell et al. 2010). MLXIPL, encoding a basic–helix-loop-helix leucine zipper (bHLHZip) transcription factor, carbohydrate responsive element binding protein (ChREBP), and STX1A have all been related to glucose metabolism and all were suppressed in WBS patients.
Chapter 1: Introduction

Figure 1.5. The Williams-Bueren syndrome chromosome region (WBSCR) on Chromosome 7.

The top panel shows the WBSCR located between flanking block of low-copy DNA repeats or duplicons. The most common WBSCR22 deletions occur in the centromeric and medial B blocks and A blocks, respectively. The width of the rectangles roughly corresponds to the gene size. Adapted from Pober et al (Pober 2010).

1.10-2. WBSCR22/Merm1 and methyltransferase activity

Genomic sequences analysis followed by RT-PCR of a cluster of genes mapping to 7q11.23 identified wbscr22 in the WBS deleted region (Merla et al. 2002). Characterization of the WBSCR22 protein suggests that the putative protein contains a bipartitie nuclear localisation signal and an S-adenosyl-L-methionine (SAM) dependent- methyltransferase
(MTase) domain (Fig 1.6) (Doll et al. 2001). Petrossian and Clarke (Petrossian et al. 2011), presented a comprehensive analysis of human methyltransferosome using computational programs HHpred (Soding et al. 2005b), FMMMR (Eddy 2009) and multiple Motif Scanning (Petrossian et al. 2009). Aligned motif sequence search in the human proteome database identified WBSCR22 as a methyltransferase belonging to the seven-β-strand family. The seven-β-strand family (also referred to as “Class I” methyltransferases is the most abundant of methyltransferase protein superfamily and methylate a wide array of substrates with their featuring Rossmann-like structural core. Other members of this family in humans include coactivator arginine methyltransferse 1 (CARM1) that primarily methylates arginine 17 on histone H3, and also Dot1L (Dot1-like) a methyltransferase for lysine79 on histone H3.

The WBSCR22 homologue in C.elegans, C27F2.4, plays an important role in embryonic development and the RNAi-mediated knock-down of the WBSCR22 protein was reported embryonic lethal (Piano et al. 2002). Based on sequence homology, WBSCR22 encodes a gamma SAM-Mtase in which a SAM motif connects helix-α C to strand β4 (Schluckebier et al. 1995). WBSCR22 expression is mainly nuclear in A375M melanoma cells and in invasive ductal carcinomas compared to normal tissues (Nakazawa et al. 2011). However, the invasive property of the tumour was significantly lost in tissues injected with a mutated WBSCR22 which lacked the wild-type methyltransferase domain. Knock down of WBSCR22 by shRNA resulted in inhibition of metastasis through Zac1-p53-dependent apoptosis in melanoma cell lines. ChIP analysis then confirmed H3 lysine9 methylation on
Zac1 promoter region in tumour cells which was lost with Merm1 shRNA-mediated knock down. Overall, this study identified WBSCR22 as a histone methyltransferase that acts as a promoter of metastasis through strong inhibition of tumour suppressor Zac1 gene and therefore referred to WBSCR22 as metastasis-related methyltransferase 1 (Merm1).
Merm1 also contains a highly conserved DXGXGXGXXG-like motif in its SAM domain where X may be any amino acid. This motif is essential for SAM binding properties of Merm1 and is rich in uncharged amino acids like Glycine (G) and proline (P) (Martin et al. 2002).
1.11- Hypotheses and Aims:

Overall hypothesis: Novel modulators of glucocorticoid sensitivity exist which can affect
glucocorticoid receptor function through molecular interactions and dynamic
mechanisms.

Hypothesis 1: Glucocorticoid action is affected by functional interaction with as yet
unidentified cellular proteins. These interactions determine GR access to target sites and
mediate GR regulation of chromatin structure.

Aim 1: To explore the role of Merm1 as a novel modulator of GR, specifically:

- To evaluate Merm1 effects on GR functions using in vitro assays.
- To characterise Merm1’s methyltransferase activity.
- To investigate the role of Merm1 in GR-regulated gene transcription.

Hypothesis 2: The kinetics of GC delivery to target cells is an important factor regulating
the pattern of gene expression and subsequent cellular phenotype.

Aim 2: To explore temporal aspects of glucocorticoid sensitivity modulation with the
specific aims to:

- Establish a flow through culture system for pulsatile and continuous cortisol delivery to
  adherent HeLa cells and to non adherent cells (primary T cells).
- Investigate the role of pulsatile and continuous cortisol treatment on HeLa and primary
  T cell apoptosis.
- Explore the effects of pulsatile vs. continuous cortisol delivery on the expression of a
  panel of GC target genes in primary T cells.
Chapter 2:

Materials and Methods
2- Materials and Methods

2.1- Bioinformatic analysis of Merm1/WBSCR22 sequence homology

2.1-1. HHpred interactive server for protein homology

HHPred (http://toolkit.tuebingen.mpg.de/hhpred), is the most powerful and sensitive server used for remote protein homology detection and structure prediction which utilises the pairwise comparison of the hidden Markov Models (HMMs) (Soding et al. 2005a). It allows searching multiple sequence databases such as Protein Data Bank (PDB), Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST), Structural Classification of Proteins (SCOP) and Protein Families database (Pfam). Based on the predicted secondary structure, a profile HMM is generated and then compared with each HMM in the selected database. Finally HHpred produces pairwise query-template alignments and shows a ‘Score’ based on the score from the secondary structure comparison between the query and the template.

2.2- DNA manipulation and purification

2.2-1. Plasmids

TAT-3 luciferase plasmid was a kind gift from Professor Keith Yamamoto and contains three copies of the GRE from tyrosine amino transferase (gtcgacGTAGCTAGACATCCTGTACAGctcgacCTGTACAGGATGTTCTAGCTACgtcgagCTGTACAGGATGTTCTA) inserted into pODLO plasmid upstream of the minimal Drosophila distal alcohol dehydrogenase promoter (-33 to +55) and the luciferase gene.
MMTV-luciferase was made by Professor David Ray (Ray et al 1996). The MMTV long terminal repeat containing five positive glucocorticoid response elements was excised from pMSG-CAT using HindIII and Smal I and ligated into pXP-2. The C/EBPβ reporter plasmid containing a C/EBP responsive promoter fused to luciferase was a kind gift from Dr Michael Killberg.

The NRE-Luc reporter plasmid is a basal cis reporter plasmid where the luciferase gene is driven by a basic promoter element (TATA box) plus 5 defined NRE elements upstream (TGGGGACTTTCCGC) and was obtained commercially (Stratagene, LaJolla, CA, USA). Renilla luciferase plasmid (Sea pansy, Promega) was used to determine transfection efficiency. Full length human GR cloned into expression vector pcDNA has been previously described (Ray et al. 1999). The pcDNA3-SRC expression vector was a kind gift from Dr Julie Stimmel at GlaxoSmithKline, Stevenage, UK. pcDNA3 and pcDNA4/HisMaxC control vectors were acquired commercially (Invitrogen). Full length Merm1, ANP32E and PCCA cDNAs cloned into CMV-SPORT6 vector using NotI/SalI restriction sites was obtained from Open Biosystems (Thermo Scientific, UK). The HisMaxC-tagged Merm1 was generated by cutting Merm1 from CMV.SPORT6 using Kpn1 and Not1 restriction sites and inserted into pcDNA4/HisMax C. Full length ELOVL1, HNRPA1, and DR1 cloned into cmv.SPORT6 using Apa1/Sal1, Apa1/Hind1 and ECOR1 respectively were obtained from Open Biosystems (Thermo scientific, UK) and were sequenced prior to use in luciferase reporter assays.
Chapter 2: Methods

2.2-2. Transformation of competent E.COLI

2μl of β-ME was added to 45μl of XL-10 GOLD ultracompetent E.COLI (Invitrogen) and left on ice for 10 minutes to activate the bacteria. 500ng of plasmid DNA was then added to the mixture and incubated on ice for 30 mins followed by a brief heat shock at 42°C for 30 seconds. 45μl of XL-10 GOLD competent E.COLI with no plasmid was included as the negative control, while 500ng of pUC18 was added to 45μl of XL-10 GOLD ultracompetent E.COLI as a positive control. Tubes were then placed on ice for 2 mins before addition of 500μl of pre-warmed sterile LB Broth (Sigma), and then incubated at 37°C with shaking at 220rpm for 1 hour. 200μl aliquots were used to prepare streak and spread plates respectively on LB/ampicillin/agar plates (Tryptone 1%, Yeast extract 0.5%, Agar 1.5%, NaCl 8.6mM, Glucose 20mM). Plates were inverted and incubated overnight (16-18hrs) at 37°C.

2.2-3. Plasmid Preparation

Commercially available kits were used for the purification of the plasmid DNA (Qiagen). The principle of the kit is a modified aklakine lysis of cells followed by binding of the plasmid to anion exchange resin which is silica based matrix with positively charged diethylaminoethyl (DEAE) group that bind the negatively charged phosphate backbone of the DNA. The pH and salt concentration of the buffers used in subsequent steps control binding, wash stringency and elution of the DNA from the resin.

A single colony was picked from the agar plate after overnight incubation and used to inoculate 5ml of LB/ampicillin (Tryptone 1%, Yeast extract 0.5%, Agar1.5%, NaCl 8.6mM,
Glucose 20mM, Ampicillin 100μg/ml). This was grown for 16-18 hrs at 37°C in a sking incubator at 225 rpm, so that the bacterial culture in in the log phase and at the highest level of plasmid expression. The culture is used for plasmid purification, during which alkaline lysis of the bacterial cells using proprietary reagents allows for absorption of DNA onto the resin column in the presence of hight salt, and plasmid purified from 5ml culture using a Qiagen spin miniprep kit following manufacturer’s instructions. Minipreps typically produced up to 20μg of plasmid DNA in 50μl of elute. Alternatively, larger amounts of plasmid were obtained from 500ml culture inoculated with 5ml of starter culture and subjected to a second overnight incubation in a sking incubator. Plasmid was purified using a Qiagen maxiprep kit, following the manufacturer’s recommended protocol. Maxipreps produced up to 800μg of DNA in 1ml of elute.

2.2-4. Glycerol stock

Prior to purification of the plasmid with the Qiagen kits, 400μl of sterile glycerol was added to 800μl of overnight culture in a 1.5 ml microcetrifuge tube, mixed by vortexing and then transferred -70°C for long-term storage.

2.2-5. DNA quantification

Principle

The spectrophotometric method uses ultra violet light to measure the optical density of a DNA solution. DNA absorbs light at 260nm in proportion to its concentration. The level of contaminating proteins can also be monitored due to the absorbance of the light at 280nm by amino acids. Any contamination from organic solvents can be measured by
their absorbance at 270nm. Values of over 1.8 were accepted as pure enough for the downstream analysis of the sample.

**Nanodrop**

DNA was quantified using an ND-1000 spectrophotometer (NANODROP), which measures nucleic acid concentrations for 2-3700ng/μl and determines the quality of the sample by reading the 260/280 ratio. 1μl of sample was pipetted directly onto the measuring surface, surface tension used to draw a column between two optical fibres and establish the measurement optical bath. The source fibre is coupled to a xenon flash lamp and receiving fibre is coupled to a spectrophotometer using a linear CCD camera.

### 2.2-6. Agarose gel electrophoresis

**Principle**

Agarose gel electrophoresis is a method used to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). The most important factor is the length of the DNA molecule; shorter molecules move faster and migrate further than longer ones. But the conformation of the DNA molecule is also a factor. Conformations of a DNA plasmid that has not been cut with a restriction enzyme will move with different speeds (slowest to fastest): nicked or open circular, linearised, and supercoiled plasmid. To avoid this problem linear molecules are usually separated. Increasing the agarose concentration of a gel reduces the migration speed and enables separation of the smaller molecules. The higher the voltage, the faster the DNA moves. But voltage is limited by the fact that
increased current heats and ultimately causes the gel to melt. High voltages also decrease the resolution (above about 5 to 8 V/cm). The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide. It fluoresces under UV light when intercalated with DNA (or RNA). By running the DNA through an EtBr-treated gel and visualising it with UV light, distinct bands of DNA appear. Loading buffers are added with the DNA in order to visualise it and sediment in the gel well. Negatively charged indicators keep track of the position of the DNA. Xylene or Bromophenol blue are typically used. They run at about 5000 bp and 300bp respectively, but the precise position varies with percentage of the gel. Other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp. Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pairs to several megabases (millions of bases) using specialised apparatus. However, it is normally used in a range of 100 bp to 20 kpb on an approximately 1% gel. Typical run times are about an hour.

Protocol

Agarose (typically 1-2% w/v; Melford labs, UK) was dissolved in TBE buffer (89mM Tris Base; 89Mm Boric acid and 2mM EDTA.2H2O) by boiling. Ethidium Bromide (EtBr) stock solution was added to the gel mixture in a fume hood cupboard to give a final EtBr concentration of 50ng/ml. 150 ml gel solution was poured into a gel tray fitted with a well forming comb. Once set, the gels were submerged in ~1 L TBE buffer in horizontal electrophoresis tanks, DNA samples were mixed with loading buffer (30% Glycerol; 2.5% Bromphenol Blue) and carefully pipetted into the sample wells. Calibrated DNA markers
were loaded onto the gel (Hyperladder 1, Bioloine) and electrophoresed alongside the samples for size determination of the samples. Typically electrophoresis was carried out at 90 volts for 0.5-1hrs at room temperature until the dye front had reached 5mm distance from the edge of the gel. Since ethidium bromide intercalates between the DNA bases and fluoresces under UV light, the gel was placed on a UV light box and an image of the fluorescent ethidium bromide stained DNA was captured using a charge couple device camera.

2.2-7. Gel extraction

Gel was visualised using a UV transilluminator and bands excised from the gel using a clean scalpel blade. Individual bands were transferred to 1.5ml microfuge tubes and the DNA extracted using a Qiagen Gel Extraction kit, following manufacturers’ instructions. Briefly, the gel slice was dissolved in 3 volumes of Qiagen buffer QG at 50°C for 10 minutes. 1 volume of isopropanol to the sample and mixed before decanting into a qiaquick column and centrifuge at 13,000 rpm for 1 minute. To remove all traces of agarose, the column was washed with 0.5ml of buffer QG and centrifuged for 1 minute, followed by washing with 0.75 ml of buffer PE. The column was centrifuged for 1 min to remove buffer. To elute the DNA 40μl of Tris-Cl (10mM) was added to the centre of the qiaquick membrane and allowed to stand at room temperature for 1 min before collection by centrifugation at 13, 000 rpm for 1 min. DNA was quantified using a spectrophotometer and 260/280 ratio determine to establish DNA quantity.
2.2-8. Restriction digest

**Principle**

Restriction enzymes are bacterial endonucleases which cut double stranded DNA at specific DNA sequences termed restriction sites. Although some restriction enzymes leave ‘blunt’ ends with no overhangs, most commonly used enzymes recognise palindromic 6 base pair sequences and leave sticky ends with one or more dNTP overhanging at the 3’ or 5’ end of the fragment. The efficiency of enzymatic digestion is influenced by various factors such as the type of DNA template, the location of restriction sites for the characteristics of the enzyme used, e.g., supercoiled DNA will require more enzyme than linearised DNA, and restriction sites at the end of linearised DNA may be more difficult to cleave by certain enzymes. Finally enzyme activity can be influenced by temperature and salt concentration. Each enzyme has its own buffer and although may be compatible some cannot be used together in a multiple digest reaction.

**Digestion of plasmid DNA**

Typically restriction digests were set up as follows:

- Plasmid DNA: 1μg
- 10 X buffer: 5μl
- Enzyme (10U/μl): 1μl
- Total Volume: Up to 50μl with ddH₂O

When multiple enzymes were used the amount of water was adjusted accordingly. Reactions were allowed to proceed at the optimal temperature (25, 30 or 37°C depending on the enzyme) for between 1 and 4 hrs.
Where multiple digests were set up with incompatible enzymes, DNA was digested first with one enzyme for 4hrs, and then the DNA was isolated using Qiagen PCR cleanup kit before digesting the second enzyme for 4hrs.

2.2-9. Ligations

Principle of T4 DNA Ligase

T4 DNA ligase is an enzyme capable of catalysing the formation of phosphodiester bonds between neighbouring 3’hydroxyl and 5’ phosphate ends of double strand DNA. T4 Ligase can also close single stranded nicks in double stranded DNA when the vector and insert are of similar length the molar ratio of vector and fragment DNA should be 1:3 for cohesive ends. If the lengths of the vector are not similar 1:1, or 1:2 ratio can be used. For blunt ended ligation a 1:5 molar ratio of vector DNA to insert DNA is used.

Protocol

200ng of plasmid DNA was mixed with equal volumes 2X DNA dilution buffer (Roche) and volume adjusted with ddH$_2$O to produce a final volume of 10µl. Samples were mixed prior to addition of 2µl of 10x T4 DNA ligation buffer and 7µl ddH$_2$O. 1µl of T4 DNA Ligase was added and the sample mixed well by flicking the microcentrifuge tube. Samples were then incubated at room temp for 5minutes. 5µl of ligation product was then used to transform 50µl of competent DH5α cells.
2.2-10. Generation of new plasmids

2.2-10.1. Merm1 deletion constructs

ΔMethT was generated using site directed mutagenesis (Stratagene, UK) by cleavage of methyltransferase (MethT) domain (amino acid 18-38) from Merm1. ΔSAM was generated by cleaving the S-adenosyl-L-methionine (SAM) domain (39-200) from Merm1. ΔNL was generated by cleaving the nuclear localisation (NL) domain (266-282) from Merm1. The details of primers used are summarised in Table 3.

Table 3. Primers for site-directed mutagenesis of Merm1 domain deletions

<table>
<thead>
<tr>
<th>Deletion domain</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyltransferase</td>
<td>CATGGCGGACCCCCAGAGCTGTTTATGGCTGGGCGAGCATTGGAG</td>
<td>CTCCAATGCTCGCCCAGCCATAAACAGCTCTGGGGGTCCGCCCATG</td>
</tr>
<tr>
<td>SAM</td>
<td>TCACGGATGATTGATCCAGGAGGAGTCTCCTGTTTCTGTTTCTGGGCTT</td>
<td>AGGCCAGAAAACAAGCAGAGGTGATACCAGGATGCAATCATCCTCCGTGA</td>
</tr>
<tr>
<td>Nuclear localisation</td>
<td>TCGAGGGCGGGGAATGGTGAGGGAGATGGTGAGTACCGCTTATGCA</td>
<td>GTCAGGTCTCGACTTCCTACCATTCCCGCCTCGA</td>
</tr>
</tbody>
</table>
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2.2-10.2. His-tagged Merm1 vector

The Merm1-HisMax C vector was generated by cutting Merm1 insert from a CMV.SPORT6 backbone using NotI and KpnI. The insert was ligated into a polyHisMax C vector using the same restriction enzyme sites.

2.3- Cell culture

2.3-1. Cell lines

HeLa cells, a human cervical carcinoma cell line from human cervix epitheloid carcinoma, obtained from ECACC (No 93021013) are well characterised cell known to express GR and respond well to steroids in culture. They have the advantage of giving high transfection efficiencies in comparison to some other adherent cell lines like A549s.

A549 cells, a human lung epithelial cell line derived from human lung carcinoma, obtained from ECACC (No 86012804) are well characterised cells known to express GR and also respond well to steroids in culture.

HEK293 cells, a human cell line derived from human embryonic kidneys, obtained from ECACC (No 85120602) are well characterised with endogenous GR expression. As the receptor is defective, the cells only respond to high levels of steroids in culture.

CEM C7A, a non-adherent human T lymphoblastoid cell line derived from the peripheral blood of an individual with acute lymphoblastic leukaemia, obtained from ECACC (No 85112105) express endogenous GR.

THP1 cells, a non-adherent human monocytic leukaemia, derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia are obtained from ECACC (No
88081201) express endogenous GR and are steroid responsive. They can also differentiate into macrophages in culture using for example DMSO.

2.3-2. Primary T cells

2.3-2.1. Isolation of peripheral blood mononuclear cells (PBMCs)

7mls of 100% Ficol/ (GE Helathcare) was added to 30ml universal tubes. Lymphocytes were isolated from whole blood by first diluting the whole blood ½ with RPMI 1640 media and then slowly layering 14mls of the diluted blood over the Ficol layer. This was then carefully placed in a centrifuge and spun at 400g for 25 minutes at room temperature. The white cell layer was then taken out of the tube using a squeeze pipette and collected in a 50ml falcon. Cells were filled up to 50mls using the RPMI 1640 media and then centrifuged again at 400g for 10mins at 4°C. The suspension was removed and the PBMCs were resuspended in 10mls of RPMI1640 supplemented with 10% FBS. Cells were counted on a haemocytometer and the volume was adjusted to a concentration of 1x 10^6 cells/ml.

2.3-2.2. Primary T cell culture

PBMCs at a concentration of 1x 10^6 cells/ml were maintained for 5 days 37°C with 5% CO₂ and humid atmosphere, and cultured in RPMI 1640 media + Glutamax I (Invitrogen, Paisley, UK), supplemented with 10% FBS and with penicillin and streptomycin (P/S). 10μg/ml PHA (Sigma-Aldrich Poole, UK) was added to cells. The cells were counted on a haemocytometer every two days and their media replaced with fresh culture media containing the PHA and P/S.
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2.3-3. Routine cell maintenance

With the exception of CEM C7A cells and THP1 cells, all cell lines were maintained in an incubator at 37 C with 5% CO2 and humid atmosphere and stocks cultured in Dulbecos Modified Eagles Medium + Glutamax 1 (Invitrogen, Paisley, UK) supplemented with 10% Fetal Bovine Serum (Invitrogen, Paisley, UK) in T75 vented flasks (Corning). Suspension CEM C7A and THP1 cells were maintained at 37C with 5% CO2 and humid atmosphere, and cultured in RPMI 1640 media + Glutamax I (Invitrogen, Paisley, UK), supplemented with 10% FBS in T175 flasks.

To ensure no residual steroids were present during the experiments, cells were grown in media supplemented with 10% charcoal dextran stripped serum (Hyclone) 24 hrs prior to the experiment.

2.3-4. Cell passage

2.3-4.1. Adherent cells

At 70-80% confluence media was removed from cells in the T75 culture flasks and the cell sheet was first washed with 5ml of phosphate buffered saline, (0.16M NaCl;0.003M KCl;, 0.008M Na2HPO4; 0.001M KH2PO4). PBS was aspirated from the cells and the cell sheet was then covered with 1.5 ml of 10X trypsin/EDTA (PAA, The cell culture company). Cells were then incubated at 37°C for approximately 5 minutes, checking regularly until cells began to round up and detach from the flask surface. The trypsin was then deactivated using media with 10% FBS and the cells were resuspended in 10 ml of growth media. Cells were then pelleted by centrifugation at 1000g for 5 minutes in a Mistral 1000 bench top
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centrifuge. Neutralized trypsin solution was aspirated and cell pellet was resuspended in fresh growth medium. The cells suspension was then split 1:5 into fresh T75 flasks, liquid volume adjusted to 10ml and returned to the incubator.

2.3-4.2. Suspension cells

Once cells reached a growing density of $1 \times 10^6$ cells/ml, cells were split by removing 90% of the growth of the cell suspension and replacing it with equal volume of fresh growth medium to keep the cell density between 1-2x $10^5$ cells/ml.

2.3-4.3. Cryogenic freezing

All cells lines were routinely frozen and stored in Liquid Nitrogen at -196°C. Cells were frozen down at low passage numbers in a culture medium containing 50% FBS and 10% dimethyl-sulfoxide (DMSO) as cryopreservat to reduce the size of any ice crystal that form. T75 flasks were grown to 80% confluence and then trypsinised as described in the previous section. Cells were pelleted and then resuspended in 3mls of freezing medium. 1ml of the cell suspension was aliquoted into cryotubes (Nunc). The cryotubes were held in the nitrogen phase for 24hrs before transfer to recorded locations in the liquid nitrogen storage tanks for long term storage.

2.3-5. Retrieving Cells from Nitrogen

Cells were removed from liquid nitrogen storage and allowed to stand at room temperature for a minute before being place in the 37°C incubator for 2-3 minutes to thaw. Once the cells were completely thawed, the content was resuspended in 5 mls of
media. The cells were pelleted and the freezing media was removed to remove the excess DMSO on cells. Cells were then resuspended in 10 mls of media and stored in a T75 flask.

2.4- Specialist culture for experiments

2.4-1. Principle of the flow-through culture system

In order to deliver ultradian cortisol pulses to primary T cells, a flow-through culture system was modified from that previously described (McMaster et al. 2011). This system was designed to replicate the ultradian rhythms described in the section 1.9 of the introduction. As primary cells are non-adherent in nature, the flow-through system had to be designed so the cells were not perturbed by the pressure of solution flowing over them. All parts of the flow-through system were sterilized using autoclave or ultra-violet (UV) irradiation. The flow-through system was maintained in a 37°C incubator. An extremely controlled pinch valve allowed the flow of medium to switch between alternative reservoirs at timed intervals (Fig 2.2). The flow-through model has been modified from the original model designed by previous PhD student Andrew McMaster (McMaster et al. 2011) and was adapted in this project to make it suitable for experiments on non-adherent primary T cells.

2.4-2. Optimisation of the flow-through system using hanging inserts:

In the first model of the flow-through system, a 6-well culture plate was drilled with a 0.25mm drill bit. A 0.25mm bore marprene tube was passed through the wall of the 6-well plate at one end and also linked to a 175ml media bottle at the other end of the tube. The culture media was transferred from the media bottle into the well of the 6-well
culture plate through the marprene tube with the peristaltic pump driving the movement of media. The output tubing returned the media inside the well via the peristaltic pump to a waste bottle. The well was filled with 6mls of media. A hanging insert (Millipore) was then soaked with culture media and placed into the well. 2mls of culture media was then added to the insert so the culture media within the insert levelled with the media in the outer compartment in the well. The bottom of the the hanging inserts consisted of a 1µm porous polyethylene terephthalate (PET) membrane which allowed the free diffusion of media and cortisol across the membrane without the cells diffusing across the membrane and into the outer compartment (Fig 2.1).

Figure 2.1. The PET hanging inserts used in the first model of flow-through system.
The hanging inserts are designed to fit 6-well plates.
2.4-3. The modified model of the flow-through culture system

Inlet and outlet holes were drilled in a 60mm culture dish with a 0.25mm drill so the outlets were higher in position than the inlets. This allowed the perfusion and removal of flow-through culture media without agitating the cells sitting on the surface of the dish. The dish was then washed and sterilized with 70% ethanol and placed inside the hood to dry. Two 0.25mm marprene tubes connected the media bottles to the dish through the inlets and the 0.5mm marprene tube removed the media from the dish and into the waste bottle. The peristaltic pump drove the movement of the media into and out of the tissue culture dish. HeLa cells were plated at a density of $1 \times 10^6$ cells/ml in the tissue culture dish and incubated overnight at 37°C to adhere. Alternatively for primary T cells, the surface of the tissue culture dish was coated with 100μg of BD Cell-Tak (BD Biosciences, Oxford, UK) according to the manufacturers’ instructions and left for 20 min for the cell-tak to adsorb onto the surface. The cells were then placed on the dish and allowed to adhere to the surface for 30 minutes.

Finally, non-adherent cells were removed by aspiration and fresh media was added to the plate before carrying out the flow-through experiment.
Figure 2.2. Outline of flow-through system.
Medium flow is driven using a peristaltic pump from alternative reservoirs into a 60 mm cell culture dish. Effluent medium is taken to the waste via the peristaltic pump. The flow-through system is maintained at 37°C in an incubator. An extremely controlled pinch valve allows switching the flow of medium from alternative reservoirs. The model has been adapted from (McMaster et al. 2011).
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2.5 - Transfection

2.5-1. Principal

Introducing DNA or RNA into eukaryotic cells is known as transfection. Transfection involves opening transient “holes” or gates in the cells to allow the entry of extracellular molecules, typically supercoiled plasmid DNA as well as siRNA. Transfection can be carried out using calcium phosphate, by electroporation. More commonly, transfection is carried out by mixing a cationic lipid with the nucleic acid to produce liposomes (Fig 2.3). It is thought that the excess positive charge of the lipids aids binding to sialic acid residues on the cell surface and the uptake of the nucleic acid by the cell through an endocytosis pathway (Wrobel et al. 1995; Zabner et al. 1995). The nuclear envelope is one of the barriers to efficient transfection and so cells going through cell division where the nuclear envelope breaks down have much higher transfection efficiency (Mortimer et al. 1999).

2.5-2. Reporter assay transient transfections

5mls of cells were seeded into 60mm plate a night before transfection so the cell confluence would be 40-50% the next day. The following day, 11μl Fugene 6 (Southhampton, UK) was mixed with 150μl of serum free RPMI 1640 in a microcetrifuge tube and centrifuged at room temperature for 5 minutes. 2μg of reporter gene construct 0.6μg coactivator or cDNA and 0.1μg of Renilla vector was added to Fugene 6 /RPMI 1640 complex and incubated at room temperature for 20 minutes before adding to the cells in a dropwise manner. The transfection mix was left on the cells overnight and removed prior to trypsinising cells and reseeding into 24-well plates. Cells were returned to the
incubator and allowed to settle before addition of dexamethasone (Sigma) to cells. Cells were incubated for another period of 16hrs before harvesting and analysis for luciferase activity.

Figure 2.3. Schematic representation of cationic lipid based transfection. The cationic lipid based reagent is first complexed with the negatively charged nucleic acid which neutralizes the negatively charge on the DNA. Lipid-DNA complex bind receptors on the cell surface membrane and this triggers endocytosis. The endosome brings in the DNA and lipid based reagent into the cell and the content is released intracellularly where the DNA enters the nucleus. Exogenous DNA can then be transcribed and translated into protein.
2.5-3.  **Plasmid DNA transfections**

HeLa cells were seeded into 6-well plates before the experiment so they were 90-95% confluent for the transfection. 2μg of either control empty vector or expression plasmid was mixed with 250μl of serum free low glucose DMEM. 5μl Lipofectamine 2000 reagent (Invitrogen) was also mixed with 250μl serum free media and allowed to incubate for 5 min at room temperature. The DNA/Lipofectamine complex were then slowly mixed and incubated at room temp for 20 minutes before being added to the cells in a dropwise manner. Growth medium was replaced with fresh DMEM+10% FBS 4-6hrs post transfection to prevent cell death caused by toxic Lipofectamine 2000. HeLa cells were transfected for a total number of 24hrs. Cells were subsequently lysed in protein or RNA extraction buffer.

2.5-4.  **Stable transfections**

**Principal**

Whilst transient transfection is advantageous for rapid analysis of genes, the introduced DNA does not become integrated into chromatin and is lost during mitosis. Stable transfection allows integration of DNA into host chromatin ensuring long term reproducible and defined gene expression. Genes of interest are usually transfected into cells along with an antibiotic resistance gene like neomycin or zeocin resistance to allow for positive selection of cells which have had the gene incorporated in their heterochromatin.
Protocol

To knock down Merm1 expression in a cell line, HeLa cells were transfected with either the empty Sigma Mission shRNA plasmid, pLKO.1-puro, (Sigma, UK) or each of the five Merm1 Sigma Mission shRNA plasmids (TRCN0000139958, TRCN0000140056, TRCN0000140572, TRCN0000144133, TRCN0000275218) which contain puromycin resistance gene. The plasmids were first lineralised with EcoR I overnight before the addition of 1/10 volume of 3M sodium acetate (Sigma Aldrich, UK) and 3 volumes of 100% ethanol (Fischer scientific) and then incubated at -20°C overnight. Plasmids were collected by centrifugation at 10,000g for 30 minutes in a microcentrifuge at 4°C and the supernatant removed. DNA was washed once with 70% ethanol and then centrifuged at 10,000 g for 30 minutes. The ethanol was removed and the pellets were air dried inside the cell culture hood for 5 minutes before resuspension in 20μl serum free medium and used for transfection following method outlined above for transient transfections. 48hrs post transfection the growth medium was replaced with fresh medium containing 300ng/ml puromycin (Invivogen) which is toxic to non-transfected cells. This selective medium was replaced every 3 days and the cells were grown continually in this medium for 2-3 weeks until clear colonies of resistant cells had formed. Once the colonies were identified, the medium was removed from the cells, and the colonies were picked up using trypsinised sterile filter paper and resuspended in growth medium in 24-well plates and allowed to expand. Clones were then screened for the knock-down and then stock frozen in liquid nitrogen for long term storage.
2.5-5. siRNA transfection

Principal

Small interfering RNA (siRNA) occurs naturally in cells and serves to silence transposable elements, repetitive genes and some viruses (Cerutti et al. 2006). RNA interference (RNAi) is evolutionally conserved process of post-transcriptional gene silencing (PTGS) by which double stranded RNA (dsRNA), causes the degradation of mRNA sequences. While the mechanism was first observed in plants and also Caenorhabditis elegans, more recent studies have shown that RNAi is present in a wide variety of eukaryotic organisms including mammals (Elbashir et al. 2001). Generation of siRNA is the result of an ATP-dependent process, where an RNase III-like enzyme, Dicer, cleaves the dsRNA into small interfering RNAs (siRNAs) (Fig 2.4). The siRNAs become integrated into a multi-subunit protein complex, known as RNAi-induced silencing complex (RISC), consisting of an Argonaute (Ago) protein as one its components, which guides the siRNAs to the target RNA sequence (Nykanen et al. 2001). The Ago component unwinds the siRNA duplex and it appears that antisense stand remains bound to RISC and directs degradation of the complementary mRNA sequences by a combination of endo and exonuclease (Martinez et al. 2002).
On entering the cell, long dsRNA acts as a trigger for RNAi process. In an ATP-dependent reaction, RNase III enzyme, Dicer cleaves the dsRNA into siRNAs. siRNAs integrate with RISC which cleaves the siRNA and guides the antisense siRNA to the target mRNA for cleavage.
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Protocol

HeLa cells were transfected with 10, 25 or 50nM of Lamin siRNA (Ambion) or IFI16 siRNA (Ambion) using Lipofectamine RNAmax (Invitrogen) and left to incubate for 24 and 48hrs before RNA and protein extraction. 24hrs prior to the transfection, HeLa cells were split into the 6-well culture plates so they were 40-50 % confluent on the day of the experiment. 2.5μl IFI16 siRNA or Lamin siRNA was mixed with 250μl Opti-MEM, reduced serum media (Gibco). 4μl of transfection reagent, Lipofectamine RNAiMAX (Invitrogen) was also slowly mixed with 250 μl of Opti-MEM. The siRNA/Lipofectamine complex was incubated at room temperature for 20 minutes before adding to the cells in a dropwise manner. Following transfection media was first removed by aspiration, cells were washed twice with phosphate buffered saline (PBS) and then lysed with 1x Netn lysis buffer for protein extraction. Alternatively, cells were lysed in RLT buffer for RNA extraction with RNeasy mini Kit (Qiagen).

2.5-6. Assay for gene regulation: Firefly Luciferase Assay

Principal

Reporter gene constructs are routinely used in cell biology to study gene expression. Firefly luciferase is a commonly used reporter due to its sensitivity and rapid translation as it does not undergo post-translational modifications for its activity. The firefly luciferase enzyme (Photinus pyralis) catalyses the oxidation of D-Luciferin to oxyluciferin and produces light as a byproduct. When plasmid constructs containing the luciferase gene are transfected into cells, the expression of the luciferase gene can be measured by...
incubating whole cell lysates with its substrate D-luciferin and ATP. The amount of light produced is measured using a luminometer and is directly proportional to the number of Luciferase molecules and therefore a direct measure of gene activity. The sea pansy (Renilla reniformis) luciferase enzyme, a monomeric 36kDa protein, catalyzes coelenterate-luciferin (coelenterazine) oxidation to produce light allowing for separate measurements of respective bioluminescent reactions.

Protocol
Following transfection, cells were first washed briefly with 5mls of PBS twice. Cells were then lysed in 250μl of Passive Lysis Buffer (Promega) and incubated in the lysis buffer for 20 mins on a rocking platform at room temp. 40μl from each well was transferred to a 96-well luminometer plate (Greiner). During this time an appropriate amount of luciferase assay reagent was prepared by resuspending the lyophilized Luciferase Assay Substrate in 10ml of the supplied Luciferase Assay Buffer II. Stop & Glo Renilla luciferase reagent was prepared by resuspending 50x Stop & Glo substrate in Stop & Glo reagent. 40μl of luciferase reagent was added to 40μl if lysate in a 96 well plates and cumulative bioluminescence was quantified over a period of 4 seconds using a Mithras LB40 automated analyser (Berthold Technologies). To normalise for the transfection efficiency, 40μl Renilla Stop & and Glo reagent (Promega) was added to the lysates. Bioluminescence was measured using the Mithras LB40 automated analyser (Berthold Technologies). The stop and glow substrate decays slowly over the course of the measurement. Light production was measured for 1 second and the background automatically subtracted.
Following analysis of firefly and Renilla luciferase expression, firefly units were divided by Renilla units to normalise for transfection efficiency.

2.6- **Protein Detection and quantification**

2.6-1. **Antibodies**

Anti-GR raised in mouse (clone 41, used at 1:1000 dilution) was obtained from BD Biosciences; anti-GR raised in rabbit (M20, used at 1:1000) and anti-TFIIB raised in rabbit (used at 1:1000-5000) were obtained from Santa Cruz technology; anti Merm1 raised in mouse (used at 1:1000) was from Abcam, anti-Merm1 raised in rabbit (used at 1:1000) was from GeneTex, Anti-α-tubulin (mouse) and anti-β-actin (rabbit) were both used at 1:1000 and were from Sigma and Abcam respectively. Anti-trimethylated H3K4 raised in rabbit (used at 1:1000), anti-dimethyl H3K79 (rabbit at 1:1000), and anti-acetyl histone H3 lysine 9 and lysine 14 (rabbit at 1:1000) were obtained from Millipore. Anti-Pan methyl H3K9 raised in rabbit (1:1000 dilutions) was from Cell Signaling. Normal mouse and rabbit IgG were obtained from Millipore. Horseraddish peroxidase conjugated anti-mouse (1:5000) and anti-rabbit (1:5000) were from Amersham (Buckinghamshire, UK). Flourophore conjugated anti-mouse (Alexaflour 546 and 488) were from Invitrogen (CA, USA).

2.6-2. **Preparation of whole cell extracts**

Whole cell extracts were prepared by first washing cells in ice-cold PBS (Sigma, UK). Cells were then scarped into NETN buffer (2.5% NP-40, 5mM EDTA, 250mM Tris-Cl (pH 8.0), NaCl (600mM) containing protease (Calbiochem, San Diego, CA, USA) and phosphatase
inhibitors (Sigma-Aldrich) before centrifugation for 10 minutes at 16000 g at 4°C. The supernatant was harvested and assayed for protein content (BIO-RAD, CA, USA) and diluted in reducing loading buffer (0.125M TrisCl ph 6.8, 0.1% SDS, 20% glycerol, 0.2% β-mercaptoethanol, 0.001% bromphenol blue) before boiling at 95°C for 5 minutes.

2.6-3. Protein estimation using Bradford assay

Principal

The assay is a dye binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine. The assay is useful since the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range.

Protocol

For protein analysis, 3µl is diluted into 1ml of 1x Bradford reagent and incubated at room temperature for 15 minutes. 200µl was then added to each well of a 96 well plate in triplicates alongside BSA standard dilution series from 0-0.8mg/ml and the absorbance was read at 595 nm.
2.6-4. Immunoblot analysis

Principal

SDS-PAGE (Gel Electrophoresis) is used to separate molecules based on the size, shape or isoelectric point. This technique, together with western blotting is useful to detect the presence or abundance of a given protein in a sample. The acrylamide gel is across-linked polymer matrix which allows the passage of protein molecules (either native or denatured), where the gel density can be controlled by varying the monomer concentration. Gels can either be of a constant density throughout or can be variable also known as gradient gels. Samples are boiled and loaded into small well in the gel. Electric current is then passed across the gel allowing the negatively charged proteins to migrate through the matrix towards the positive electrode. Proteins need to be denatured and negatively charged using a detergent molecule such as sodium dodecyl sulfate (SDS). The amount of SDS bound to the proteins is proportional to the size of the protein and proteins have a similar charge to mass ratio. A calibrated molecule of known weight marker that produces band of known size is used to identify the abundance of protein of interest. Once the protein samples have sufficiently separated on the gel by electrophoresis, they can be transferred to a PVDF or nitrocellulose membrane. The transfer process uses the sample principle as SDS-PAGE although this time the electric current is applied at 90 degrees to the gel and the proteins will therefore be transferred across from the gel onto the membrane. Once the transfer is complete and bound to the membrane, specific monoclonal or polyclonal primary antibody to the target protein is
added to the membrane. Western blotting is then performed which involves incubation of the membrane with the primary antibody followed by the detection of the antibody with the horse-radish peroxidase-linked secondary species specific antibodies. When substrate containing the luminal and hydrogen peroxide is added the membrane, light is emitted where the antibodies are bound and this can be detected on light sensitive photographic film.

Protocol

Whole cell extracts (25-50µg protein) were electrophoresed loaded in Novex Tris-Glycine Mini Gels and run in XCell SureLock Electrophoresis cells (Novex mini-cell, Invitrogen) and transferred to 0.2 micron nitrocellulose membranes overnight at 4°C or in the cold room for 2hours. Membranes were blocked for a minimum of 3 hrs (0.15M NaCl, 1.2% dried milk, 0.1% Tween 20) and incubated with primary antibody (diluted in the blocking buffer) overnight at 4°C. Membranes were washed 3 times, 10 min each, with wash buffer (50μM Tris HCl, 25μM Tris Base 0.25% dried milk, 0.1% Tween 20), membranes were incubated with a species specific horseradish peroxidase-conjugated secondary antibody (diluted in wash buffer) for 1 hour at room temperature and washed a further 3 times for 10 minutes each. Immunoreactive proteins were visualized on Kodak Biomax MR film using enhanced chemiluminescence ECL Prime western blotting reagent (GE healthcare) or Pierce ECL western blotting substrate (Thermo scientific) and the protein molecular weight established using the calibrated full range (10-250kDa) Precision Plus standards (BIO-RAD).
2.6-5. **Stripping and re-probing nitrocellulose membrane**

Membranes can be stripped of antibodies using solutions that disrupt the pH environment in which the antibody binds to the antigen on the membrane. Membranes can then be re-probed for additional proteins after chemiluminescence detection. Following chemiluminescence, membranes are first washed in PBS for 45 minutes followed by washing in 0.2M NaOH for an additional 5 minutes on a rocker at room temperature. Finally the membrane is washed in PBS before blocking for an additional 3 hours and re-probing with the primary antibody.

2.6-6. **Nuclear and cytosolic fractionation**

**Principal**

Subcellular fractionation is a useful technique for assessing protein localization or producing extracts enriched for proteins from specific cellular compartments. It is based on the principal that components of the cytosolic fractions are bigger in size and weight than the nuclear components and will therefore be separated in a solution depending on the speed of centrifugation. For this purpose, cells were first lysed with buffer A which destroyed the plasma membrane but kept the nuclear envelope intact. This brought the cytosolic compartment into the solution. Cells were then spun down at a specific speed to pellet the large plasma membrane pieces and nuclear fractions. The supernatant was kept as the cytosolic fraction. The pellet however, was resuspended in buffer B which lysed the nuclear membrane by osmotic pressure and allowed the resuspension of all nuclear fragments which were subsequently collected by high speed centrifugation.
Chapter 2: Methods

Protocol

Buffer A (10mM HEPES, 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.05% NP40 pH 7.9) was prepared with added cocktail of protease and phosphatase inhibitors and kept on ice. Cells were first washed briefly in ice cold PBS and scraped into 500μl buffer A and spun down at 2000 rpm for 5 minutes at 4°C. The supernatant was kept at the cytosolic fraction. The pellet was resuspended in 200μl of buffer B (5mM HEPES, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 26% glycerol, 300mM NaCl, pH 7.9) and homogenized on a vortex for 20-30 seconds. The nuclear lysates were then spun down on a centrifuge at 24,000g for 20 minutes at 4°C.

2.7- Enzyme Linked Immunosorbent Assay (ELISA)

Principal

ELISA is a technique allowing the sensitive detection of antigens or antibodies in biological samples. The concept is to couple an enzyme to an antibody and an immunosorbant substrate is added. The resultant colour is proportional to the amount of antigen in the sample (Fig 2.5).
Figure 2.5. The principle of ELISA.

The resultant colour produced by the addition of substrate solution is inversely proportional to the concentration of target protein in the sample

**Cortisol assay**

A competitive binding assay was used to quantify the cortisol levels in a flow-through cell culture system. This assay is based on the competitive binding technique in which cortisol present in a sample competes with a fixed amount of horseradish peroxidise (HRP) -labelled cortisol for sites on a mouse monoclonal antibody. Samples were collected from the 60mm dish every 5-15 mins for up to 4 hours. The samples were diluted with
Calibrator Diluent RD5-43. A 100µl of the samples were incubated with monoclonal antibody specific for cortisol. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution was added to the wells to determine the bound enzyme activity. The color development was stopped and the absorbance was read at 450 nm. The intensity of the color was inversely proportional to the concentration of cortisol in the sample. This assay was performed using Cortisol Parameter Assay Kit (KGE008, R& D systems).

2.8- Quantification of cell death using fluorescence-activated cell sorting (FACS)

Principle

FACS analysis is a technique by which fluorescence of individual cells within a population can be measured. In this case analysis was made using two particular fluorophores, Annexin V-APC and human anti-CD3+-Alexaflour488 antibody. Annexin A5 or annexin V is a probe used in molecular biology for detection of cells undergoing apoptosis and is excited at 595-605nm. Annexin V works by binding to the phosphatidylserine on the cell surface of apoptotic cells (Koopman et al. 1994). Detection of Annexin V conjugated to fluorochromes such as allophycocyanin (APC), on the flow-cytometry machine resulted was indicative of cell death. Anti-CD3+ staining is used for detection of human T cell CD3 antigen and is excited with an excitation source of 488nm. Detection by flow-cytometry allows for purification or quantification of CD3+ primary T cells from a population of primary blood mononuclear cells.
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Protocol

At the end of a treatment period, the media over the HeLa cells were removed and collected in a 50ml falcon tube. Cells were washed twice with PBS and added to the floating cell suspension. Cells were then trypsined using a 1x Trypsin/EDTA (PAA, The cell culture company) and were added to the cell suspension as soon as they had lifted off the culture plates. For primary T cells this was not necessary as T cells are non-adherent. Cells were then centrifuged for 10 minutes at 800g and the cell pellet containing typically 5x10^5-1x10^6 cells, was resuspended in 200μl binding buffer (10mM HEPES, 10mM NaOH, 140mM NaCl, 25mM CaCl). Cells were then strained using a 40 μM cell strainer to remove large debris from cells. 2-5μl of Annexin V-APC and/CD3-Alexaflour488 was added to the samples. Cells were incubated for 15 minutes at room temperature before another 200μl of binding buffer was added. Flourescene was quantified on a Beckman Coulter Cyan ADP FACS machine.

2.9- Analysis of protein interaction and cellular localisation

Principal

Co-immunoprecipitation (Co-IP) is a popular technique to identify protein-protein interactions by using target protein-specific antibodies to indirectly capture proteins that are bound to a specific target protein. Co-IP utilizes the antibody properties of bacterial protein A or protein G beads attached to agarose or sepharose beads. The principle of the assay is based on that, an antibody (monoclonal or polyclonal) against a specific target protein forms an immune complex with that target in a cell lysate. The immune complex
is then captured, or precipitated, on a beaded support to which an antibody-binding protein is immobilized (such as Protein A or G), and any proteins not precipitated on the beads are washed away. Finally, the antigen is eluted from the beads and analyzed by SDS gel electrophoresis (SDS-PAGE).

Protocol

Cell extracts (100-500μg protein) were precleared with 50μl of protein G-sepharose beads (Sigma) for 30 minutes at room temperature. The beads were pelleted by centrifugation at 5000g for 20 seconds and discarded. In the target sample, supernatant was incubated with 2-3μg of protein specific antibody and 50μl of protein G beads and in the control sample, supernatant was incubated with 50μl beads and IgG from non-immunised animal. Following the overnight incubation, both samples were centrifuged (5000g for 20 seconds) to pellet the protein G sepharose beads. The pellet was washed three times in ice cold PBS and then resuspended in 50μl of SDS loading buffer and boiled for 5 minutes. The beads were pelleted prior to immunoblot analysis by centrifugation (5000g, 20 seconds).

2.9-1. Immunoflorescence

Principle

Cells are permeabilized and incubated with a primary antibody raised against the protein of interest. A secondary antibody is then used to visualize the protein of interest. Secondary antibodies are conjugated with either Alexaflour 546 (red) or Alexaflour 488
(green) (molecular probes). The fluorescent labeled proteins are visualized on an Axio Imager.A1 Mat microscope. Cells were excited with a laser 488nm for green, 546 for red or 405 for DAPI. The absorbance and emission maximum of each of the flours are distinct enough to visualize each separately.

**Protocol**

Cells were seeded onto 13 mm coverslips at a density of 1 x 10^6 cells/ml and left to attach overnight. Following treatment, cells were fixed with 4% formaldehyde at room temperature for 10 minutes followed by permeabilisation with PBS containing 0.25% Triton X-100 for 5 minutes and then blocked in PBS.Tween (PBST) with 3% serum from species the secondary antibody was raised in. Cells were then incubated with 1:250 dilution of primary antibody overnight at 4°C. Subsequently, the coverslips were washed three times in PBS, 5 min each. Following 2hrs incubation with the secondary antibody at room temperature, coverslips were washed and mounted. Slides were allowed to dry for a few hrs before visualization on the Axio Imager A1 fluorescent microscope.

2.10- **RNA purification and quantification by polymerase chain reaction (PCR) analysis**

2.10-1. Principle of RT-PCR

Real-time PCR is an extension of PCR technology that allows logarithmic amplification of short DNA sequences (usually between 100-600 bases) after each amplification cycle to be monitored. Using the number of amplification cycles and the amount of PCR end product, it is possible to calculate the initial quantity of DNA. The DNA is quantified after
each round of amplification using a fluorescent dye (SYBR Green) that intercalates with the double stranded DNA. Initially SYBR green dye is intercalated with the double strand DNA. During denaturation process the dye is released, annealing of the primers takes places and extension begins. When polymerisation is completed, the dye intercalates with the double-stranded DNA product and fluoresces. Once bound, the SYBR green dye emits a fluorescent signal vastly stronger than produced by unbound dye. A melting curve analysis of PCR products is used to eliminate the possibility of non-specific amplification.

qRT-PCR consists of 3 steps:

Denaturation: Double stranded DNA is heated to 94°C-98°C for 10 minutes. During this step the double stranded DNA melts into two single stranded templates.

Annealing: The reaction is cooled to 45-65°C. Single stranded primers anneal to single stranded DNA templates. During this cycle, DNA polymerase attaches to the primed template and begins to incorporate complementary nucleotides.

Extension: The temperature is raised gradually to 65-75°C. The optimal temperature for Taq polymerase is 72°C. DNA polymerase extends the DNA sequence using nucleotides complimentary to the DNA template yielding a double–stranded DNA. SYBR green dye then incorporates the polymerised DNA double helix and fluoresces. The amount of fluorescence is measured at the end of each cycle and is subtracted from the background fluorescence produced. The relative intensity of the fluorescent light is the used to quantify the newly generated DNA.

These three steps are repeated 40 times ± cycles.
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2.10-2. Isolation of RNA from cells

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions for ‘Purification of total RNA from animal cells using spin technology’ procedure. On-column DNase digestion with DnaseI (Qiagen) allowed the removal of any genomic DNA contamination. All the workplace, glassware and pipettes were sprayed with RNaseZap to ensure an RNase-free environment. RNA quality and quantity was measured on Nanodrop ultra-low volume spectrophotometer (Nanodrop Technologies). 1.5μl RNA samples were measured at an absorbance of 260nm. The A260/A280 ratio determined the purity of the RNA and if it was smaller than 2.0 indicated the presence of protein or other contaminants in the sample. A secondary purity measure is the A260/230 ratio which also had to be greater than 2.0 for the samples to be suitable for experiments.

2.10-3. Reverse transcription (RT)

High Capacity cDNA Reverse Transcription Kit (Invitrogen) was used to generate first strand cDNA. 10μl of 2x RT buffer,

1μl RT Enzyme Mix,

200ng-1μg RNA and

Nuclease-free water to 20μl

For -ve RT samples no RT enzyme Mix was added to mixture and the volume was made up to 20μl with DPEC-treated water. Reaction was carried out on a DNA Engine tetrad 2 Peltier Thermal cycler (BIO-RAD) on the following programme: 37°C for 60 mins, 95°C for
5 mins followed by incubation at 4°C for 20 mins. cDNA samples were then stored at -80°C until qRT-PCR step.

2.10-4. Running the qRT-PCR

Samples to be tested were run in triplicates in 96-well MicroAmp optical reaction plates (Applied Biosystems, USA). cDNA samples of approximately 1μg/μl were diluted 1 in 10 for the qRT-PCR reaction. In a 25μl reaction, 12.5ng cDNA (2.5μl of the diluted cDNA sample) was added to 12.5μl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.5μl of each Forward and Reverse primer (final 200nM). The mixture was then made up to 25μl with DPEC-treated water (Sigma). The preparation of a master mix is crucial for reduction of pipetting errors. The qPCR plate was then sealed with an ABI prism optical adhesive cover (Applied Biosystems) and centrifuged for 30 seconds at maximum speed. Applied Biosystems StepOnePlus Real Time PCR Systems (Applied Biosystems) was used for the qPCR amplification according to the following parameters:

95°C for 10 minutes hold

40°C cycles of

95°C, 15 seconds

58°C, 15 seconds

72°C for 45 seconds
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The melting curve analysis for the amplified DNA was performed by increasing the temperature from 60°C to 90°C. This would confirm the production of a single amplification product.

2.10-5. Primer Design

All qPCR primers were designed using Roche Applied Sciences Assay design centre (https://www.rocheappliedscience.com/servlet/RCConfigureUser?URL=StoreFramesetView&storeId=10305&catalogId=10304&langId=-1&countryId=uk). The primers closest to a specific gene probe set (Affymetrix HG U133A chip) on the mRNA sequence were selected for the qPCR experiment. The primers sequences were also compared against the whole genome on the NCBI BLAST search engine to ensure then specificity of the primer sequences.

2.10-6. qRT-PCR data analysis

The amplification plot produced after a qRT-PCR reaction is analyzed by the comparative Ct method comparing the Ct values of test samples to that of a control. The Ct value is the cycle number at which the fluorescence produced by the amplified PCR product reaches a level higher than the background fluorescence known as the ‘threshold level’. Threshold level corresponds to an exponential increase of the PCR product. Therefore, the smaller the Ct value is, the more gene product is detected. In the negative control samples, with no template, there was no detectable signal after 40 cycles. The Ct values calculated by the ABI 7300 system were first imported to a Microsoft Excel spreadsheet and mean Ct values were calculated. The mean ΔCt was calculated according to the following:
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Mean ΔCt = Mean target gene Ct - Mean GAPDH (control gene) Ct

Mean ΔCt was used to calculate the ΔΔCt as the following:

ΔΔCt = ΔCt (sample) – ΔCt (Calibrator or control sample of the experiment)

The amount of amplified target gene is presented by $2^{-\Delta\Delta Ct}$, which is a measure of relative quantification and is used to find the fold increase or decrease in mRNA expression of the samples relative to the control. The mean relative fold induction of the genes was presented as bar charts in Graphpad Prism 4 (Hearne Scientific Software, UK) with the standard deviation of the mean presented as error bars.

2.11- Chromatin immunoprecipitation (ChIP) assays

Principle

ChIP assays are powerful tools to identify links between genome and proteome through investigation of histone or transcription factor-DNA binding interactions. ChIP utilizes the antibody binding properties of proteins to find interactions between specific proteins with regions of genomic DNA such as the regulatory regions. The assay begins with the cross-linking of the protein-DNA complexes using formaldehyde. Subsequently cells are lysed and cross-linked lysates are brought into solution. The lysates are spun down and resuspended in a cell lysis buffer to shear the plasma membrane. This allows the cellular components to be released into solution. Once spun down, the pellet containing the protein-bound DNA material is resuspended in a nuclear lysis buffer and sonicated into smaller fragments (between 200-800bp). The samples are then incubated with the
protein ChIP-specific antibodies overnight to capture the protein-DNA complex. The specific antibody bound protein-DNA complex was separated from the other complexes using ChIP-grade protein G magnetic beads. Proteinase K cleaves the carboxy-side of aliphatic, aromatic or hydrophobic residues to reverse the cross-linking of proteins from DNA fragments. Finally, the DNA is washed and cleaned up and amplified on a PCR machine using target region specific primers (Fig 2.6).
Figure 2.6. ChIP assay as a tool for identifying protein-DNA interactions.

Chromatin IP experiments require a variety of steps and molecular biology methods including cross linking with formaldehyde, cell lysis, nucleic acid shearing through sonication, antibody-based immunoprecipitation, DNA sample clean-up and PCR.
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**Protocol**

HeLa cells were fixed using 1% PFA for 10 min at room temperature. The cross-link was quenched using 0.125 M Glycine in PBS and cells were rinsed and scraped in ice cold PBS, collected by centrifugation. Cells were first lysed in cell lysis buffer (10mM HEPES, 0.5mM EGTA, 10mM EDTA, 0.25% Triton X100) and incubated on ice for 5 min. The nuclei were then collected by centrifugation and washed in nuclear wash buffer (10mM HEPES, 0.5mM EGTA, 1mM EDTA, 200mM NaCl). Nuclei were then lysed in nuclear lysis buffer (50mM Tris-HCl pH 8.1, 10mM EDTA and 1% SDS) and sonicated for 15 min (30 sec intervals) on medium intensity at 4°C. Cell debris was removed by centrifugation at 13200 rpm for 10 min at 4°C. The sheared chromatin was then incubated with specific antibodies or immunoglobulin from non-immunised animal (IgG) (Millipore) on a rocker overnight in the cold room. ChIP-grade protein G magnetic beads (Cell Signaling) were added to the samples and the incubation was continued for 4 hours. The beads were washed twice in wash buffer (20mM Tris-HCl pH 8.1, 2mM EDTA, 50mM NaCl, 0.1% SDS and 1% Triton X-100), 15 min each at 4°C. The beads were washed once more in wash buffer 2 (10mM Tris-HCl pH 8.1, 1mM EDTA, 250mM LiCl, 1% NP40, 1% sodium deoxycholate) for 15 min and twice in TE buffer (10mM Tris-HCl pH 8.1 and 1mM EDTA) 15 min each in the cold room. Following the washes the beads were incubated in elution buffer (1% SDS, 100mM NaHCO$_3$) for 45 min at room temperature. Following overnight incubation with 5M NaCl at 65°C, samples were incubated with 10mg/ml proteinase K, 40mM Tris-HCl pH 6.5 and 100μM EDTA for 1hrs at 45°C. DNA was cleaned up using PCR purification kit (Qiagen)
and eluted in 50μl water. Primers used in the ChIP assay are shown in table3. Dilutions of input were amplified by qRT-PCR. Real-time PCRs were carried out in triplicates on each of the immunoprecipitated and input DNA sample. To account for the DNA quantity, ΔCt value was calculated for each sample by subtracting the Ct value of the immunoprecipitated sample from the Ct value of the input. Raising 2 to the ΔCt power yielded the relative amount of PCR product. Data were then presented as percentage enrichment of immunoprecipitated sample relative to the input.

2.12- Statistical analysis

Data were analyzed in multiple samples after n=3 determinations using SPSS software package (SPSS Inc., Chicago, IL, USA) and, where appropriate, are expressed as means ± SD. For reporter gene assays and qRT-PCR assays, means were compared by independent samples T test and P≤0.05 was considered statistically significant. When comparing between groups, means were compared by one way ANOVA followed by the Bonferroni post hoc test as specified in the results section.
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Merm1 and GC sensitivity
3- Merm1 and GC sensitivity

A previous microarray study, by our group, of transcriptome profiling of lymphocytes identified a panel of n=20 discriminatory genes that showed small but significant changes in expression between GC-sensitive and GC-resistant groups (Donn et al. 2007). Further in vitro evaluation of six of the discriminatory genes was conducted. This confirmed interferon-inducible protein 16 (IFI16) and bone morphogenetic protein receptor type II (BMPRII) as novel modulators of GC sensitivity and GR function (Berry et al. 2010; Donn et al. 2007). An additional 5 genes from the original panel of 20 i.e, human elongation of very long chain fatty acids-like 1 (ELOVL1), heterogeneous nuclear ribonucleoprotein A1 (HNRPA1), down-regulator of transcription 1 (DR1), propionyl CoA carboxylase, alpha polypeptide (PCCA), acidic (leucine-rich) nuclear phosphoprotein 32 family, member E (ANP32E) were selected herein for further in vitro evaluation.

3.1- Effect of Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA on GC action

3.1- Confirmation of identity of the expression vectors

The five genes identified from the microarray study (Donn et al. 2007) were ordered as culture of E.coli cells transformed with an expression vector containing the cDNA of interest. Thermo Fisher Scientific (clone distributor) is part of the IMAGE consortium (http://image.hudsonalpha.org/). They provide pre-sequenced cDNA clones, and errors in sending the wrong clones very rarely occur during handling and moving of the clone arrays. ELOVL1, HNRPA1 and DR1 were obtained cloned into pOTB7. Following amplification and purification plasmids were digested to excise the full length cDNA.
cleave the insert from the vector, ELOVL1, HNRPA1 and DR1 were cut out of the pOTB7 backbone (1815bp) using Xhol and EcoRl. CMV.SPORT6 was digested with Sall and Notl enzymes. The digested fragments were run on agarose gel alongside hyperladder I and fragment size calculated. The ELOVL1 insert is 1480bp, HNRPA1 insert is 1160bp and DR1 is 3220bp. The identity of the ELOVL1, HNRPA1 and DR1 were confirmed using enzymatic digestion of each clone (Fig 3.1). The ANP32E and PCCA cDNA clones, in a CMV.SPORT6 backbone, were not initially checked by enzymatic digest. The ability of the ELOVL1, HNRPA1, DR1, ANP32E and PCCA to regulate GC sensitivity was then examined by reporter assays. Subsequent sequencing of all of the clones confirmed the identity of ELOVL1, HNRPA1, DR1 and PCCA. However the cDNA clone delivered by gene services as ANP32E was infact WBSCR22/Merm1. An error by the cDNA provider resulted in the ANP32E being replaced by WBSCR22/Merm1.
Figure 3.1. Restriction digest of DR1, ELOVL1 and HNRPA1 constructs in pOTB7 backbone.

DR1, ELOVL1, HNRPA1 were cut using EcoRI and XhoI. The cut out plasmid were electrophoresed on agarose gel. Hyperladder I was run as marker in lane M.
3.1-2. Overexpression of Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA and GR transactivation on a TAT3-Luc reporter in HeLa cells

The expression vectors for TIF2A, Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA were transiently transfected into HeLa cells then cultured with dex for 18 hrs. As illustrated in figure 3.2, overexpression of TIF2A, co-activator of GR and the positive control of the transfection assay, and Merm1 enhanced the luciferase in response to dex, when compared to CMV.Sp6 empty vector. In contrast to Merm1 and TIF2A, ELOVL1 significantly reduced dex-driven GR-mediated transactivation of TAT3-Luc compared to control backbone vector and antagonised GR transactivation of TAT3-Luc reporter. None of the remaining panel of genes significantly affected the luciferase response.
Figure 3.2 GR-mediated TAT3-Luc transactivation in HeLa cells and regulation by Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA.

HeLa cells were transiently transfected with 2μg of TAT3-Luc reporter gene, 0.5μg of Renilla reporter control construct and 0.6 μg of TIF2A, Merm1, ELOVL1, HNRPA1, DR1, PCCA or empty cmv.SPORT6 vector. Cells were incubated with (0.1-10) nM Dex for 24hrs and assayed for luciferase. Graphs depict means ± SD of triplicate samples from 3 independent experiments. RLU, relative luminescent unit. Data were compared using 1 way ANOVA followed by Bonferroni’s post hoc test. ** indicates p<0.001.
3.1-3. GR transrepression of a NRE-Luc reporter and regulation by overexpression of Merm1, ANP32E, ELOVL1, HNRPA1, DR1, PCCA

The ability of the plasmids to regulate GR-mEDIATE transrepression was studied using a TNF-α activated NF-κB response element (NRE) reporter assay as previously described (Berry et al. 2010). The NRE-Luc reporter construct was transiently transfected into HeLa cells and the regulatory effect of Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA was then analysed in the presence of 0.5 and 5 ng/ml TNF-α. 0.5ng/ml TNF-α maximally activated the NRE reporter in HeLa cells. Increasing TNF-α concentration to 5ng/ml impaired the GC inhibitory response; mimicking the acquired GC resistance seen in inflammation loci. Neither TIF2A, nor Merm1 showed any significant effect on GC repression of NF-κB activity (fig 3.3a). Furthermore, none of the remaining panel of GC sensitivity genes produced any significant effect on the TNF-α induced NRE assays (Fig 3.3b). As Merm1 consistently enhanced GR-mediated transactivation of TAT3-Luc reporter, it was therefore selected for further analysis of effects on GR functions.
Figure 3.3 Modulation of GR transrepression by Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA overexpression in HeLa cells.

HeLa cells were transiently cotransfected with 2μg of NRE-Luc, 0.5 μg and 0.6 μg of each of the Merm1, ANP32E, ELOVL1, HNRPA1, DR1 and PCCA. Twenty four hours post transfection, cells were incubated with 10nM Dex and 0.5 or 5ng/ml TNF-α for 16hrs before luciferase analysis. Graphs shown are representative of three independent experiments.
3.2- Over-expression of Merm1 increases nuclear hormone receptor transactivation of TAT3-Luc reporter.

In order to investigate the specificity of Merm1 for GR transactivation, reporter experiments examined other nuclear hormone receptors (NRs). HEK293 cells were transiently transfected with TAT3-Luc reporter construct together with each of the mineralocorticoid receptor (MR), progesterone receptor (PR) and androgen receptor (AR). The ability of Merm1 to drive NR-driven transactivation of TAT3-Luc was then examined in the presence of the relevant ligands i.e. corticosterone, progesterone and dihydrotestosterone (DHT). As is seen in figure 3.4, MR, PR and AR all activated the TAT3-Luc reporter in a ligand dependent manner. Overexpression of Merm1 however significantly enhanced the studied reporters suggesting that Merm1 potentiated transactivation through these examined NRs.
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Figure 3.4 Overexpression of Merm1 induces nuclear hormone receptor-mediated TAT3-Luciferase response.

HEK cells were transiently co-transfected with 0.5μg of TAT3-Luc reporter, 0.01μg Renilla reporter, 0.3 μg of Merm1 or cmv.SP6 empty vector (EV) and 0.5 μg of each of MR, PR or AR. Twenty four hrs after transfections, cells were incubated with corticosterone (10nM), progesterone (10nM) or dihydrotesterone, DHT, (10nM) for 16hrs and assayed for luciferase. Graphs depict means ± SD of triplicates wells, representative of 3 independent experiments. WB, WBSCR22/Merm1 plasmid; RLU, relative luminescent unit. ** p<0.001 data compared using independent samples t test.
3.3- Merm1 overexpression induces CCAAT-enhancer-binding protein (CEBP)-Luc activation in HeLa cells

To explore the role of Merm1 in regulating non-nuclear hormone related transcription factors, HeLa cells were co-transfected with CEBP-Luc reporter construct together with either Merm1 or empty cmv.Sport6 vector. As can be seen from the (Fig 3.5), Merm1 significantly potentiated CEBP-Luc sensitivity in HeLa cells. This indicates that Merm1 not only acts through the nuclear receptor family but also functions globally through non-nuclear receptor family receptors as well to modulate their sensitivity and actions.

Figure 3.5 Merm1 overexpression induces CEBP-Luc sensitivity in HeLa cells.

HeLa cells were transiently transfected with 2μg of C/EBP-Luc, 0.6 μg of empty vector or Merm1 and 0.1μg of Renilla reporter construct. Cells were incubated for 24 hrs and assayed for luciferase. Graphs depict means ± SD of triplicates wells, representative of 3 independent experiments. RLU, relative luminescent unit. Independent samples T test were used to compare the luciferase relative to empty vector. ** indicates p<0.001.
3.4- Merm1 methyltransferase domain is responsible for effects on GR functions

Merm1 is a putative methyltransferase protein consisting of an S-adenosylmethionine (SAM) dependent methyltransferase domain and a nuclear localisation (NL) domain (Fig 3.6a) (Doll et al. 2001; Nakazawa et al. 2011). To explore the mechanism involved in the actions of Merm1 on GR transactivation properties, site directed mutagenesis was performed to delete each of the SAM, or Methyltransferase (Meth-T) or nuclear localisation signal domain of the Merm1 protein. Deletion of each of the domains was confirmed through sequencing. Restriction digest of the wild type and the deletion constructs are shown in (Fig 3.6b). The wild type Merm1 insert is 1.243Kbp. The MethT domain deleted construct is 1166bp, SAM domain deleted is 760bp, and NL domain deleted Merm1 is 1189bp.

To investigate the domain responsible for enhancing GR-mediate transactivation, TAT3-Luc reporter construct was co-transfected into HeLa cells in the presence of either wild type Merm1 construct or each of the Meth-T, SAM or NL deletion constructs. HeLa cells were incubated with either vehicle or 10nM dex for 16hrs before luciferase analysis. Fig 3.6c indicates that wild type Merm1 increased the GC sensitivity of TAT3-Luc transactivation in the presence of 10nM dex. In contrast to the NL deletion vector, both Meth-T and SAM domain deletion constructs showed a significant loss of the luciferase signal relative to the wild type. This suggests that the methyltransferase domain of the Merm1 plays an important role in regulating GC sensitivity of the GR.
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The HHpred interactive server for protein homology detections and structural prediction was utilised that revealed significant structural homology between Merm1 and Dot1-like (Dot1L), a histone H3 lysine79 methyltransferase based on their amino acid and secondary protein structures (See appendices). This is inline with findings from Petrossian et (Petrossian et al. 2009) where they had categorised Merm1 with Dot1L and also CARM1, in the seven-β-strand family of methyltransferases. In addition, Merm1 was found to be tightly maintained in evolution from *Saccharomyces cerevisiae* (baking yeast) and *Caenorhabditis elegans* (Fig 3.7). Further investigations were subsequently undertaken to characterise Merm1 and explore its cellular functions as a histone methyltransferase.
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Figure 3.6 Merm1 modulates GR actions via SAM domain and Meth-T domain.

a) Diagrammatic representation of Merm1 protein and its functional domains. b) MethT, SAM and NL domain of Merm1 was cut out using site directed mutagenesis. The deletion constructs were separated on agarose gel. Representative image is shown. c) Merm1 functional domain deletion constructs were prepared. HeLa cells were transiently cotransfected with 2μg of TAT3-Luc reporter gene, 0.5μg of Renilla reporter and 0.6 μg of either wild type Merm1, or each of the ΔMethT, ΔSAM, ΔNL Merm1 deletion constructs in a cmv.SP6 empty vector backbone. Cells were incubated with either vehicle or Dex (0.1-10 nM) for 24hrs and analysed for luciferase. Graphs show mean ± SD of triplicate wells representative of three independent experiments. RLU, relative luminescence unit. *P<0.01, **P<0.001 as measured by independent sample T test.
Figure 3.7 Evolutionary conservation of WBSCR22/Merm1. Conservation GeneTree of \textit{wbscr22} gene reveals that WBSCR22/Merm1 is conserved in evolution. WBSCR22/Merm1 in humans is shown in red. Adapted from EnSEMBL GeneTree images.
3.5- Interaction between Merm1 and GR

3.5.1. Subcellular localisation of Merm1 and GR within HeLa cells

Since Merm1 possesses a nuclear localisation signal domain it was hypothesised that Merm1 resides in the nucleus. Subcellular localisation of Merm1 and GR was therefore analysed by immunoflourescence. Figure 3.8 demonstrates the endogenous expression of Merm1 and GR in HeLa cells. As can be seen from figure 3.8a Merm1 had a predominant nuclear localisation in HeLa cells and its localisation was not affected by dex. GR however, was homogenously distributed in both cytoplasmic and nuclear compartments under basal conditions. Following the addition of 100nM dex to HeLa cells, the majority of GR translocates into nucleus as expected. Interestingly, cells showing strong Merm1 expression demonstrated weak GR staining. The opposite was also seen in cells with strong GR staining (Fig 3.8a).

To confirm the pattern of Merm1 expression, HeLa cell lysates were separated into cytosolic and nuclear fractions following incubation with 100nM dex. Nuclear fractionation confirmed the nuclear localisation of Merm1 under both basal and 100nM dex conditions. GR localisation was both cytosolic and nuclear under basal conditions and largely nuclear after 1 hr of dex treatment (Fig 3.8b).
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Figure 3.8 Merm1 and GR subcellular localisation.

a) HeLa cells were grown on coverslips overnight. Following treatment with either vehicle or 100nM Dex for 1hr, cells were fixed and then double-labelled with antibodies against GR (green) and Merm1 (red). Nuclei were counterstained with DAPI (blue). Yellow arrows show reciprocal Merm1 and GR expressed cells. b) HeLa cells expressing endogenous Merm1 were treated with either vehicle or 100nM Dex for 1hr. They were then lysed and separated into cytosolic and nuclear fractions. Cellular fractions were separated by gel electrophoresis and immunoblotted using antibodies against β-actin, TFIIB, GR and Merm1. Representative images are shown. Experiments were done in triplicates.
3.5-2. *In vitro interaction between GR and Merm1*

Although the immunoflorescent studies have shown Merm1 to reside in the nucleus and co-localise with GR upon GC hormone addition, this co-localisation does not necessarily confirm a physical interaction between the two proteins. Therefore, the possibility of a direct physical interaction was examined through co-immunoprecipitation. HeLa cells were incubated with 100nM dex over night and then lysed. GR was isolated with a specific antibody and the resulting precipitate was probed with Merm1 antibody (Fig 3.9). GR was immunoprecipitated from HeLa cell lysates as shown in figure 3.9, but no Merm1 was co-immunoprecipitated as an immune complex with GR. Anti-rabbit Merm1 antibody (Fig 3.9b) as well as His-tagged Merm1 antibody (not shown) were also tested and failed to show direct binding of the two proteins in a co-immunoprecipitated complex. Different stringency buffers used in HeLa cell lysis also did not seem to affect the pull-down of the Merm1 protein (data not shown).
Figure 3.9 CoIP analysis of Merm1 and GR interaction.

HeLa cells were treated with either vehicle or 100nM dex for 1hr and lysed. Cell extracts were immunoprecipitated (IP) with an antibody raised against GR. Precipitates were immunobloted (IB) with anti-GR antibody and both anti-mouse Merm1 (a) and anti-rabbit Merm1 (b). Samples immunoprecipitated with mouse IgG were included as control for the CoIP experiment. Blots shown are representatives of three separate experiments.
3.5-3. **In vitro regulation of Merm1 protein expression**

To determine what regulates Merm1 protein expression, HeLa, CEM C7A and THP-1 cells were incubated with inflammatory and cytotoxic reagents. CEM C7A cells are acute lymphoblastic leukaemia cell line derived from T lymphocytes while THP-1 cells are acute monocytic leukaemia cell lines and therefore these two cell types provide a good model for investigation of inflammatory stimuli on protein expression of Merm1. Effects on GR were also investigated in parallel to Merm1 expression. Dexamethasone notably decreased GR expression in HeLa and THP-1 cells while increasing the GR levels in CEM C7A cells as previously reported (Gruber et al. 2009). None of the inflammatory stimuli i.e., PMA, TNF-α, or dexamethasone affected the expression of Merm1 in HeLa, THP-1 and CEM C7A cells (Fig 3.10a). Forskolin, Trichostatin A (TSA), leptomycin B (LMB), and 5-aza-deoxycytidine (Decitabine) also failed to show any consistently significant effects on Merm1 expression of HeLa, CEM C7A or THP-1 lysates. Overnight incubation with nocadazole, antimitotic reagent and cycloheximide, an inhibitor of protein translation, both reduced Merm1 in HeLa cells indicating that the protein synthesis was inhibited through these cytotoxic reagents (Fig 3.10b). However, the effects were not specific for Merm1 and affected GR expression as well while not affecting total protein levels since cellular tubulin expression was unchanged. None of the other cytotoxic reagents affected Merm1 expression.
Figure 3.10 Merm1 modulation by inflammatory and cytotoxic compounds.

a) HeLa, CEM C7A and ThP-1 cells were incubated with 100nM dex, 1 μM decitabine (5-aza-deoxycytidine), 10ng/ml TNF-α, 100nM PMA, 10nM leptomycin B (LMB), 500nM trichostatin A (TSA) and 1μM forskolin for 16 hrs. DMSO treatment was used as the vehicle control. Cells were then lysed and immunoblotted for Merm1, GR and alpha tubulin. b) HeLa cells were treated with 4hrs or 24hrs of 150μM H2O2, 50μM cycloheximide, 1 μg/ml aphidicoline and 200nM nocodazole for 16hrs. Cells were lysed and immunoblotted as before for Merm1, GR and tubulin. Images shown are representative of 3 separate experiments.
3.6- Merm1 modulation of GR-regulated gene expression

3.6-1. Selection of a panel of GC target genes for qRT-PCR analysis

RNAi interference and qRT-PCR assays are well-characterised tools for investigating the effects of a gene knock down on expression of other target genes. A panel of GC target genes were selected for quantification by qRT-PCR to explore the role of Merm1 in regulating GR target gene modulation. Previous microarray expression profiling identified the most highly GC-regulated target genes in primary T lymphocytes and HeLa cells respectively (Donn et al. 2007; McMaster et al. 2011). Genes that occurred in both expression arrays, with the most highly significant p values, were identified and the top 7 genes from this combined list were then selected to design primers for subsequent investigation by qRT-PCR analysis. The primers closest to a specific gene probe set (Affymetrix HG U133A chip) on the mRNA sequence were selected for the qPCR experiment.

3.6-2. Merm1 regulates basal levels of GC target genes independent of GR

Site-directed mutagenesis showed the Merm1 methyltransferase domains to be involved in regulating GC sensitivity of GR in TAT3-Luc transactivation (Fig 3.6). Merm1 regulation of GR target genes was explored to further discover whether Merm1 is involved in regulating GR functions. For this purpose, Merm1 expression in HeLa cells was first knocked-down using specific siRNA and the expression of a panel of GR target genes was then quantified using qRT-PCR.
Figure 3.11a illustrates the representative blot for Merm1 knock-down. The siRNA-mediated knock down completely abolished the endogenous Merm1 protein following 48hrs incubation. GR expression was unchanged with Merm1 knock-down removing the possibility that Merm1 might regulate total GR protein concentration. HeLa cells were analysed for relative expression levels of a panel of GC target gene transcripts (Fig 3.11b). In the absence of ligand, Merm1 knockdown did not show any significant effect on the expression of any of the methallothioneine 1X (MT1X), FK506 binding protein 5 (FKBP5), TSC22 domain family 3 (TSC22D3, GILZ), glutamate ammonia ligase (glutamine synthase (GLUL), myosin 1B (MYO1B), or aspartate β-hydroxylase (ASPH) transcript levels. Expression of interleukin-6 signal transducer (IL6ST) however, significantly increased with Merm1 knock-down, indicating that Merm1 has a GR-independent effect on expression of IL6ST.
a) HeLa cells, transfected with 10nM Merm1 or scrambled siRNA for 48 hrs. Cells were treated with either vehicle or 100nM dex overnight and were lysed and immunoblotted for Merm1, GR and α-tubulin. Representative images are shown. b) HeLa cells were transfected with 10nM Merm1 or scrambled siRNA, and then lysed and RNA processed. Effect on basal expression of 7 GC-mediated genes (ASPH, FKBP5, MT1X, IL6ST, MYO1B, GLUL and GILZ) was analysed by qRT-PCR. Graphs depict means ±SD of triplicate well, representative of 3 independent experiments. Data were compared using independent samples T test with * showing P<0.01.

Figure 3.11 Merm1 regulation of endogenous genes independent of GR
3.6-3. **Merm1 regulates expression of GC-dependent GR-activated and GR-repressed genes**

The effect of Merm1 knockdown on GC-mediated expression GR target genes was then examined. For this study, two well characterised GR-activated genes, FKBP5 and GILZ and three GR-repressed genes, IL-6, IL-8 and chemokine (C-C motif) ligand 2 (CCL2) were examined. Both FKBP5 and GILZ expression were significantly induced by dexamethasone, as expected. Merm1 knock-down did not cause a significant effect on the expression of basal levels of FKBP5 and GILZ transcripts (Fig 3.12). However, Merm1 knockdown significantly impaired the dex-mediated induction of both FKBP5 and GILZ indicating the role of Merm1 in GR potentiating GR transactivation.

The dex-mediated inhibition of IL-6, IL-8 and CCL2 were also measured by qRT-PCR following Merm1 knockdown in HeLa cells. Dexamethasone significantly inhibited the expression of IL-6, IL-8 and CCL2, as expected (Fig 3.13). GR-mediated fold-repression of both IL6 and IL8 genes was impaired with Merm1 knockdown showing the role of Merm1 in GR-mediated transrepression. Furthermore, Merm1 knockdown also inhibited the basal expression of IL6 indicating a role in regulating IL6 independently of the GR. These results provide evidence for both GR-dependent and GR-independent actions of Merm1 on GR transregulation.
Figure 3.12 Merm1 alters GR transactivation of endogenous genes.

HeLa cells were transfected with 10nM Merm1 or scrambled siRNA, treated with vehicle or 100nM Dex for 6hrs and then lysed and RNA processed. Effect of Merm1 siRNA on Dex-mediated induction of GILZ, FKBP5, IL6, IL8 and CCL2 was analysed by qRT-PCR. Graphs depict means ±SD of triplicate well, representative of 3 independent experiments. *P<0.01, **P<0.001.
Figure 3.13 Merm1 alters GR regulation of endogenous genes.

HeLa cells were transfected with 10nM Merm1 or scrambled siRNA, treated with vehicle or 100nM Dex for 6hrs and then lysed and RNA processed. Effect of Merm1 siRNA on Dex-mediated repression of IL6, IL8 and CCL2 was analysed by qRT-PCR. Graphs depict means ±SD of triplicate well, representative of 3 independent experiments. Results were analysed using One-way ANOVA followed by Bonferroni post hoc correction. * shows p<0.01, ** shows p<0.001.
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3.7- Chromatin immunoprecipitation analysis of Merm1 and GR at the GILZ locus

3.7-1. Selection of GILZ as an index gene for the ChIP assay

Although the co-immunoprecipitation studies failed to show a direct interaction between GR and Merm1, the qRT-PCR analysis confirmed that Merm1 modifies the GC sensitivity of GR in regulating the well characterised GR target genes GILZ, FKBP5, IL-6 and IL-8. Therefore, the aim of the next study was to investigate the indirect interaction of GR and Merm1 through co-binding to a GR index gene. GILZ, as a highly activated GR target gene has been well studied by chromatin immunoprecipitation (ChIP) assays and the GR binding sites on GILZ have been defined in detail (Blind et al. 2008; Chen et al. 2006; Asselin-Labat et al. 2005; Wang et al. 2004a).

3.7-2. Identification of GREs on the GILZ promoter region for the ChIP assay

Initially, the GR binding sites on GILZ promoter upstream of the transcription start site were selected based on a previous publication by Chen et al (Chen et al. 2006). A region of 5000 base pairs upstream of the transcription start site (TSS) of the GILZ gene was selected to confirm the selected regions for GR target sites. The genomic sequence of GILZ was extracted from UCSC genome browser. The selected GREs used for the ChIP assay were compared against the GREs found through TRANSFAC® 7.0 transcription factor binding site prediction database. Four sets of primer pairs were then designed for the ChIP assays (GL-1 to GL-4) (Table 4) (Fig 3.14). GL-1 served as the negative control primer pair for the ChIP assay as the region amplified by the primer did not harbour any GR binding sites within and it extended into the TSS. GL-2 and GL-3 both were used as
positive controls for ChIP assay and had 1 and 3 known GR binding sites respectively within the amplified region (Chen et al. 2006). GL-4 was designed more distant (1000bp upstream) from the rest of the primer pairs and did not hold a well-characterised known GREs although it did harbour a potential GRE based on primary sequence analysis.

Figure 3.14 Schematic representation of GILZ promoter upstream of (TSS).

GL-1, 2, 3 and 4 are primer pairs used in the ChIP assay. The regions with glucocorticoid response elements (GREs) are shown in red. GL-1 served as the negative for the ChIP assay and did not contain any GR binding sites (Chen et al. 2006). TSS is shown by the black arrow.

Table 4. ChIP assay primer pairs for GILZ promoter region

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<td>TCCCAAACAGATAGCTTTCT</td>
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3.7-3. **Merm1 and GR both co-localise to the same elements on GILZ promoter**

Role of Merm1 in regulating GR recruitment to regulatory regions was examined using Merm1 specific siRNA. mRNA transcript of Merm1 was significantly decreased following Merm1 siRNA treatment for 48hrs (Fig 3.15a). Dexamethasone did not affect the expression of Merm1.

To examine whether Merm1 and GR bind to the same regulatory regions on GILZ gene, HeLa cells were incubated with vehicle or 100nM dex for 1 hour and lysed and processed for ChIP assays. The binding of Merm1 and GR to four regions upstream of the transcription start site of the GILZ gene was analysed by DNA gel electrophoresis (Fig 3.15b) and then quantified by qRT-PCR (Fig 3.16). As expected, GL-1 served as the negative control region for the ChIP assay with no IgG, GR or Merm1 binding detected at this region. GR binding to GL-2, GL-3 and GL-4 was also detected by DNA gel electrophoresis analysis and GR recruitment was enhanced with 100nM dex. Similar finding was observed for Merm1 at GL-2, GL-3 and GL-4 although no obvious changes were seen following dex treatment. H3K9 acetylation was also detected at all the loci examined and was significantly enriched by dexamethasone, indicating a positive mark for active chromatin (Fig3.15b).

The chromatin from the ChIP assay was quantified by qRT-PCR (Fig 3.16). Merm1 binding was detected at three putative regulatory regions GL-2, GL-3 and GL-4. The binding of Merm1 was not affected by dex treatment and as expected, and siRNA specific Merm1 knock-down resulted in the loss of Merm1 binding to the chromatin (Fig 3.16). GR
binding onto GILZ GREs was detected on GL-2 and GL-3 and also on GL-4 and this binding was enhanced by dex, although this increase in binding was not seen for the GL-4 (Fig 3.16). To explore whether Merm1 affects GR binding onto GILZ promoter, endogenous Merm1 expression was inhibited using specific siRNA (Fig 3.15a). Loss of Merm1 inhibited the dex-dependent recruitment of GR onto GILZ GREs demonstrating that Merm1 potentiates GC-driven GR recruitment to GILZ GREs (Fig 3.16). Dex-dependent acetylation of histone lysine 9 (H3K9 acetyl) was observed on all the regulatory regions studied as a positive control for open chromatin. Loss of Merm1 did not appear to significantly attenuate H3K9 acetylation on all GILZ promoter regions although a tendency for a decrease in H3K9-acetyl binding was observed on GL-3 and GL-4 regulatory regions.
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a)

![Graph showing fold induction of Merm1](image)

b)

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<tr>
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Figure 3.15 Merm1 and GR ChIP to the same region of GILZ promoter.

a) HeLa cells were grown and transfected with 10nM Merm1 specific siRNA. Scrambled siRNA was used as control. Cells were incubated for 48hrs and analysed for Merm1 transcript levels by qRT-PCR. b) HeLa cells were treated with vehicle or 100nM Dex for 1hr. They were then fixed using formaldehyde and ChIP assay was performed using specific primer pairs. The PCR products were resolved on agarose gels to determine the viability of the ChIP assay. The results shown are representative of three independent experiments. Data were compared using student’s t test. ** indicates p<0.001.
Figure 3.16 Merm1 regulates GR binding to GILZ GREs.

Control and siRNA-mediated Merm1 knocked-down HeLa cells were treated with vehicle or 100nM Dex for 1hr. Cells were fixed by formaldehyde for 10 minutes and lysed and processed for ChIP analysis. The chromatin was quantified using qRT-PCR. The PCR signal for input was set as 1. Percentage enrichment was calculated relative to the input. ChIP assays were performed in triplicates. One-way ANOVA was used to compare the values. * indicated p<0.01 and **p<0.001.
3.8- Merm1 regulates histone methylation patterns

3.8-1. Merm1 and histone 3 methyl marks

Nakazawa et al (Nakazawa et al. 2011) demonstrated that Merm1 is a methyltransferase responsible for repression of Zac1 gene through methylation of histone 3 lysine 9, a repressive methyl mark. Results in figure 3.13 confirm that Merm1 potentiated GR binding to GREs at GL-2 and GL-3 and enhanced GR-mediated transactivation of both endogenous genes (Fig 3.12) and GR reporter gene constructs (Fig 3.2). Loss of Merm1 by RNAi knock-down inhibited dex-mediated GR binding to regulatory elements. Therefore, it was hypothesised that Merm1 is a histone methyltransferase involved in mediating GR-transactivation through modulation of activating histone methyl marks. Since H3K4 trimethylation and H3K79 di-methylation are activating methyl marks for gene transcription (Heintzman et al. 2007; Im et al. 2003b), Merm1 methylation of these lysine residues were subsequently examined. Merm1 regulation of H3K9 methylation, a repressive methyl mark and H3K9 acetylation, a positive control for active state chromatin were also included in as positive controls for the experiments. The effect of Merm1 knock down on global pattern of H3 methylation was therefore tested by in vitro protein analysis of HeLa cell lysates (Fig 3.17). Although Merm1 overexpression did not have a significant effect on any of the methyl marks, siRNA-mediated knockdown of endogenous Merm1 strongly inhibited the global state of H3K9me, H3K4me3 and H3K79me2. Acetylation state of H3K9 did not alter with overexpression or knockdown of Merm1 protein. These results provide evidence for a role of Merm1 on either direct or indirect in global methylation state of
histone 3 lysine residues and further assist in characterisation of Merm1 as a histone methyltransferase.

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**Figure 3.17 Merm1 modulates histone H3 methylation.**
HeLa cells were split, grown to confluence and transfected with either 2μg of Merm1 plasmid or 10nM Merm1 siRNA and incubated for 24 or 48 hrs respectively. pCMV.SPORT6 and control scrambled siRNA were used as the controls for transfection. Cells were then lysed and immunoblotted for Merm1, tri-methylated H3K4 (H3k4me3), acetylated H3K9 (H3K9 acetyl), methylated H3K9 (H3K9me), di-methylated H3K79 (H3K79me2) and β-actin. Experiment was repeated three times. Representative images are shown.
3.8-2. **Merm1 and modulation of methylation at GR target gene promoter**

The ability of Merm1 to modulate the methylation marks at the GILZ locus was then examined. HeLa cells were incubated with Dex for 1 hour and then lysed. H3K4me3, H3K79me2 and H3K9me recruitment to GILZ loci was examined in a ChIP assay and the immunoprecipitated chromatin was then analysed by qRT-PCR. Merm1 was immunoprecipitated on GL-2, GL-3 and GL-4. SiRNA knock down of Merm1 abolished the binding of Merm1 at all the three elements (Fig 3.18). Expectedly, H3K4 tri-methylation was significantly enhanced in the presence of dex on all three GL-2, GL-3 and GL-4 on GILZ promoter (Fig 3.18). Loss of Merm1 significantly attenuated both the basal and the dex-induced tri-methylation of H3K4, an active methylation mark, on GILZ regulatory elements indicating the significance of Merm1 in potentiating GR- transactivation. Dex decreased H3K79me2, while loss of Merm1 significantly abolished dex-mediated reduction of H3K79me2 recruitment at all three regulatory regions studied. Binding of H3K9me to GL-2, GL-3 and GL-4 was also detected by qRT-PCR and was increased in a dex-dependent manner. Loss of Merm1 had a template dependent effect on methylation of H3K9 at the three regulatory regions. The data here demonstrate a role for Merm1 in regulation of GR-mediated transcription through methylation of both active and repressive methyl marks.
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**Figure 3.18** Merm1 modulates basal and GC-directed methylation of H3 lysine residues on GILZ promoter. Cells were transfected with 10nM Merm1 specific siRNA and incubated for 48hrs. Control and transfected HeLa cells were incubated with 100nM Dex for 1hr and then fixed and processed for the ChIP assay. Sonicated chromatin was immunoprecipitated with antibodies against GR, Merm1, H3K4me3, H3K79me2 and H3K9me. The DNA quantity was measured by qRT-PCR. ChIP assays were performed in triplicates. Results are shown as means of three independent experiments. Data were compared by One-Way ANOVA followed by Bonferroni post hoc test. * and ** indicated p<0.01 and p<0.005 respectively.
3.9- Merm1 expresion is modulated by cytokine-induced glucocorticoid resistance in A549 cells

Previously I have shown that Merm1 increased GR-mediated transactivation of both reporter constructs (Fig 3.2) and endogenous target genes (Fig 3.12). Regulation of Merm1 expression by different stimuli could point towards a mechanism to regulate GR function, both by regulating GR access to target sites and also by affecting GR mediated methylation of histones. Combination of a TNF-α and IFN-γ has been previously shown to be present in sites of inflammation and also a combination shown to cause GC insensitivity (Tliba et al. 2008b). Merm1 expression was not affected with either TNF-α or IFN-γ alone. However, a combination of the two cytokines together markedly reduced Merm1 expression (Fig 3.19a). In order to examine the effect of Merm1 concentration on cytokine-induced GC resistance, A549s were also transiently transfected with Merm1 expression vector or empty vector together with TAT3-Luc reporter plasmid and treated with TNF-α and IFN-γ in the presence of vehicle or 10 nM Dex. Dex significantly induced TAT3-Luc activation in A549s as expected (Fig 3.19b). This activation significantly increased with overexpression of Merm1. TNF-α/IFN-γ significantly inhibited dex-induced GR activation of TAT3-Luc luciferase. The cytokine-induced GC insensitivity was rescued with Merm1 overexpression plasmid through restoration of the Merm1 protein in A549s (Fig 3.19b). The effects of TNF-α/IFN-γ on GR expression and modulation by Merm1 were tested by western blot analysis. Dex caused a marked decrease in total GR as expected although TNF-α/IFN-γ combination did not notably change the expression of basal or ligand-treated GR. Endogenous and over-expressed Merm1 protein were both reduced
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with cytokines relative to control (Fig 3.19c). The overexpression however was sufficient to restore GC sensitivity.
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a)

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b)

Figure 3.19 GC resistance model for modulation of Merm1 expression
A549s cells were treated with 10ng/ml TNF-α or 500U/ml IFN-γ or a combination of TNF-α/IFN-γ. Cells were incubated for 16hrs and the lysed and immunobloted for Merm1 expression. b) A549s cells were co-transfected with 2μg of TAT3-Luc, 1.2μg cmv.SP6 or Merm1 and 0.1μg of Renilla. Following overnight incubation, cells were treated with 10ng/ml TNF-α and 500u/ml IFN-γ. 100nM Dex was added to the cells 6hrs prior to cell lysis and luciferase analysis. c) A549s were cotransfected with 1.2μg cmv.SP6 or Merm1 plasmid overnight and then treated with 10ng/ml TNF-α or 500U/ml IFN-γ before 100nM dex was added. Cells were lysed and immunobloted for GR, Merm1 and tubulin. Representative image is shown. Experiments were performed in triplicates. Results were compared using independent samples T test. * and ** indicate p<0.05, p<0.001 respectively.
3.10-Cytokine-induced GC-resistance inhibits GR and trimethyl-H3K4 recruitment to GILZ promoter

To determine the underlying mechanism of cytokine-induced GC resistance on GR target gene expression, A549s were treated with cytokines overnight in the presence of dexamethasone and then fixed and processed for ChIP analysis. GR binding to the GILZ promoter at GL-3 was investigated by qRT-PCR analysis. To confirm the cytokine-mediated insensitivity to GC, expression of a highly regulated GR target gene, GILZ was analysed following treatment with TNF-α/IFN-γ. Dexamethasone significantly induced GILZ gene expression while combination of TNF-α and IFN-γ inhibited both basal and GR induction of GILZ transcript levels (Fig 3.20a). The previously described GL-3 region harbouring three GREs was selected for ChIP analysis (Fig 3.14). There was a significant increase in the recruitment of GR to GL-3 in the presence of 100nM dex. Cytokines induced recruitment of GR to GILZ GREs at GL-3 region under basal conditions but significantly repressed dex-induced binding of GR. There was a marked induction of H3K4me3 with dex while cytokines significantly inhibited the GC-driven tri-methylation of H3K4me3 at GL-3. Interestingly, cytokines caused a significant induction in the basal H3K4me3 indicating a role for cytokines in mediating chromatin conformation and gene expression (Fig 3.20b).
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Figure 3.20 Cytokine-induced GC resistance inhibits GR binding to GILZ promoter.

a) A549s were treated with a combination of 10ng/ml TNF-α or 500U/ml IFN-γ and incubated for 16hrs. 100nM Dex or vehicle was added to the cells 6hrs prior to RNA extraction. The effects of TNF-α and IFN-γ on GILZ transcripts were measured by qRT-PCR analysis. b) A549s were treated with combination of cytokines for 16 hrs. 100nM dex was added to cells 1hr prior to ChIP analysis. Chromatin was quantified by qRT-PCR and the results were normalised relative to input. Data were analysed in triplicates and were compared using One-Way ANOVA followed by Bonferroni post hoc test. * shows p<0.01 and **p<0.005.
3.11-Summary

Activated GR is a transcription factor with chromatin remodelling activity that binds to constitutive (pre-determined) sites on the genome. Merm1 is a histone methyltransferase previously shown to mediate cancer cell metastasis through methylation of histone H3 at lysine 9 (H3K9me) on the Zac1 promoter leading to the suppression of Zac1 gene. In our initial transient transfection screening assays, Merm1 was found to consistently potentiate GR activation of a TAT3-luc GR reporter gene. In an attempt to find the functional domain responsible for this activity, site direct mutagenesis was used and this identified SAM and methyltransferase domains to be responsible for the GR potentiation. Merm1 knockdown using specific siRNA significantly impaired both GR transactivation of GILZ and FKBP5, as well as transrepression of IL-6 and IL-8 endogenous target genes. Since this effect was not through the regulation of total available GR, a possible model for Merm1 affecting GR binding to promoter binding elements was suggested. Both GR and Merm1 bound to the GILZ promoter and Merm1 facilitated ligand-induced GR recruitment to promoter GREs showing a role for Merm1 in regulating chromatin through its methyltransferase activity. Indeed, Merm1 knock-down abrogated global histone H3 lysine 4 tri-methylation (H3K4me3) and H3 lysine79 di-methylation (H3K79me2), both of which are activating methyl marks, while it also inhibited H3K9 methylation in line with a previous publication on Merm1-regulated metastasis formation (Nakazawa et al. 2011). At the GILZ locus, GR induced H3K4me3 and repressed H3K79me2. Merm1 was required for methylation of basal H3K79me2, and Merm1 knock-down impaired both the induction of H3K4me3 as well as the reduction of H3K79me2 in the presence of ligand. These
coordinated histone modifications suggest H3K4me is the driving event and that Merm1 regulation of these two methyl marks plays a role in GR recruitment to target sites through modification of histones and chromatin remodelling. Merm1 was found to be strongly regulated by TNF-α/IFN-γ, cytokines known to induce GC resistance. These cytokines inhibited the GR activation of the TAT3-Luc plasmid and Merm1 overexpression rescued sensitivity to GC through Merm1 protein restoration. The reduction in Merm1 expression reduced GC-dependent GR recruitment to GL-3 GREs, which was also associated with reduced H3K4me3 at GL-3. In conclusion, Merm1 regulates chromatin structure to affect GR recruitment, and mediates GR actions of transcription by histone modifications. A proposed model summarising these effects of Merm1 is shown in figure 3.21.
Figure 3.21 Schematic representation of Merm1 as a co-modulator of GR signalling.

Under basal conditions, multiple components of the transcriptional machinery such as histone methyltransferases and histone demethylases work closely to produce a steady state in which chromatin accessibility is tightly regulated by the extent of methylation and demethylation of histone lysine residues such as H3 lysine4 and lysine 79. Pre-existing chromatin remodelling sites partly available by Merm1 are host to GR binding upon hormone treatment. GR transactivation is aided through recruitment of Merm1, which in turn increases GR dependent H3K4 tri-methylation and inhibits H3K79 di-methylation. This leads to a conformational change in chromatin and recruitment of BRM and BRG1-dependent Swi/Snf chromatin remodelling complexes that rearrange chromatin structure into a more permissive state for transcriptional activation.
Chapter 4: Results

Novel pharmacokinetic approach to modulating GC sensitivity
4- Development of a flow-through culture system

4.1- Overview

An ultradian rhythm of GC delivery exists in humans, with a pulse of hormone production every 1-2 hours (Veldhuis et al. 1990a; Young et al. 2004). In order to investigate the differential effects of pulsatile vs continuous cortisol delivery on the pattern of gene expression and cell proliferation in adherent HeLa cells and non-adherent primary T cells, a flow-through culture system was modified from that previously designed (McMaster et al. 2011) that allowed for the delivery of pulsatile or dose-equivalent continuous synthetic cortisol (hydrocortisone (Hc)).

4.1-1. Investigation of cortisol concentrations in the hanging insert model of the flow-through system

To attempt to replicate the ultradian rhythm of endogenous cortisol for delivery to non-adherent primary T cells, the flow-through system was infused with pulses of cortisol in regular intervals. Previous experiments in the flow-through system had been established and optimised to deliver hourly cortisol pulses to adherent HeLa cells in culture (McMaster et al. 2011). To replicate those findings in the hanging insert model of the flow through system, the same parameters were used to carry out the ELISA experiment. The hanging insert (1µm pore size) was filled with 2mls of growth media and placed into 6mls of media in a 6-well culture plate. 1µM cortisol was infused at a rate of 30rpm for 10mins followed by 50 mins wash out period. Samples were taken from the insert every 10 mins and assayed for cortisol concentrations using cortisol ELISA. Figure 4.1 shows a gradual
rise in the concentration of cortisol between the first and second hour. Although individual cortisol peaks are observed at 70 and 130 mins, the peak at 130 mins (27ng/ml cortisol) is higher than at 70 mins (17ng/ml), suggesting a gradual build-up of cortisol concentration inside the hanging inserts.

![Figure 4.1. Pulse modelling in the hanging inserts.](image)

The cell chamber was infused with 1µM cortisol medium for 10 minutes, and then the input reservoir was switched to medium without cortisol. ELISA was performed on samples taken every 10 mins to replicate the cortisol treatment schedules on HeLa cells.
4.1-2. **Investigation of cortisol concentrations in the flow-through culture system**

In the modified flow-through system for non-adherent primary T cells, a cortisol ELISA was used to calibrate the flow through system to replicate endogenous cortisol pulses (McMaster *et al*. 2011). The culture dish was infused with the Hc flow-through medium for 10 minutes, and then input was switched to the cortisol-free flow-through medium for 50 minutes (Fig 4.2a). This produced a pulse that closely replicated the delivery of endogenous corticosterone rhythm seen *in-vivo* in frequently sampled rats (Lightman *et al*. 2000; Windle *et al*. 2001) (Fig 4.2b). Both 0.25mm and 0.5mm bore diameter outlet tubing produced the same graph of pulsatile cortisol in the flow-through culture system. This was particularly important as it showed that the bigger diameter tubing could replace the smaller outlet tube, which had a greater risk of blocking by cellular debris and overflowing the flow-through tissue culture system.
Figure 4.2 Establishment of flow-through culture system to analyse pulsatile glucocorticoid action.

a) Photographic image of the flow-through system showing the two cell chambers and input reservoirs for delivery of conditioned media through a peristaltic pump. b) The cell culture chamber (60mm culture dish) was infused with 1μM cortisol (Hc) for 10 min. Then the input reservoir was switched to medium with no Hc for 50 minutes. Effluent medium was collected every 5 minutes and assayed for cortisol concentrations with ELISA. Samples were collected for up to two hours. Two types of outlet tubing were compared; black (0.5mm bore diameter) and red (0.25mm bore diameter). Graph shows mean of three ELISA experiments with mean ± SD.
4.2- Differential effects of pulsatile and continuous hydrocortisone on HeLa cell proliferation

Initial assessment of effects of cortisol dynamics on HeLa cell proliferation and viability was carried out. Pulsatile Hc caused a significantly bigger reduction in live cell survival compared to continuous exposure, reflecting a complex cell response to cortisol delivery kinetics. To determine the contribution of apoptosis to the variation in cell viability observed, HeLa cells were exposed to the identical treatment regime as used for the cell viability assay, and then Annexin v staining determined by FACS. The results confirmed that the reduction in cell viability and cell proliferation observed was due, at least in part, to increased apoptosis (Fig 4.3).
Figure 4.3 Altered GC delivery dynamics differentially affects cell proliferation in HeLa cells.

a) HeLa cells were seeded at $2 \times 10^6$ per well and subjected to control (normal flow through medium, no cortisol), (100ng/ml) pulsatile cortisol or dose-equivalent continuous flow with cortisol 100ng/ml. After 16 hours cells were counted using a haemocytometer. The experiment was performed in triplicate, bars indicate mean and SEM. b) Cells were seeded at $2 \times 10^6$ /ml overnight. The cell chamber was infused with pulsatile (100ng/ml), continuous (100ng/ml) or control (no Hc) for 12 hrs. The cells were then washed with PBS and labelled with APC conjugated-Annexin v and analysed with FACS. Data shown are the relative fold increase in induction of apoptosis by pulsatile treatment compared to the continuous Hc. Graph is mean of n=3 experiments. One sample t test was performed to compare the means. ** indicates p<0.001.
4.3- Differential activation of MMTV-Luc reporter gene assay in HeLa cells with altered GC delivery dynamics

Previously, McMaster et al (McMaster et al. 2011) utilised a microarray expression system to explore the differential pattern of gene expression between pulsatile and dose-equivalent continuous Hc delivery for HeLa cells. Several transcription factor binding sites were enriched in the differentially regulated target genes as discovered by the transcription factor (TF) networks analysis of the microarray data (McMaster et al. 2011). CCAAT-displacement protein (CDP) was of interest as it regulates the mouse mammary tumour virus (MMTV) through binding to multiple sites on the MMTV long terminal repeat (Zhu et al. 2002). Since MMTV is also GC responsive, a MMTV-Luc reporter gene assay was performed to analyse the effects of cortisol delivery dynamics on regulation of the MMTV gene. Interestingly, continuous and pulsatile Hc delivery exerted significantly different effects on the induction of MMTV gene as predicted (Fig 4.4).
Figure 4.4 Differential activation of MMTV-Luc reporter gene assay in HeLa cells by continuous and pulsatile cortisol delivery.

2x10^6 HeLa cells were plated in five 60mm plates. The cells were transfected with 2μg of MMTV-Luc plasmid and 0.1μg of Renilla-Luc plasmid. Cell chamber was infused with either 100ng/ml pulsatile or dose-equivalent continuous cortisol (Hc) or no Hc control for 12 hrs. Subsequently cells were washed twice with PBS and lysed in passive lysis buffer. Cells were then assayed for luciferase signal. Relative Luminescence Units (RLU) was calculated as the ratio of luciferase to Renilla luciferase. Continuous and pulsatile groups were compared using an independent samples T test. The graph is a representative of 3 independent experiments. **indicates p<0.001.
4.4- Optimisation of the flow-through system for primary T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as described previously in the Materials and Methods section 2.3-2.2 (primary T cell culture), and expanded by T cell mitogen 10 μg/ml PHA (Sigma) (Donn et al. 2007) for five days in primary tissue culture incubator. Mature parimary T cell population count was verified by FACS sorting using CD3+ staining and found to be >95% as measured on three different occasions (Fig 4.5a). Also, a viable cell count was performed following 5 days expansion period with 10μg/ml of PHA. Bromphenol blue staining of the T cells on a haemocytometer also indicated a steady increase in the proliferation of cells by day 5 followed by a plateau phase in the viable cell count demonstrating that 5 days incubation with PHA is optimum for maximal proliferation of mature T cells (Fig 4.5b).
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a)

![Graph showing % of CD3+ T cells with PHA.](image)

**Figure 4.5 Proliferation of primary T cells with PHA.**

a) PBMCs were isolated from whole blood and then expanded using the T cell mitogen PHA (10 µg/ml) and incubated for 5 days. Following incubation period, mature T cells were stained with anti-human CD3 antibody and analysed by flow-cytometry.  
b) PBMCs were isolated from whole blood and then spun down and resuspended to a final concentration of 1 x 10^6 cells/ml. 4 mls of cells were then incubated with 10µg/ml PHA. Primary cells were stained with bromphenol blue everyday for 5 days and counted on a haemocytometer. Graphs depict means of three independent measurements. Results were compared using independent sample T test. ** indicates p<0.001.
4.5- **Differential effects of pulsatile and continuous hydrocortisone delivery on proliferation in primary T cells**

Having investigated the differential effects of pulsatile vs continuous delivery of Hc in HeLa cells, the effects on primary T cells were then explored as a more physiologically relevant cell type in inflammation. To discover the role of Hc-mediated apoptosis, primary T cells were exposed to the identical regime as described for HeLa cells in section 4.2. The primary T cells were labelled with CD3+ and Annexin v and sorted by flow-cytometry to determine the extent of apoptosis in the CD3+ primary T cell population.

No significant differences were observed between the two delivery modes (Fig 4.6).
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Figure 4.6 Effects of pulsatile and continuous Hc on induction of apoptosis in primary T cells.

Cells were seeded at 2x10^6 cells/ml overnight. The cell chamber was infused with pulsatile (100ng/ml), continuous (100ng/ml) or control (no Hc) for 12 hrs. The cells were then washed with PBS and labelled with APC-conjugated Annexin v and Alexa488-conjugated CD3+ and analysed with FACS. Data shown are the relative fold increase in induction of apoptosis by pulsatile treatment compared to the continuous Hc. Graph is mean of n=3 experiments. One sample t test was performed to compare the means.

4.6- Differential effects of pulsatile and continuous hydrocortisone delivery on gene expression in primary T cells

The differential effect of continuous and pulsatile GC delivery on endogenous GC target genes was then explored in primary T cells. First, the effects on the expression of 15 GC target genes, FK506 binding protein (FKBP5), glucocorticoid-induced leucine zipper (GILZ), adrenomedullin (ADM), IL6 signal transducer (IL6ST), myosin1 beta (MYO1β), metallothionine 1X (MT1X), metallopeptidase domain 12 (ADAM12), aspartate beta
hydroxylase (ASPH), collagenase 7A1 (COL7A1), GATA binding protein 3 (GATA3), inteleukin 8 (IL8), glucocorticoid receptor (NR3C1), nuclear receptor subfamily 6, group A, member 1 (NR6A1), RAP1 interacting factor1 (RIF1), glutamate ammonia ligase (GLUL) were investigated under static conditions in the presence of 100ng/ml Hc. The genes selected were among a panel of most significantly regulated GC target genes as found by expression profiling of primary lymphocytes (Donn et al. 2007). From the panel of 15 genes studied, 5 demonstrated consistent pattern of regulation by GC treatment under static conditions. Hydrocortisone, significantly unregulated GILZ, FKBP5, GLUL and IL6ST as expected and significantly repressed IL8 expression (Fig 4.7a). The differential effects of pulsatile vs continuous Hc delivery on GILZ, FKBP5, GLUL, IL6ST and IL8 expression levels was subsequently investigated in the flow-through system. Both pulsatile and continuous Hc delivery of the same total concentration (100ng/ml) significantly increased GILZ, FKBP5, GLUL and IL6ST transcripts (Fig 4.7b). Moreover, continuous Hc induced FKBP5 and GILZ expression significantly more than the pulsatile Hc delivery, indicating a potential role for Hc delivery dynamics on target gene expression. IL8 was significantly repressed by both continuous and pulsatile Hc and no significant difference was observed between the two delivery modes (Fig 4.7b).
Figure 4.7 Differential regulation of GC target genes with pulsatile and continuous Hc delivery in primary T cells.

a) Mature primary T cells were treated with vehicle or 100ng/ml Hc overnight. Cells were then washed and RNA processed. qRT-PCR graph of five Gc-regulated genes, GC-induced leucine zipper protein (GILZ), FK506 binding protein 5 (FKBP5), glutamate-ammonia ligase (GLUL), interleukin-6 signal transducer (IL-6ST) and interleukin-8 (IL-8) are shown. b) Mature primary T cells were infused with either pulsatile (100ng/ml) or continuous (dose-equivalent) cortisol for 12hrs in the flow-through system. Cells were subsequently washed and RNA extracted. Transcript levels of FKBP5, GILZ, GLUL, IL6ST and IL8 were analysed by qRT-PCR. Results shown are representative of three separate experiments. Data were compared using independent samples T test. * and ** indicate p<0.01, p<0.001 respectively.
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4.7- Summary

A flow-through culture system was modified to explore the temporal dynamics of GC delivery on adherent HeLa cells and non-adherent primary T cells. A cortisol ELISA was used to calibrate the flow-through cell chamber for delivery of cortisol pulses to cells to replicate the endogenous pulsatile cortisol rhythm. Infusion of 1μM cortisol for 10 minutes followed by 50 minutes wash out period produced cortisol oscillations (peak of 100ng/ml) that closely replicated the endogenous rhythm. Effects of cortisol dynamics on HeLa cell proliferation and viability was first carried out. There were significantly fewer cells with pulsatile cortisol delivery compared with the continuous delivery following 16hrs treatment period. This cell loss was found to be due to higher apoptosis levels in the pulsatile group compared to the continuous group, as found by Annexin V staining, indicating the role of delivery dynamics in mediating cellular response to the GC ligand. Microarray profiling of HeLa cells also identified genes that were differentially regulated between pulsatile and continuous cortisol delivery in HeLa cells. Transcription factor (TF) binding site analysis of the microarray data identified CCAAT-displacement protein (CDP) as a common TF binding site in the differentially regulated target genes. Mouse mammary tumour virus (MMTV) gene is regulated by CDP and also GC responsive. Therefore, MMTV-Luc reporter assay was done in order to investigate the effects of cortisol delivery dynamics on regulation of the MMTV gene. Interestingly, altered cortisol delivery dynamics produced quite different effects on the regulation of the MMTV reporter.

Since primary T cells are important cell types in modulating anti-inflammatory effects of GCs during inflammation, T lymphocytes were chosen for subsequent studies in the flow-
Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and the T cell population was expanded using the T cell mitogen PHA. 5 days incubation with 10μg/ml PHA yielded a 95% CD3+ T lymphocyte population in culture. Primary cell count also confirmed a maximal cell proliferation under these conditions. Altered cortisol delivery dynamics on apoptosis in primary T cells was investigated. No significant differences were observed between the two delivery modes as shown by Annexin v staining of the CD3+ T cells. To find out the role of pulsatile vs continuous cortisol delivery on the expression of GC target genes, a panel of 15 highly regulated GR target genes were identified. GC regulation of the selected genes was validated by qRT-PCR analysis under static conditions. GILZ, FKBP5, IL6ST and GLUL were consistently unregulated by cortisol whereas IL8 was significantly repressed under no flow conditions. These genes were then taken forward for investigation within the flow-through system. As expected, FKBP5 and GILZ were up-regulated by GC, although continuous delivery exerted a more pronounced induction than the pulsatile delivery. IL6ST and GLUL transcripts were both equally induced by the continuous and pulsatile delivery while IL8 was also equally repressed by both pulsatile and continuous cortisol. Clearly, these data define a role for rapid oscillations in GC concentration on target gene regulation, and provides a possible biological impact for the physiological fluctuations in serum glucocorticoid concentrations.
Chapter 5:
Discussion and
Future Directions
5- Discussion and future directions

5.1- Significance of glucocorticoid sensitivity modulators in physiology and disease

Endogenous GC production is required for maintaining basal energy homeostasis. In addition, GCs have profound immune modulating activity and synthetic GCs are the most potent anti-inflammatory drugs currently used. However, variation in response to GCs is significant and may present on either side of an optimal range as hypersensitivity or resistance. Hypersensitivity to GCs can result in metabolic diseases such as visceral obesity-related insulin resistance and ischaemic heart disease (van Rossum et al. 2004). On the other hand, several autoimmune and inflammatory diseases such as rheumatoid arthritis, Crohn’s disease and ulcerative colitis and also systematic lupus erythematosis are often associated with localised resistance to GC. PBMCs from patients with glucocorticoid resistant rheumatoid arthritis showed a poorer anti-proliferative and cytokine response to corticosteroids in vitro than cells from patients with GC sensitive disease (Sliwinska-Stanczyk et al. 2007). A gene expression profiling study in primary lymphocytes from 100 healthy volunteers that compared expression of circulating genes between the 10% of extreme GC responders and the 10% of non-responders, as determined by cortisol measurements following a low dose dexamethasone suppression test, dentified 20 genes that showed a difference in GC response between the groups (Donn et al. 2007). A bone morphogenetic protein receptor, type II (BMPRII) gene enhanced the glucocorticoid responsiveness when analysed by luciferase reporter gene assays in HeLa cells. Furthermore, in a follow up study of 20 genes with potential GC
modulating effect, interferon-inducible factor 16 (IFI16) was investigated for modulating GC sensitivity by in vitro assays (Berry et al. 2010). In fact IFI16 enhanced GR-mediated transactivation of TAT3 gene while it also rescued GR-mediated transrepression of a NF-κB reporter construct. Co-immunoprecipitation assays showed that GR and IFI16 interacted in vitro and that GR ligand binding domain is required for IFI16 binding. Also, IFI16 enhanced GR-regulated genes making IFI16, a novel modulator of GR and GC sensitivity (Berry et al. 2010).

5.2- Identification of Merm1 as a methyltransferase and a novel GC sensitivity modulator

Using the same functional analysis as employed previously in our lab for BMPRII (Donn et al. 2007) and IFI16 (Berry et al. 2010), we set out to evaluate if ELOVL1, HNRPA1, DR1 and PCCA or ANP32E, identified as being part of a discriminatory panel of genes, were capable of affecting GR action (Berry et al. 2010; Donn et al. 2007). As explained previously in the Results section 3.1-1, Merm1 became included, in addition to ANP32E due to inaccuracies in the clone arrays handling by the clone distributor, Open Biosystems.

GC responsive reporter TAT3-Luc, which contains 3 copies of the glucocorticoid response element (GRE) from the tyrosine amino transferase 3 plasmid, was utilised to investigate the capability of the above genes in modulating GR-transactivation with TIF2A plasmid as the positive control. Overexpression of Merm1 potentiated GR transactivation of TAT3 relative to empty vector control, while overexpression of ELOVL1 inhibited TAT3-Luc activation. The other remaining genes did not show any significant effect on TAT3-Luc activation. This transfection system was previously shown by work in our lab to be a
robust screening assay for novel GR co-modulators (Berry et al. 2010; Donn et al. 2007). Inhibition of TAT3-Luc by ELOVL1 overexpression, further confirmed that the transfection was successful and the consistent potentiation of TAT3 by Merm1 was accurate.

Merm1 is part of variably deleted region of chromosome 7 which causes Williams Beuren Syndrome (Pober 2010). In addition, it has recently been identified in a genetic screen as a regulator of cancer cells metastasis by transcriptional repression of Zac1 gene (Nakazawa et al. 2011). In order to examine whether the Merm1 or other potential GC sensitivity co-modulators affected GC-mediated repression their effect on repression of an NF-κB reporter was examined. Neither Merm1 nor other genes, showed any regulation of TNF-α driven NF-κB reporter. TIF2A, a well characterised GR co-factor and also a positive control for the NRE assay, also failed to show any significant restoration of dex-induced repression of NF-κB. IFI16, a co-modulator of GR, which has been previously shown by Berry et al. (Berry et al. 2010) to restore GC repression could have been a better control for this assay (See Future research section 5.7-1 Merm1 and GR transrepression). This however, does not question the capability of Merm1 in functioning as a co-factor for GR since transactivation in itself is an important property of GR in modulating important aspects of inflammation. GR-activation of glucocorticoid-induced leucine zipper (GILZ) protein inhibits both T-cell receptor (TCR)-induced interleukin-2 (IL-2)/ interleukin-2 receptor (IL-2R) expression and NF-κB activity through inhibition of nuclear translocation of NF-κB and reduced DNA binding (Ayroldi et al. 2001). Further investigation was therefore set out to find the role of Merm1 in regulating GR functions.
Our GR reporter assays with TAT3-Luc plasmid presented Merm1 as a strong candidate for modulating GR transactivation. Analysing Merm1/WBSCR22 cDNA sequence confirmed that it contains three main functional domains i.e. a methyl-transferase domain, an S-Adenosyl Methionine (SAM) domain and a nuclear localisation domain. Merm1 shares structural homologies with the N-terminal domain of the histone H3 lysine 79 (H3K79) methyltransferase, Dot1L as explained in Introduction section 1.10-2. It also belongs to the seven-β-strand family of methyltransferases (Petrossian et al. 2011) which includes coactivator-associated arginine methyltransferase 1 (CARM1), a coactivator of nuclear receptors (NRs) such as androgen, estrogen receptor (Chen et al. 2000; Chen et al. 1999; Teyssier et al. 2002) and also glucocorticoid receptor (Ma et al. 2001).

The enzymatic activity of the methyltransferases is required for the effects mediated by methyltransferases. Chen et al (Chen et al. 1999) mutated the S-adenosylmethionine binding domain of CARM1 by substitution of three alanines (AAA) for three amino acids, valine 189, leucine 190, and aspartic acid 191 (VLD) and observed a substantial reduction of the methyl transferase activity of CARM1 on histone H3. This was also associated with a loss of the transcriptional activation of the reporter gene by the mutant CARM1 vector when compared to wild type. CARM1, as a second co-activator, potentiated MMTV promoter, responsive for androgen, estrogen and thyroid hormone receptor-β through interaction and methylation of the COOH-terminal domain of the NR-bound SRC2/GRIP1 activation domains AD1 and AD2. In another study, protein arginine methyl transferase 1 (PRMT1) also demonstrated a methyltransferase-dependent enhancement of the nuclear receptor activity in transient transfection assays in CV-1 cells as the mutation in the C
terminus of GRIP1 abrogated the NR activity when compared with wild type GRIP1 (Koh et al. 2001).

In our experiments, we used site directed mutagenesis to delete each of the methyltransferase, SAM motif or the nuclear localisation signal domain of the Merm1 in order to identify the functional domains responsible for potentiating GR activity. In fact, deletion of both methyltransferase and the SAM regions abolished the effects on GR transactivation of the TAT3-Luc reporter indicating a role for the conserved methyltransferase domain of Merm1 for affecting GR activity, although in vitro histone methylation assays were unsuccessful (Nakazawa et al. 2011), suggesting a requirement for another unidentified factor to allow the methyltransferase enzymatic function.

5.3- In vitro analysis of Merm1 and GR interaction

Nuclear localisation experiments using cellular fractionation showed Merm1 to be predominately nuclear and there was no evidence of trafficking with ligand induced translocation of GR. Merm1 and GR shared cellular heterogeneity whereby in the presence of ligand, cells rich in GR expressed low levels of Merm1 and vice versa, raising the possibility of Merm1 and GR reciprocal regulation. Our Co-IP studies did not confirm direct protein: protein interaction of GR and Merm1 in vitro, contributing to the hypothesis that Merm1 and GR require a chromatin template for indirect interactions. An extensive study of endogenous human regulatory proteins utilised a large spectrum of primary antibodies in order to identify hierarchical organization of protein complex networks in mammalian cell regulation. Merm1/WBSCR22 was identified as a potential
interacting protein in the nuclear receptor transcriptional regulator complex which was detected in 4 separate immunoprecipitation experiments although this study failed to identify the protein through which Merm1 has been pulled down (Malovannaya et al. 2011). This study might add to our hypothesis of template dependent Merm1 and GR interaction and provide new insights into GR/Merm1 interactions through other nuclear receptor co-activators. Other possible co-activators such as SRC/p160 or CBP/p300 could be intermediary binding partners for Merm1 and GR. It is known that following activation, nuclear receptors recruit transcriptional steroid receptor co-activators (SRC1, SRC2/GRIP1, and SRC3) which in turn provide a platform for binding of other transcriptional co-factors such as CREB-binding protein (CBP)/p300 and protein arginine methyl transferase 4 (PRMT4/CARM1) (Chen et al. 2000; Glass et al. 2000; McKenna et al. 1999; Xu et al. 1999; York et al. 2010). More recently, it was shown that CARM1-dependent methylation of CBP and p160 co-activators was required for transcriptional activation of estrogen target genes (Ceschin et al. 2011). Immunoprecipitation studies of CARM-/- mouse embryonic fibroblasts (MEFs) failed to show any methylated CBP which was associated with much decreased histone acetyltransferase activity of CBP in MEFs and lower estrogen receptor-α (ER-α)-mediated recruitment of CBP to target gene promoters.

Merm1 knockdown did not reveal any change in GR protein by immunoblot analysis suggesting that Merm1 does not alter GR activity through GR expression. These findings suggested that Merm1 regulated histone methylation and thereby altered access of the GR to target gene binding sites. Hence, the effect of Merm1 on GR endogenous target
gene expression was further investigated. In line with previous GR reporter transfection assays, Merm1 potentiated ligand-driven GR-induction of GILZ and FKBP5 genes, and it also potentiated GR-inhibition of IL-8, IL-6 and CCL2 genes showing that both GR transactivation and transrepression mechanisms were in part dependent on Merm1.

Merm1 knockdown also induced basal IL6ST expression independent of ligand-activated GR. IL6ST (gp130) is a signal transducer shared by many cytokines, including IL6, ciliary neutrophobic factor (CNTF), leukemia inhibitory factor (LIF), and oncostatin M (OSM). This protein functions as part of a cytokine receptor complex and is part of a key signalling pathway central to multiple inflammatory processes (Heinrich et al. 2003). Merm1 knockdown also repressed basal IL-6 expression, a central player in the stress induced and inflammatory processes which is strongly inhibited by GCs and is induced by NF-κB during inflammation (Ding et al. 2010; Grassl et al. 1999). Modulation of basal IL6ST and IL6 expression independent of GR by Merm1 might be a potential target towards novel management of inflammatory pathways. In a study by Chen et al (Chen et al. 2009), G9a, a histone H3 lysine 9 methyltransferase, directly interacted with the NF-κB transcription factor, RelB and mediated the transcriptional silencing of the IL-1β through RelB-induced facultative heterochromatin formation. The accumulation of G9a methyltransferase at the IL-1β promoter was required for the direct binding with RelB and gene silencing.

To find the mechanism of action of Merm1 on GR-mediated gene expression, ChIP analysis was performed to investigate whether Merm1 interacts with GR on the same target gene promoter. Merm1 was recruited to GR regulatory elements on GILZ promoter and this binding was independent of GC ligand. Merm1 recruitment was confirmed by
ChIP analysis onto GL-2 and GL-3 regions, known to harbour well characterised GR binding sites (Chen et al. 2006), and also on GL-4 but was not detected on GL-1 negative control (in the region of transcription initiation) for the ChIP assay. The directed recruitment of Merm1 not to all but to specific regulatory regions on GILZ promoter might be facilitated through cooperative interactions with other factors and binding partners. Astrand et al (Astrand et al. 2009) reported a reciprocal binding of transcription factor 1 (Oct1) and nuclear factor 1 (NF1) to MMTV long terminal repeat which was associated with an increase in histone H3 and H4 acetylation. This facilitated a preset chromatin for enhanced hormone-dependent GR-DNA interaction. Furthermore, Forkhead boxA1 (FoxA1) was shown to act in concert with NF1 and Oct1 and to have a dominant effect in the absence of hormone. MMTV promoter analysis indicated that the maintenance of enlarged nucleosome by FoxA1 together with Oct1 and NF1 –dependent nucleosome organisation lead to the stepwise increase in transcriptional activation contributing to FoxA1 as a nucleosome remodelling molecule prior to hormone induction and GR activation (Belikov et al. 2009). FoxA1 also acts as a pioneer factor in the recruitment of ERα to several regulatory elements in the genome and plays a role in subsequent chromatin remodelling and recruitment of transcription factors during transcription of ERα target gene expression. Genomic analysis and ChIP seq revealed that FoxA1 binding sites on the MC7 breast caner cell line genome significantly overlapped with ERα regulatory elements (Lupien et al. 2008). FoxA1 recruitment was also associated with an increase in H3K4 mono- and di-methylation both of which are active histone marks. H3K4 methylation at the FoxA1 binding regions was unchanged following FoxA1
silencing although it significantly impaired target gene transcription indicating interplay between FoxA1 as a pioneer factor and H3K4 methylation at enhancer for an efficient gene transcription. The evidence points to the important role of methyltransferases, such as Merm1, in making a methylation signature across the genome for directing the binding of transcription factors like FoxA1 as pioneers for nucleosome remodelling and NR-mediated gene transcription. Furthermore, it would be of high relevance to more fully investigate the mechanisms by which Merm1 is directed to specific binding sites on the target DNA sequence. Genomic wide chromatin immunoprecipitation (ChIP-seq) of Merm1, GR and other transcription factor partners such as FoxA1 could be a step towards understanding this pathway.

As expected, GR was recruited to GL-2, GL-3 and GL-4 regions on GILZ promoter and not GL-1 control region. GR recruitment increased significantly upon hormone induction at GL-2 and GL-3 but not GL-4 confirming the binding of GR to the specific GR binding sites following GR activation and nuclear translocation. Interestingly, Merm1 silencing abolished GR binding to the binding elements. The observation that GR protein expression was unchanged by Merm1 knock-down removes the possibility of reduced GR binding to GREs to be due to lower total concentration of available GR (Fig 3.11a).

5.4- Role of Merm1 on GR-mediated conformation of chromatin architecture

A number of genomic analysis studies have contributed to the discovery of histone modification marks for active transcriptional states of chromatin as well as repressive states (Bernstein et al. 2007; Kouzarides 2007). It is now well understood that in addition
to acetylation, certain histone methyl marks are also associated with euchromatin and active transcriptional state. Arginine17 (R17) methylation by histone methyltransferase CARM1 is associated with accessible chromatin at sites of chromatin remodelling complex occupancy. Lysine residues which include H3K4-trimethylation (Heintzman et al. 2009; Muramoto et al. 2010; Schnetz et al. 2009) and H3K79 di-methylation (Barski et al. 2007) have also been associated with active transcriptional state in mammalian cells. Histone H3 methylation at lysine 9 represent a signature of the promoter of a silenced gene (Bannister et al. 2001).

As Merm1 potentiated GC-driven GR recruitment to GREs, we set out to explore the Merm1 regulation of chromatin remodelling pre-and post-ligand. Dex increased acetylated histone H3 lysine 9 (H3K9), a positive mark for open chromatin, however, Merm1 knock-down did not substantially affect the acetyl-H3K9 on any of the GL-2, GL-3 or GL-4 regions indicating that in addition to Merm1 other histone modifications enzymes might also be responsible for maintaining the active state of the chromatin at the GILZ locus, compensating for Merm1 silencing. We therefore set out to explore the role of Merm1 in regulating other methyl marks in vivo in order to better characterise Merm1 as a methyltransferase in regulating GR binding to binding sites. Merm1 knock down in HeLa cells caused a significant loss of methylation at H3K9, a repressive methyl mark, which was in line with previous findings from Nakazawa et al (Nakazawa et al. 2011), in which they showed that Merm1 was mediating cancer cell metastasis through H3K9 methylation and suppression of tumour suppressor Zac-1 gene. In our studies, Merm1 potentiated GR transactivation of GILZ and FKBP5 expression and so the effects of Merm1, as a histone
methyltransferase on two active methyl marks, H3K4me3 and H3K79me2, were also investigated. Merm1 knock-down attenuated both H3K4 and H3K79 methylations demonstrating that Merm1 cannot only serve to repress but also to activate the chromatin for transcription. Methyltransferase activity is generally regarded as highly specific with target sites being specified by the flanking amino acid residues. Merm1 is therefore unusual in directing both activating and repressing methyl marks. However, it is possible that Merm1 catalyses one marks in a primary manner, with further changes being mediated by other enzymes in a secondary response (Barski et al. 2007; Daniel et al. 2005). Since Merm1 knock down caused a global loss of methylation on H3K4, K9 and K79, it could be proposed that loss of Merm1 could have a toxic effect on the pattern of methylation or it could be deduced that Merm1 acts as a master regulator of methyltransferases, which mediate methylation at the studied locion histone H3 lysine residues. This however requires more detailed analysis and could be made clear through methylation assays or genome wide ChIP analysis. Since previous attempts to show direct methylation of target histone by Merm1 in vitro have failed (Nakazawa et al. 2011), a requirement for additional cellular factor(s) is also suggested.

Regulation of global histone methylation status by Merm1 demonstrates a role for Merm1 in regulating histone modification, but the changes at a target gene locus are more important to determine the function, since GR acts to both activate and repress gene transcription. Therefore Merm1 methylation of methyl marks at H3 lysine residues was analysed by ChIP at the GILZ locus. Merm1 significantly potentiated both GR-induced induction of H3K4me3 and also caused a reduction of GR-mediated H3K79me2 mark.
Merm1 knockdown abolished H3K4me3 while it also impaired basal H3K79me2. The coordinate increase in H3K4 tri-methylation with reduction in H3K79 di-methylation in response to GR activation by ligand, and DNA binding suggests coordinated histone modification whereby one change influences another. Loss of Merm1 inhibited both changes, suggesting that the driving event is the gain of methyl marks on H3K4. Interestingly, GR activation also caused an induction in the H3K9 methylation, a repressive methyl mark. Loss of Merm1 produced differential effects on H3K9me at the GILZ loci with reduction of H3K9me at GL-3 and no effect on GL-2. The observation that loss of Merm1 attenuated GR binding as well as H3K4me3, points to the required role of Merm1 for facilitating GR recruitment to GREs and suggests that glucocorticoid receptor functions more as an enhancer protein than a classic transcription factor with pioneering potential for nucleosome remodelling.

It has been long believed that nuclear receptors such as GR (Richard-Foy et al. 1987) act as pioneer factors capable of binding to genomic target sites and mediate chromatin remodelling which is associated with nuclear receptor-mediated modifications of histones. Genomic analysis of estrogen-regulated promoters revealed strong positive correlations between ERα regulated genes and acetylated histone levels as well as RNA polymerase II occupancy and steroid receptor co-activator binding and ERα recruitment to the promoter target genes (Kininis et al. 2007). A more recent study also showed that ERα-specific enhancers that are subject to methylation by coactivator-associated arginine methyltransferase 1 (CARM1) action are also associated with active gene expression. Similarly, peroxisome proliferator-activated receptor γ (PPARγ) induced H3K9 acetylation
at genomic regions close to PPARγ binding during adipogenesis. H3K9 acetylation was increased in 67% of the PPARγ-binding regions in adipocytes compared to preadipocytes suggesting that PPARγ bound at these sites might be recruiting HATs (Lefterova et al. 2008).

However, genome wide analysis of transcription factor binding profiling and ChIP-seq have recently shown that nuclear receptor (NR) binding almost always takes place at pre-existing hypersensitive sites. Sam John et al (John et al. 2011) showed that GR binding is localised to regions of open chromatin that are open prior to hormone treatment (constitutive sites). Functional interaction of NRs with other transcription factors plays an important role in maintaining the constitutively open chromatin for DNA binding. Emerging evidence from genome-wide studies suggest that activating protein 1 (AP1), acts on chromatin before glucocorticoid receptor binding (Biddie et al. 2011). Ablating AP1 binding attenuated chromatin accessibility, suggesting a fundamental role for AP1 in the maintenance of chromatin accessibility and, hence in the recruitment of GR to chromatin. However, not all regions of accessible chromatin were dependent on AP1 occupancy implicating the activity of other transcription factors in the maintenance of chromatin accessibility. The target sites and regulatory elements bound by NR and their binding partners are associated with active histone methyl marks. A recent study showed that in macrophages and B cells, interactions of the common factor PU.1 with macrophage- or B cell lineage-determining transcription factors, established nucleosome remodelling which was associated with H3K4 methylations. Since PU.1 binding and nucleosome remodelling was not sufficient for deposition of H3K4 methyl mark, it was
suggested that other transcription factors such as CCAAT enhancer binding protein (C/EBPβ) and histone methyltransferases are required for nucleosome displacement and targeting of H3K4me1 deposition around PU.1 binding sites (Heinz et al. 2010).

These observations together with the findings herein of Merm1 and assisted GR recruitment to GREs suggest that GR binding sites are predetermined and there is a strong interplay between Merm1 as a methyltransferase and the acquired chromatin remodelling for GR-DNA binding which contradicts the pioneering model of GR. Further genome wide Merm1 binding analysis will help explore the role of Merm1 in directing GR binding across the genome.

5.5- Role of Merm1 on cytokine-induced GC resistance

We sought to explore how Merm1 expression is regulated, in order to investigate how Merm1 regulation can affect GR actions. Although none of the inflammatory and cytotoxic stimuli affected Merm1 expression, combination of TNF-α and IFN-γ, also known to induce GC resistance (Tliba et al. 2008a), markedly reduced Merm1 expression in A549s. It was therefore hypothesised that GC resistance could be mediated through Merm1 and overexpression of Merm1 could rescue the cytokines-induced resistance to GCs. A GC-responsive TAT3-Luc reporter gene assay was utilised for this purpose. TNF-α and IFN-γ inhibited the GR transactivation of TAT3-luc gene, used in the initial screening for Merm1, and Merm1 overexpression rescued the inhibition caused by cytokines through increase in the Merm1 expression. Investigating the protein levels of Merm1 following TNF-α and IFN-γ treatment revealed that cytokines not only inhibited the
endogenous Merm1 expression but also attenuated Merm1 protein, suggesting a post-translational mechanism of action. A role for proteasome complexes is suggested which could be analysed further using the proteasome inhibitor MG132.

In the ChIP assays, Dex-induced GILZ expression was associated with lower GR recruitment to GL-3 region and smaller H3K4 methylation which could be explained through a reduction of Merm1 protein concentration. In the absence of ligand however, an increase in GR binding was observed which was also associated with a gain of methyl mark at H3K4. Considering the lower basal expression levels of Merm1 with the TNF-α and IFN-γ, we can deduce that, other co-factors and methyltransferases mediate the rise in GR-induced binding and H3K4 methylation at the GILZ locus. A study by Verhoog et al (Verhoog et al. 2011) showed that TNF-α induced a GC-independent increase in GR recruitment to IL6 promoter which was associated with higher GRIP1/TIF2 recruitment as well. Since GRIP1 is a well-characterised co-activator of glucocorticoid known to interact with chromatin remodelling enzymes such as CBP/p300 and CARM1, it might be possible to speculate that cytokine-induced GRIP1 recruitment to IL6 promoter could be associated with recruitment of other known H3K4 methyltransferases such as mixed lineage leukemia 3 (MLL3) (Ananthanarayanan et al. 2011) and MLL1 (Kinyamu et al. 2007), both of which have been shown to mediate H3K4 methylation at a GC responsive promoter to account for the TNF-α and IFN-γ-induced increase in H3K4 methyl mark. MLL-mediated methylation could therefore in part contribute to the increase seen with H3K4me3 at the GILZ GL-3 region despite the cytokine-mediated attenuation of Merm1 protein. Investigating GR recruitment and H3K4 methylation at the IL6 promoter by ChIP
analysis could potentially point out an important mechanism through which unliganded GR could alleviate TNF-α induced inflammation and mediate cytokine-stimulated GC resistance.

5.6- Summary

In this study a novel GC sensitivity modulator is proposed and examined. GR is a transcription factor capable of DNA binding in areas pre-determined by chromatin structure and is in itself a factor with chromatin remodelling activity through histone and DNA modifications.

Merm1 is a newly identified histone methyltransferase which has been previously shown to mediate cancer metastasis through methylation of histone 3 lysine 9 (H3K9me). The function of Merm1 as a GR co-modulator was therefore investigated. Merm1 potentiated GR transactivation of a reporter gene which was found to be dependent on its SAM and methyl-transferase domains, showing a role for the methyltransferase enzymatic activity of Merm1 for this effect. Merm1 also facilitated GR transactivation and transrepression of endogenous genes including GILZ. Both Merm1 and GR were recruited to GR binding elements on GILZ promoter and Merm1 regulated ligand induced GR recruitment.

Merm1 was also found to regulate methylation of H3K4me3 and H3K79me2, activating methyl marks, and also H3K9me which is a repressive mark. At the GILZ locus GR induced H3K4me3 and repressed H3K79me2. Merm1 was required for the maintenance of basal H3K79me2 and also for the GR induction of H3K4me3. The coordinated increase in H3K4me3 followed by H3K79me2 suggested that H3K4me3 is the driving event. It is
therefore concluded that Merm1 regulates chromatin structure to affect GR recruitment and mediates GR functions through histone modifications.

5.7- Future research

This study has identified Merm1 as a novel histone methyltransferase with GR potentiating activity. More detailed investigation will help fully characterise Merm1 protein and explore its role on GR functions.

5.7-1. Merm1 and GR transrepression

Even though the qPCR findings showed that Merm1 knockdown in HeLa cells regulated GR dependent target gene expression of both transactivated and transrepressed genes, Merm1 failed to show a rescue of the GC-responsive NF-κB reporter (NRE-Luc). This was also associated with a lack of response from the TIF2A, a GR co-activator known to be relieve the NF-κB-mediated repression of GR activity (Sheppard et al. 1998). Therefore to clarify whether this means that NF-κB reporter transfection assays are true readings, IFI16, previously shown by Berry et al (Berry et al. 2010) to rescue NF-κB-mediated repression of GR acitivity, should be included as a positive control for the NRE-Luc transfection assays.

Our findings point towards a potential role for Merm1 in both potentiating GR transactivation as well as transrepression. However, our ChIP studies have mainly focused on recruitment of Merm1 and GR on a transactivated GR target gene and so therefore it is as important to explore Merm1 recruitment to a transrepressed GR target gene (e.g. IL-6 or IL-8) and investigate the methyltransferase activity of Merm1 in a context of GR-
transrepression. This will also confirm the findings by Nakazawa et al (Nakazawa et al. 2011) where the introduced Merm1 as a promoter of cancer cell metastasis through methylation of histone 3 lysine 9, a repressive methyl mark, on Zac-1 promoter and hence inhibiting the expression of the Zac-1 gene.

5.7-2. Merm1 and GR interactome

In my studies, Merm1 potentiated GR transactivation of target genes like GILZ through chromatin binding and histone methylation. Identification of Merm1 binding partners through CoIP or ChIP will help better understand the mechanisms by which Merm1 enhances GR activation. Co-activators such as SRCs and CBP have been shown to interact directly with GR and enhance GR-mediated transcription (Ding et al. 1998; Kamei et al. 1996). Recently, Merm1 was identified as one of the proteins forming an immune complex with steroid receptor co-factors in four separate immunoprecipitation assays (Malovannaya et al. 2011). Also a distant relative of Merm1, CARM1 has been shown to form complexes with transcription factors such as GR and nuclear receptor co-activators like SRCs, CCAAT-enhancer binding protein (CBP) to modulate nuclear receptor target gene expression (Chen et al. 2000). This interaction is necessary for nuclear receptor recruitment to target gene promoters (Ceschin et al. 2011). Also, ChIP analysis with Merm1 and RNA polymerase, Pol II, on a target gene promoter such as GILZ, will contribute to an understanding of the role of Merm1 as a facilitator of GR-mediated gene transcription through chromain remodelling and histone modification.
5.7-3. **ChIP-seq for GR and Merm1**

In our studies, Merm1 has been found to be required for GR-mediated H3K4 methylation and GR target gene expression. Furthermore, Merm1 also maintained the basal H3K79 methylation. Our data suggest that Merm1 regulates chromatin structure to affect GR recruitment, and mediates GR actions of transcription by histone methylation. Recently genome–wide analysis revealed that the majority of GR binding occurs to constitutively accessible sites (John *et al.* 2008). These sites not only include classical GRE elements, but are also enriched for other transcription factor binding sites, notably AP-1 (Biddie *et al.* 2011). Performing a genome wide ChIP-seq analysis of Merm1 and GR will firstly confirm whether GR and Merm1 bind same sites on the genome and secondly whether Merm1 is required for maintenance of constitutive sites in DNA pre-hormone treatment and facilitates de novo GR binding after hormone induction.

5.7-4. **Merm1 differential regulation in a model of inflammatory disease, RA**

Previously Merm1 has revealed differential expression in healthy vs cancer states with higher expression in invasive breast cancer cells leading to metastasis formation. The differential expression of Merm1 in other inflammatory disease states has not been investigated. The underlying mechanism giving rise to development of inflammatory diseases like rheumatoid arthritis (RA) is not fully characterised. Comparison of Merm1 RNA and protein expression patterns between healthy volunteers and patients with RA will confirm whether Merm1 is differentially expressed between in RA and also will indicate whether this differential expression is regulated through the Merm1 gene or
post-translational modifications of the Merm1 protein. Since primary T cells are important mediators of inflammation and cytokine release in inflammatory disease like RA, Merm1 expression in these cells lines will be a good indicator of the role of Merm1 on the modulation of inflammation. The primary T cells for this study could be provided by the ARUK national repository which consists of a large number of primary blood mononuclear cells taken from both healthy and RA affected individuals.

5.7.5. **Merm1 knock-out mice**

The glucocorticoid receptor mediates physiological immune and inflammatory functions and also regulates glucose and bone homeostasis in the body. It is necessary for normal development of respiratory systems as well as the brain and the HPA axis in developing embryos. Since Merm1 is a co-activator of GR and GR-mediated transactivation as well as transrepression, it is hypothesised that loss of Merm1 in Merm1 knockout animals will have a profound effect on GR signalling and hence on physiological growth and development of mouse embryos. RNAi-mediated knock-down of Merm1 in *Caenorhabditis elegans* has been reported as embryonic lethal indicating that Merm1 is vital for development. Generation of mouse models of Merm1 knockout will clarify both the importance of Merm1 in development and also the effects of Merm1 on GR functions in vivo.
5.8- Novel pharmacokinetic approach to the modulation of GC sensitivity

5.8-1. Significance of pulsatility in maintenance of biological responses

Ultradian rhythm of GC secretion has been shown to alter the diurnal pattern of synthesis of several cytokines, involved in inflammation, through inhibition of their target gene expression (Hermann et al. 2006; Petrovsky et al. 1997; Petrovsky et al. 1998). In addition to the diurnal oscillations seen in the HPA axis and ultradian rhythm of cortisol release, a number of recent studies have also investigated the significance of biological oscillations of other molecules and proteins, including P53 (Geva-Zatorsky et al. 2006), NF-kB (Nelson et al. 2004), Crz1 (Cai et al. 2008), FuS3 MAP kinase (Hilioti et al. 2008) and SOS stress response systems (Friedman et al. 2005), in the regulation of target gene expression in a wide range of human and other eukaryotic cell lines.

Nuclear Factor kappa B (NF-kB) is a family of dimeric transcription factors involved in regulation of cell division, apoptosis and inflammation. Signalling by NF-kB involves its release from inhibitor kappa B (IκB) in the cytosol, followed by translocation into the nucleus. IκB is a transcriptional target for NF-kB, creating a negative feedback loop that drives oscillations in NF-kB translocation. Nelson et al (Nelson et al. 2004) used fluorescence imaging of NF-kB (RelA) (fused at the C terminus to red fluorescent protein (DsRed)) and IκB (fused at the C terminus to the enhanced green fluorescent protein (EGFP)) to study oscillations in RelA nuclear-cytoplasmic localisation in HeLa cells and SK-N-AS (human S-type neuroblastoma cells that have been associated with deregulated NF-kB signalling) cells. TNF-α caused asynchronous oscillations in NF-kB (RelA) localisation.
that decreased in frequency with increased IkB transcription. Persistent oscillations in NF-kB localisation coupled to cycles of RelA and IkB phosphorylation maintained NF-kB-mediated gene expression.

Also Ashall et al (Ashall et al. 2009), transiently transfected SK-N-As cells and mouse embryonic fibroblasts (MEFs) with a vector expressing RelA fused to the Discosomasp.red fluorescent protein dsRed-Express (RelA-dsRedxp). Single-live cell imaging of both cell types showed robust cycles of RelA-dsRedxp nuclear translocations. To mimic the pulsatile inflammatory signals, they treated cells with repeated short pulses of TNF-α at various intervals. Although all TNF-α stimulations caused synchronous cell responses, short-interval stimulations (e.g. 100- or 60- min intervals) produced significant reductions in the magnitude of RelA-dsRedxp fusion protein translocation. RT-PCR analysis of four TNF-α induced gene transcripts i.e. IkBα, IkBε, MCP-1 and RANTES produced altered gene-expression profiles in response to varying frequencies of TNF-α.

NF-kB is a master regulator of inflammatory processes with a growing list of cancers that need its aberrant activity (Greten et al. 2004; Jackson-Bernitsas et al. 2007; Karin 2006). Live cell imaging of cultured head and neck squamous carcinoma cells (HNSCCs) revealed oscillations in TNF-α-induced NF-kB activity (Sung et al. 2008). Bortezomib is an anticancer drug which inhibits NF-kB through stabilisation of IkBα, which sequesters NF-kB in the cytoplasm. When administered to TNF-α activated cancer cells, it caused dampening of the oscillations of NF-kB inducible interleukin-8 promoter driven reporter. These findings suggest that network dynamics and intracellular pharmacokinetics have to be
critically analysed before developing strategies or therapeutic interventions for oncogenic and possibly other disease pathways.

The negative feedback loop between tumour repressor, p53, and the oncogene, Mdm2, is one of the best studied protein circuits in human cells. P53 transcriptionally activates mdm2, while mdmd2 negatively regulates p53 by both inhibiting its activity and enhancing its degradation rate (Momand et al. 2000). Following stress signals, p53 activates transcription of several hundred genes involved in growth arrest, apoptosis and DNA repair. Dynamic measurements of p53-cyan fluorescent protein (CFP) and Mdm2-yellow fluorescent protein (YFP) in the nucleus of gamma-irradiated breast cancer MCF7 cells over several days revealed prolonged undamped oscillations which lasted for at least 3 days (Geva-Zatorsky et al. 2006). Increasing the doses of irradiation from 0.3 to 5 Gy resulted in a higher fraction of cells that performed Mdm2-YFP oscillations. Although the central role of oscillations is not established yet, it is speculated that these oscillation may play a general role in stress or damage response.

5.8-2. Significance of pulsatile GC release on GC sensitivity and actions

It has been some time since the existence of pulsatile GC delivery from the adrenal cortex was described (Windle et al. 1998a). The downstream effects of ultradian GC secretion in terms of GC sensitivity and actions on target tissues have not been fully explored. However, in pathophysiological conditions such as long-term stress, chronic disease and affective disorders such as adjuvant-induced arthritis, the glucocorticoid pulse frequency over the 24-hour period is increased (Windle et al. 2001). Also altered pulse size and/or
frequency have been reported in conditions such as acute stress (Windle et al. 1998b) and obstructive apnoea (Henley et al. 2009).

To explore the biological consequences of hourly fluctuations in glucocorticoid concentrations for the target cell response, a flow-through culture system was optimised from that previously described (McMaster et al. 2011), for investigations in HeLa cells and non-adherent primary T cells.

The ultradian rhythm of endogenous GC release has been reported to be altered in certain inflammatory and pathological conditions such as acute physiological stress and adjuvant-induced arthritis (Windle et al. 2001). Primary T cells are important immune and inflammatory modulators and are therefore a more relevant cell type to explore the role of differential dynamics of GC delivery (Flammer et al. 2011). Pulsatile compared to continuous cortisol delivery to HeLa cells produced a bigger decrease in cell numbers which was found to be due to higher apoptosis. In the primary T cells however apoptosis rates from pulsatile delivery of cortisol did not differ significantly from that of dose-equivalent continuous cortisol treatment. This could at least in part be attributed to the low sample number used for this experiment (n=4) and inter-sample variability of gene expression seen in primary T cells taken from individual volunteers. Induction of the two up-regulated GC target genes, GILZ and FKBP5, were significantly less with pulsatile cortisol than the same total concentration of cortisol given continuously. It is striking that also in the earlier work by Stavreva et al (Stavreva et al. 2009) continuous corticosterone consistently had a greater effect on target gene induction, and subsequent protein accumulation compared to pulsatile delivery. As shown in the study by Stavreval et al
(Stavreva et al. 2009), pulsatile delivery of the rodent physiological GC, corticosterone, resulted in rapid oscillations of GR on and off target genes. These cycles correlated with pulses of gene transcription, or “gene pulsing” showing that pulsatile GC delivery has important implications in altering the expression of target cell genome and ultimately the cellular phenotype. In line with these studies, it would be of relevance to investigate the interaction of GR with the GR binding sites on GILZ and FKBP5 promoter regions in primary T cells exposed to the pulsatile and continuous GC. The feasibility of a ChIP experiment within the flow-through system however, needs to be taken into account before carrying out such experiments. The non-adherent nature of primary T cells has made it extremely challenging to contain these cells in a culture system with a dynamic flow of media without compromising the flow-through status. The high variability in the gene expression profile in the primary T cells from different individuals also makes it difficult to have a clear and consistent read out for any experiment. A much larger number of volunteers for lymphocyte analysis, together with the complicated process of flow-through preparation make it unfeasible to carry out more relevant and detailed experimental analysis which is both consistent and statistically significant. More recently, ultradian corticosterone secretion in the rat brain produced cycling of GR activation and DNA binding onto Per1 promoter, a well characterised clock gene (Conway-Campbell et al. 2010). ChIP analysis revealed cyclic GR recruitment to GREs on Per1 promoter with pulsatile hormone release. Following each glucocorticoid pulse there was a burst of Per1 leading to the accumulation of the mature mRNA transcript during exposure to pulsatile corticosterone (Lightman et al. 2010). It is the accumulative effects of pulse-driven bursts
in Per1 transcription that leads to rapid transcription of Per1 mRNA in the hippocampus providing a remarkably sensitive signalling system for rapid and efficient responses to changes in the plasma steroid levels.

The design system utilised by Stavreva et al (Stavreva et al. 2009) as well as by lightman et al (Lightman et al. 2010) and by Conway-Campbell et al (Conway-Campbell et al. 2010) differs importantly from our model flow-through system since in all the three studies mentioned above pulsatile corticosterone delivery to cells was achieved through manual pipetting of GC containing media to cells in timed intervals. This design did not replicate the endogenous GC oscillations as occurs in vivo. Furthermore, GC pulses given to the cells were compared against the same concentration given continuously over a longer time interval, resulting in a reduction in the total cumulative GC dose to the cells. In our system the conditioning effect of pulsatile and equivalent concentration matched continuous cortisol cumulative doses were achieved by the flow-through culture system using a pinch valve-controlled peristaltic pump with equal total GC dose given in both treatment schedules i.e, pulsatile and continuous treatments. However, mathematical modelling of GC release and activity in peripheral tissues has also revealed that the non-linearity in receptor-ligand kinetics, through constant GC release, produces the differential expression of GC-responsive genes in response to different patterns of glucocorticoid secretion, even when the total amount of glucocorticoid exposure is held constant (Scheff et al. 2011). This indicates that it is not the accumulative concentration of cortisol but rather the pulsatile delivery of GC hormone which renders the gene pulsing through cyclic
GR-target DNA sequence interactions. These findings together with data from the flow-through work presented within this thesis by McMaster et al (McMaster et al. 2011) define a role for rapid oscillations in GC concentration on target gene regulation and provide a possible biological impact for the physiological fluctuations in serum GC concentrations.

In conclusion, my findings define a role for rapid oscillations in GC delivery on target gene regulation, and provide a potential biological impact for the ultradian rhythm of endogenous GCs on the target cell phenotype. I describe an important frequency modulation signal encoded by the pulsatile release of GCs capable of delivering differential information to target cells when compared to the continuous cortisol exposure.

5.8-3. Summary

In this section of my thesis, the biological consequence of cortisol pulsatility has been explored in Hela cells and primary T cells. A flow-through culture system was modified for delivery of pulsatile and continuous endogenous GC cortisol to both adherent HeLa cells and to non-adherent primary T cells. In HeLa cell, pulsatile cortisol caused a significant reduction in cell survival compared to continuous exposure of the same cumulative dose, which was due to increased apoptosis. Primary T cells exposed to pulsatile as well as dose-equivalent continuous cortisol delivery produced higher induction of GILZ and FKBP5 levels with continuous cortisol compared to the same total concentration given in pulses indicating that the oscillations in GC delivery have important implications in altering the
expression of a target cell gene expression and ultimately the cellular phenotype. These differences are not due to cumulative cortisol exposure.

5.8-4. Future work

GCs are used frequently for the use of inflammatory diseases such as RA, although their use is limited due to their extensive side effects. Developing GC therapeutic interventions with fewer side effects and better clinical outcomes will benefit the treatment of many inflammatory diseases. The in vitro findings in this thesis on HeLa cells and primary T cells reveal differential modulation of GR-mediated gene expression and differential cellular response between pulsatile and continuous cortisol. It is therefore potentially relevant to explore pulsatile vs continuous cortisol delivery in vivo to ultimately extrapolate these findings into a clinical context. A translational study was proposed by our group, utilising a novel GC delivery system of a subcutaneous medtronic pump, for infusions of continuous or pulsatile GC hormone cortisol of equivalent dose into both control and RA patients. The resulting changes in the inflammatory, metabolic and bone biomarkers were to be compared following the treatment period in RA patients by either pulsatile or continuous cortisol delivery. The ability to accurately deliver GC pulses to human volunteers has not been well accepted by grant referees. The possibility of extending our preliminary in vitro findings for translational benefit has not been achievable.
5.9- Concluding remarks

The work presented in this thesis has identified a novel histone methyltransferase, Merm1, as a GC sensitivity modulator acting through histone methylation and chromatin remodelling. Furthermore, a pharmacokinetic approach to GC delivery has suggested GC induction of the GR leads to altered gene expression and cellular phenotype.

The first part of the project focused on characterisation of Merm1 as a co-modulator of the glucocorticoid receptor. This study showed that Merm1 potentiated a GR reporter gene and enhanced GR transactivation of GC target genes as well as transrepression. These effects were dependent on methyltransferase activity of Merm1. Merm1 was also found to bind to the same regulatory elements as the GR and mediate GR mediated GR expression through histone methylations. Merm1 mediated global H3K4 trimethylation and H3K79 dimethylation and a H3K9 monomethylation. At the GILZ locus, Merm1 maintained basal H3K79me2 and also regulated GR dependent H3K4me3. Gain of methylation of H3K4 followed by loss of methylation of H3K79 showed that the H3K4me3 was the driving event. Our results suggest that Merm1 is regulating GR recruitment to target sites and mediates chromatin remodelling activity previously associated with binding of activated GR to DNA. In a physiological context, our results implicate Merm1 in cytokine-induced GC resistance, with Merm1 expression significantly reduced in the presence of TNF-α and IFN-γ combined. The cytokines also inhibited GR activation of the reporter gene, a reflection of GC insensitivity, which was associated with lower Merm1 expression. Restoring Merm1 expression through an overexpressing Merm1 vector also restored GC sensitivity and GR reporter gene activation. These findings implicate Merm1,
at least in part, in cytokine-induced GC resistance, a phenomenon common to multiple chronic inflammatory diseases such as RA.

The second part of the project has focused on the potential role of pulsatile versus continuous GC delivery to target cells in bringing about a differential target cell response. Our results show that pulsatile GC treatment of HeLa cells produced significantly more apoptosis than continuous treatment indicating that pulsatile delivery of GC mediates differential pattern of gene expression in the target cells giving rise to a differential phenotype.

Although no significant difference was observed in apoptosis between pulsatile and continuous treatment in primary T cells, transcript level analysis revealed a higher induction of two GC-regulated GR target genes, GILZ and FKBP5, with continuous GC delivery causing significantly (p<0.01) greater induction than pulsatile GC of equivalent dose. These findings indicate a role for differential modulation of gene expression through frequency modulation. This might have implications for therapeutic glucocorticoid drug design and also for the administration of glucocorticoid drugs.
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