Mechanisms of Altered Glucocorticoid Sensitivity

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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ABCB</td>
<td>ATP-binding cassette sub-family B member</td>
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<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
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<td>BCL2</td>
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<td>BMI</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CBG</td>
<td>Corticosteroid-binding globulin</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>CDK</td>
<td>Cyclin-dependant kinase</td>
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<td>cDNA</td>
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<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>Cholesterol</td>
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<td>Chronic obstructive pulmonary disease</td>
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<td>Deoxyribonucleic acid</td>
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<td>DUSP</td>
<td>Dual specificity phosphatase</td>
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<td>EB</td>
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<td>ENaC</td>
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<td>Luciferase</td>
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<td>Mitogen-activated protein kinase</td>
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<td>NRF</td>
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<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside triphosphate</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OVL T</td>
<td>Organum vascularis laminas terminalis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PER</td>
<td>Period circadian protein homolog</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
</tr>
<tr>
<td>PLA</td>
<td>PhospholipaseA</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>POL</td>
<td>Polymerase</td>
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<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
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<td>PTB</td>
<td>Phosphotyrosine-binding</td>
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<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<td>PWM</td>
<td>Position-weight matrices</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RANKL</td>
<td>Stimulating nuclear factor-kB ligand</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RSAT</td>
<td>Regulatory sequence analysis tools</td>
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<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>SCCD</td>
<td>Semicircular canal duct</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SF</td>
<td>Steroidogenic factor</td>
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<tr>
<td>SGK</td>
<td>Serum/glucocorticoid induced kinase</td>
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<tr>
<td>SGRM</td>
<td>Selective GR modulator</td>
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<tr>
<td>SNO</td>
<td>S-nitrosylation</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SP</td>
<td>Specificity protein</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
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<tr>
<td>SRF</td>
<td>Serum response factor</td>
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<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
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<tr>
<td>TAD</td>
<td>Transactivation domain</td>
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<tr>
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<td>TATA box binding protein</td>
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<td>TCF</td>
<td>Ternary complex factor</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRIS</td>
<td>Hydroxymethylaminoethane</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>TTS</td>
<td>Transcription termination site</td>
</tr>
<tr>
<td>UCSC</td>
<td>University of California Santa Cruz</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low-density lipoprotein</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>Zinc sulfate</td>
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ABSTRACT

Glucocorticoids (GCs) are the most potent, and widely used anti-inflammatory agents known. Despite their wide therapeutic use over many years much remains unclear about how GCs mediate their broad spectrum of cellular effects. The reasons why some patients fail to respond to GCs are also poorly understood.

GCs mediate their cellular effects through the glucocorticoid receptor (GR) which is a ligand activated transcription factor. After binding GC, GR translocates to the nucleus to modulate transcription either by direct DNA binding (transactivation), or by tethering to other DNA bound transcription factors such as AP1 and NF-κB (transrepression).

My findings show that raised cellular cholesterol selectively attenuates GC sensitivity. GR transactivation of MT1X and FKBP5 were impaired, but not GR transrepression of IL-6 or IL-8. The impaired MT1X induction was rapidly reversed by washout into regular medium for 4 hours. The underlying mechanism was not a direct effect on GR expression or localisation, but was due to increased AP1 activity as a consequence of JNK pathway activation.

Hypoxic culture also selectively reduced cellular GC sensitivity. My results implicated this was not due to altered activity or localisation of GR, but was through altered DNA binding. To explore genome wide changes in GR binding, I developed ChIP-sequencing protocols and identified 595 highly-specific GR binding sites. Motif analysis demonstrated a template specific switch with cooperative binding with GR and KLF4 in normoxia and GR and FOXC1 in hypoxia.

Results present in this thesis identify two novel mechanisms operating within the local tissue microenvironment to modify GC sensitivity, which may be operational in inflammatory disease.
DECLARATION

I declare that no portion of the work in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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Chapter 1 - Introduction
1.1- Glucocorticoid (GC) regulation

Glucocorticoids (GCs) are steroid hormones. The name glucocorticoid (glucose + cortex + steroid) is indicative of their actions on glucose metabolism, their secretion by the adrenal cortex, and their steroidal structure (Fig. 1.1). The natural GC in humans is cortisol, and in mouse, corticosterone. In 1949, Hench et al. demonstrated the significant efficacy of cortisone in alleviating symptoms of inflammatory disease (HENCH et al. 1949). From that time on, many studies on anti-inflammatory and immunosuppressive actions of GC have been conducted. A number of synthetic GC exist including dexamethasone and prednisolone, and are widely used as potent anti-inflammatory medications in the clinic.

1.1.1 GC synthesis

GCs are synthesized in the adrenal cortex, which consists of three layers. The middle layer is known as zona fasciculata and, in humans, mainly synthesizes cortisol from cholesterol. In addition to secretion of the basal level cortisol to maintain body homeostasis, the zona fasciculata produces cortisol in response to stimulation by adrenocorticotropic hormone (ACTH) which is secreted by the anterior lobe of pituitary gland (Enyeart 2005).

Cortisol is a 21-carbon steroid hormone. The biosynthesis of cortisol requires multiple steps. A series of oxidative enzymes which are located in both mitochondria and endoplasmic reticulum are required. After transport from the cytoplasm to the inner mitochondrial membrane, the substrate cholesterol is cleaved to pregnenolone by the enzyme CYP11A1 (Fig. 1.2).

Pregnenolone is the precursor of all steroids and it is subsequently converted to progesterone by the enzyme 3βHSD. Although also synthesized in the adrenal, progesterone serves as a precursor to synthesize adrenal hormones. It can be converted to 17-α-hydroxyprogesterone which is a precursor for cortisol synthesis by the CYP17 enzyme.
Fig. 1.1 Chemical structures of GCs. Cortisol is the natural steroid in human (A). Dexamethasone is widely used synthetic glucocorticoids in the clinic (B). O oxygen, H hydrogen, F fluorine (Bledsoe et al. 2004)
Fig. 1.2 Major pathways in the biosynthesis of cortisol. Key enzymes involved in cortisol biosynthesis are shown. CYP11A1 cleaves cholesterol to pregnenolone, the first committed intermediate in the biosynthesis of cortisol. CYP11A1: Side-chain cleavage enzyme; CYP17: 17 alpha-hydroxylase; 3βHSD: 3 beta-hydroxysteroid dehydrogenase; CYP21A2: 21-hydroxylase; CYP11B1: 11 beta-hydroxylase; CYP11B2: Aldosterone synthase.
Alternatively, it is oxidized to deoxy-corticosterone by the enzyme CYP21A2 and then converted to corticosterone. Both deoxy-corticosterone and corticosterone are regarded as precursors to the synthesis of aldosterone. Actually, corticosterone can also be converted to cortisol by the action of CYP17 enzyme (Miller et al. 2011).

1.1.2 Patterns and rhythms of GC release

The secretion of cortisol is pulsatile, and follows a circadian rhythm. The highest level of cortisol occurs in the early morning (5am) in humans, and reaches its lowest level at around midnight where the concentration can be undetectable. The circulating, serum concentration of cortisol steadily decreases during the day, except for a lesser peak in the afternoon. There are changes in timing and magnitude of released cortisol, depending on different sleep and meal schedules (Linkowski et al. 1993). In addition to the circadian oscillation in serum cortisol concentration there is also an ultradian oscillation caused by pulsatile ACTH release driving pulses of cortisol release from the adrenal. These rapid pulses, approximately one every two hours, drive oscillations in GR activation which is important for appropriate regulation of GR target genes (Stavreva et al. 2009).

1.1.3 Regulation of GC release

1.1.3.1 The hypothalamic–pituitary–adrenal axis

Cortisol release is predominantly regulated by pituitary ACTH (Fig. 1.3). In response to challenge, typically a stressor to the homeostatic balance of an organism, the hypothalamus interprets descending chemical and neurological signals and releases both corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) (Papadimitriou et al. 2009).
**Fig. 1.3 Regulation of cortisol secretion.** Homeostasis of cortisol is maintained by the hypothalamic-pituitary-adrenal axis, a negative feedback mechanism. In response to change, e.g. stress, CRH binds to CRH receptors on the corticotrophs, and then stimulates ACTH secretion. ACTH stimulates cortisol secretion from the adrenal cortex. In turn cortisol inhibits the biosynthesis of ACTH precursor, proopiomelanocortin (POMC), and the secretion of CRH and vasopressin.
In turn, CRH and AVP activate the production of ACTH in the corticotroph cells of the anterior pituitary, through the synthesis and processing of proopiomelanocortin (POMC), and also the release of pre-formed ACTH stored in dense-core neurosecretory granules (Raffin-Sanson et al. 2003). In response to ACTH, synthesis of cortisol in the adrenal cortex is stimulated. Cortisol terminates its own production through a negatively feedback loop which suppresses the release of CRH and AVP in the hypothalamus, as well as inhibiting the production of ACTH from the corticotroph cells (Rhen et al. 2005).

1.1.3.2 ACTH actions on adrenal gland

ACTH plays a principle role in the HPA axis as it directly stimulates the secretion of cortisol. ACTH regulates the synthesis of cortisol through several pathways. The actions of ACTH are mediated by the MC2melanocortin receptor which is located on plasma membrane of adrenocortical cells. The MC2R, or ACTH receptor (ACTH-R), activation results in activation of adenylate cyclase, and intracellular accumulation of cAMP. This is the key regulatory biosynthesis of cortisol (Enyeart et al. 2009). The rise in cAMP increases expression of low-density lipoprotein (LDL) receptors on the plasma membrane, so promoting cholesterol uptake. In addition steroidogenic factor 1 (SF-1) is activated so promoting translocation of cholesterol into the mitochondrion, the rate-limiting step in steroidogenesis ACTH action also activates cortisol synthesis by stimulating gene expression of the cholesterol side-chain cleavage enzyme, which is a key part in the initial step of cortisol production (Miller et al. 2011).

1.1.3.3 Cytokines

As a humoral factor lying between the immune system and the HPA axis, proinflammatory cytokines play an important role in mediating HPA activation in response to inflammation.
Peripheral administration of IL-1, IL-6 and TNF-α induces augmentation of CRH in the hypothalamic-pituitary portal circulation, and CRH mRNA in the PVN of the hypothalamus. This central activation of the HPA axis, resulting in the synthesis and secretion of ACTH increases cortisol production. Many hypotheses exist to explain how cytokines regulate the HPA axis, such as via the organum vascularis laminas terminalis (OVLT) or the vagus nerve. The principal cytokine capable of regulating the HPA axis has not been identified, but is suspected to be IL-6 (Ray et al. 1997; Rivest 2001; Webster et al. 2002).

1.1.4 GC bioavailability

1.1.4.1 GC binding proteins

In the circulatory system, GC is bound by corticosteroid-binding globulin (CBG) or serum albumin, which acts as a reservoir and also limits GC bioavailability. Accordingly, more than 80% of cortisol is bound to CBG and approximately 15% bound more loosely to albumin. Therefore only 5% free cortisol is available to exert actions on target organs and tissues (Cameron et al. 2010). The interaction between cortisol and CBG is influenced by several factors such as pH and temperature (Cameron et al. 2010; Perogamvros et al. 2012). In patients with inflammatory disease CBG also serves as a substrate for neutrophil elastase. This enzyme cleaves CBG, and so releases cortisol locally at sites of inflammation (Hammond et al. 1990).

1.1.4.2 GC metabolism

11β-Hydroxysteroid dehydrogenase (11β-HSD) exists as two isoforms, termed 11β-HSD1 and 11β-HSD2, respectively. Both play an important role in the interconversion of cortisol (active) and cortisone (inactive) and therefore provide a further point for regulation of cortisol action at the cellular level.
11ß-HSD1 is a nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzyme. It is able to convert inactive cortisone into biologically active cortisol. This enzyme is mainly expressed in liver, adipose tissue and brain, which are regarded as primary metabolic tissues in vivo. Altered 11ß-HSD1 activity is widely observed among patients with obesity and insulin resistance (Tomlinson et al. 2004). Conversely, the expression of 11ß-HSD2, an NAD-dependent enzyme, is more restricted to the kidney and colon. These tissues are the main targets of mineralocorticoid action, which predominantly maintains salt and water homeostasis. The mineralocorticoid receptor (MR) has high affinity for cortisol. However, expression of 11ß-HSD2 is capable of preventing cortisol action on the MR by inactivated to cortisone (White et al. 1997).

1.2- Physiological effects of GC

GC mediates cellular effects through binding and activation of the glucocorticoid receptor (GR), a ligand activated transcription factor and member of the nuclear receptor subfamily. Once activated, GR mediates a wide range of cellular effects. GC effects can be broadly classified into two main groups, inflammation and energy metabolism. GCs are capable of regulating the homeostasis of glucose, lipid and protein due to their transcriptional activation of gene targets. As part of feedback mechanism involved in the immune system, GCs are also able to inhibit immune activity (inflammation). Consequently, GCs play key roles in the regulation of important metabolic, immunologic and cardiovascular functions, together with the homeostasis of bone and electrolytes.

1.2.1 Metabolic effects

GCs are able to mobilize amino acids from peripheral tissues as well as suppress glucose uptake in muscle and adipose tissue (Stafford et al. 2001).
GCs mainly regulate glucose metabolism and lipid metabolism in the liver (Fig. 1.4). GCs primarily promote hepatic gluconeogenesis through phosphoenolpyruvate carboxykinase (PEPCK) as well as glucose-6-phosphatase (G6Pase). The PEPCK gene has a GC response unit (GRU), two GR-binding sites as well as four accessory factor elements. All of these components are regulated by GCs (Pilkis et al. 1992). GCs and insulin, by respectively promoting and indirectly disrupting association of CBP (CREB-binding protein) and RNA polymerase II with the PEPCK promoter, reciprocally regulate PEPCK gene expression. GCs also regulate expression of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (6-PF-2-K/Fru 2, 6-P2ase) that controls fructose-2, 6-bisphosphate level, an allosteric regulator of gluconeogenic and glycolytic enzymes (Menconi et al. 2007). Due to the activation of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), the synthesis of very-low-density lipoprotein (VLDL) and triglyceride in the liver are stimulated by GCs. Meanwhile, GCs interfere with Acyl-CoA dehydrogenase (DH), to inhibit the activity of free fatty acid (FFA) β oxidation. Therefore GCs promote mediation of energy resources to the circulation (Vegiopoulos et al. 2007).

1.2.2 Anti-inflammatory actions

GC exerts multiple effects on the immune response. For example, GCs suppress the release of proinflammatory cytokines IL-1, IL-2, IL-6, IL-8, IL-11, IL-12, TNF-α, IFN-γ, and GM-CSF, which are expressed in response to inflammatory stimuli (Webster et al. 2002). GCs exert their inhibition of inflammation through several pathways (Fig. 1.5). GCs can inhibit gene expression driven by nuclear factor κB (NF-κB) and activator protein 1 (AP1) through a tethering mechanism, by binding of the activated GR to the DNA-bound target transcription factors. NF-κB and AP1 are both pro-inflammatory transcription factors, which activate expression of many inflammatory proteins. NF-κB is held in the cytoplasm, in an inactive state, by its inhibitors (IκBs).
Fig. 1.4 GC/GR-regulated metabolic pathways in the liver. A. The mechanism for GC stimulating hepatic gluconeogenesis. Transcription of gluconeogenic enzyme PEPCK gene is promoted by the binding of GR to GRU, relating to several factors/co-factors within the PEPCK gene promoter (e.g. steroid receptor co-activator 1 (SRC-1), CBP/p300). B. GC action on fat metabolism in liver (Vegiopoulos et al. 2007)
Fig. 1.5 Partial molecular architecture underlying the GC-induced antagonism of inflammation. From (Rhen et al. 2005). Inflammatory pathways are characterised by positive feedback loops (i.e., cytokines activate NF-κB, which in turn stimulates the synthesis of more cytokines) and by redundancy (i.e., cytokines also activate c-Jun–Fos). The glucocorticoid receptor inhibits these pathways at multiple points by directly blocking the transcription of inflammatory proteins by NF-κB and activator protein 1 and by inducing the expression of anti-inflammatory proteins such as IκB, annexin I, and MAPK phosphatase I. 5-LOX denotes 5-lipoxygenase, and COX-2 cyclooxygenase 2.
GCs also stimulate expression of IκB α so as to promote inactive NF-κB (Rhen et al. 2005). Furthermore, GCs are able to induce the expression of annexin I and mitogen-activated protein kinase (MAPK) phosphatase I (also termed Dual specificity phosphatase 1 or DUSP1), which are both anti-inflammatory proteins. Annexin I (also called lipocortin-1) represses cytosolic phospholipaseA 2a (cPLA 2a), one of a class of enzyme involved in the production of arachidonic acid and its downstream eicosanoids such as leukotrienes, prostacyclins and prostaglandins. GC induce and activate annexin I by inhibiting cPLA 2a as well as suppressing production of arachidonic acid and its subsequent inflammatory metabolites (Lim et al. 2007).

Additionally, GCs stimulate the expression of MAPK phosphatase I in various cell types where it dephosphorylates and inactivates members of the MAPK family, especially Jun N-terminal kinase and p38 kinase (Franklin et al. 1997). Since these proteins play a key role in the expression of inflammatory gene, GCs ultimately inhibit the expression of inflammatory factors.

1.2.3 Effects on the cardiovascular system

Endogenous GCs play a key role in the regulation of blood pressure by increasing vascular reactivity, along with maintaining renal hemodynamics. This is proved by the administration of the GC antagonist RU486. It has been observed that RU486 treatment significantly inhibits the vascular action of GCs and subsequently blunts vascular reactivity to norepinephrine and angiotensin II (Grunfeld 1990). GCs also affect the risk of developing cardiovascular diseases, due to their action on cardiovascular disease risk factors. These include promotion of type 2 diabetes mellitus, obesity, high blood pressure and dyslipidaemia. At the target tissue, GCs can exert actions through interactions with GR and/or MR, both of which are detectable in the heart and blood vessel wall (Hadoke et al. 2009; Walker 2007). Recent studies suggest that selective 11ß-HSD1 suppression may diminish the formation of atherosclerotic lesion through direct action in the arterial wall.
However, the potential mechanisms underlying this effect are not clear (Hadoke et al. 2009).

1.2.4 Actions on electrolyte homeostasis

GCs are able to mediate transmembrane ion transport through both genomic and nongenomic pathways. Considerable studies on epithelia have shown that corticosteroids are responsible for the regulation of sodium transport. The transport pathway is through the epithelial sodium channel (ENaC) which is located in the apical membrane (Champigny et al. 1994; Stockand 2002). As an effective approach to management of Meniere’s disease, GCs can also restore luminal fluid volume in the inner ear, by increasing ENaC-mediated Na\(^+\) absorption in the semicircular canal duct (SCCD) epithelia (Pondugula et al. 2004).

1.2.5 Actions on bone

GCs exhibit dramatic effects on bone, through the widely expressed GR, identified in all bone cell types (La et al. 2010). The best characterised target cell type in bone is the osteoblast. GC acts on the osteoblast to decrease synthesis of bone matrix protein and calcification (Mancini et al. 2007). Besides reduction of osteoblast mediated bone formation, GCs also stimulate osteoclast mediated bone resorption. About 50% of patients suffer from secondary osteoporosis after GCs therapy for 6 month (Mitra 2011; Weinstein 2011). The daily dosage that caused bone loss was as small as 6.0mg per day (Pearce et al. 1998). Also GCs have been shown to repress bone formation with doses as little as 5.0mg per day (Ton et al. 2005).
1.3- Glucocorticoid receptor (GR)

1.3.1 GR structure and function

GC actions are mediated by the glucocorticoid receptor (GR). The GR is a member of the nuclear hormone receptor subfamily. It is encoded by the gene NR3C1 (Niu et al. 2009). The human GR gene is located on chromosome 5q31–q32 and consists of nine exons. The GR gene comprises a number of different transcription-initiation sites. Most transcripts include exons 2 through 8 as invariable components. Exon 9 can generate alternative splice variants during transcription (Fig. 1.6). The GR comprises three major functional domains, an N-terminal domain (NTD), central DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD). The DBD and the LBD are linked by a hinge region.

1.3.1.1 GR protein subtypes

The human GR gene consists of eight protein-coding exons and multiple upstream non-coding exons, so giving rise to multiple transcripts capable of generating the same full-length protein (Presul et al. 2007; Turner et al. 2005; Zhang et al. 2004). Alternative splicing of precursor GR mRNA induces five different GR isoforms, including GRα, GR ß, GR γ, GR-A and GR-P (Gross et al. 2009b). Increased expression of GR ß, GR γ, GR-A and GR-P has been reported to contribute to GC resistance in some experimental studies (Brogan et al. 1999; de Lange et al. 2001; Haarman et al. 2004; Koga et al. 2005; Longui et al. 2000; Ray et al. 1996; Tissing et al. 2005a).

As the most abundant GR isoform, GRα is expressed in most cell types. It has a distinct 50 amino acids sequence at its carboxy end. GRα is able to bind cortisol, DNA as well as other transcription factors (McMaster et al. 2007).
**Fig. 1.6 GR structure and protein subtypes.** GR is encoded by nine exons. Variant GR protein subtypes result from alternative splicing of GR mRNA. There are five GR isoforms identified, including GR\(\alpha\), GR\(\beta\), GR\(\gamma\), GR-A and GR-P. The NTD contains AF-1 domain, has main function in transactivation. The DBD directly binds to GR elements and is also essential for dimerization and nuclear translocation. In response to ligands, the LBD undergoes conformational change inducing interaction with co-modulator proteins. The LBD and DBD are linked by a hinge region that has ill-defined function. AF, transactivation function domain; AP1, activator protein 1; DBD, DNA-binding domain; HR, hinge region; HSP, heat-shock protein; LBD, ligand-binding domain; NF-\(\kappa\)B, nuclear factor B; NTD, N-terminal domain; aa, Amino acids (Beck et al. 2009; McMaster et al. 2008b).
The GRα transcript is identified to translate into variants proteins, which are differentially expressed in cell types to exhibit diverse bioactivity on the modulation of target gene (Yudt et al. 2002).

GRβ is produced by alternative splicing of exon 9. This protein is expressed at low level in most human tissue. There are only 742 amino acids in GRβ, with 15 unique amino acids at the carboxy terminus. GRβ has a disrupted LBD, and is incapable of binding conventional agonist ligands. However, it is reported to bind the partial agonist RU486 (Lewis-Tuffin et al. 2007). GRβ may form heterodimers with GRα, which may explain the reported dominant negative activity reported (Oakley et al. 1999).

GRγ is similar to GRα but contains an additional arginine residue in the DBD, as a result of the retention from the intronic region between exons 3 and 4. GRγ consist of 778 amino acids and was originally defined as having reduced transactivation function (Rivers et al. 1999), and expression in childhood leukemia results in resistance to administered GC therapy (Beger et al. 2003; Haarman et al. 2004).

Expression of GR-A and GR-P (also known as GRδ) are increased in myeloma and leukemia. GR-A lacks exons 5 to 7 meanwhile GR-P lacks exons 8 to 9, both of which abolish ligand binding (de Lange et al. 2001; Moalli et al. 1993).

1.3.1.2 The N-terminal domain

Additional variations of GR structure and function arise from different translation initiation sites within the NTD encoded in exon 2 (Lu et al. 2004). Eight GRα isoforms reported due to internal start codons of methionines 1, 27, 86, 90, 98, 316, 331, and 336 (Fig. 1.7). These generate GRα translational isoforms designated as GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3.
Fig. 1.7 Translational isoforms of the GR. Alternative initiation of translation within the NTD generates eight GR isoforms, including GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3, due to internal start codons of methionines 1, 27, 86, 90, 98, 316, 331, and 336, respectively. Here the amino acids 187-227 shaded is the functional core region (Gross et al. 2011a).
These isoforms only differ in the length of NTD, and now appear to play a role in regulating cellular sensitivity to GCs (Gross et al. 2009a; Lu et al. 2005). In particular, the GRα-D proteins have less transcriptional activity compared with other GRα translated protein isoforms (Lu et al. 2005). In the U2-OS osteosarcoma cell line, expression of the relatively inactive GRα-D3 may contribute to GC-induced apoptosis resistance (Gross et al. 2011b).

Phosphorylation is a key factor in modulating the activity and the stability of GR, with the main sites of phosphorylation located in the NTD (Ismaili et al. 2004). The NTD of the human GR spans residues 1 to 417 and contains the transcriptional activation function-1 (AF-1) domain (Orbak 2006). AF-1 recruits diverse proteins to the GR to regulate target gene expression, including TATA-binding protein and MED14 (Chen et al. 2005; Kumar et al. 2004). The interactions are determined by a conformational change within the AF-1, which is poorly understood, in the absence of a crystal structure, but likely result in generation of novel interaction surface.

1.3.1.3 The DNA-binding domain

The DBD is located in the central amino acid sequence of the GR. Residues 418 to 487 form this domain, which bind to its DNA targets, termed GC response elements (GREs). This specific binding capability is achieved by its two highly conserved zinc fingers motifs (Luisi et al. 1991). In the first zinc finger motif, there is a “P-box” which consists of amino acids responsible for directly interacting with its recognition bases in the main groove of the DNA. The second zinc finger is able to bind bases in the minor groove of the DNA, which contributes to stabilize interaction between DBD and GRE (Hard et al. 1990) (Fig. 1.8). There is also a “D-box” which spans five residues within this domain, and accounts for the homodimerization at GREs. The GRE binds monomeric DBD with subsequent alteration in GR conformation.
Fig. 1.8 DBD binding domain of the human GR. The DNA binding domain of the GR is shown, as based on the crystal structure reported by Luisi (Luisi et al. 1991). The two alpha helices are shown, with –ve (red) and +ve (blue) charge indicated. S containing amino acids are shown in yellow. The protein and space-fill of the additional arginine in the GR γ splice variant is shown in green.
This is essential for recruitment of a second DBD domain coactivation to the GRE in the major groove, dimerizing in a head-to-head orientation (Lu et al. 2006).

1.3.1.4 The Ligand-binding domain

The LBD spans 527 to 777 at the C-terminus and adopts a complex globular tertiary structure, including eleven α helices and four short β sheet that fold as a central pocket for ligands (Fig. 1.9) (Bledsoe et al. 2002). The LBD gates ligand access, and also recruits chaperones and coactivators (Lu et al. 2006). There is a transcriptional activation function-2 (AF-2) domain towards the C-terminal end. The AF-2 consists of residues 526-556 and has significant ligand-dependent function, acting to recruit co-activator and co-repressor complexes with the motif LXXLL (Giguere et al. 1986; Glass et al. 1997; Heery et al. 1997; Horwitz et al. 1996; Kucera et al. 2002; Kumar et al. 2005; McKenna et al. 1999; Necela et al. 2003; Yamamoto et al. 1998).

1.4- Mechanisms of GR action

GCs bind with high affinity to the GR to exert their physiological actions. As a result, the bound GCs induce the dissociation of the molecular chaperones (e.g. heat shock proteins, Hsp) from the GR (Fig. 1.10A). Within the cell, the GC-GR complex represses inflammation through both direct and indirect genomic mechanisms, in addition to its non-genomic function. Chiefly, the GC-GR complex transfers into the nucleus, binding GREs located on the target gene as a homodimer, which is known as the GC-GR–GRE complex. This complex is able to recruit the proteins of either coactivator or corepressor to the regulation of target gene. Thus, this pathway will stimulate or suppress the gathering of the basal transcription machinery as well as the initiation of transcription (Fig. 1.10B).
**Fig. 1.9 The crystal structure of the GR LBD.** The ribbon model shows the 12 α helices of the GR LBD. Amino acid side chains within the ligand binding pocket are shown in yellow. The structure of dexamethasone, bound in the pocket, is shown in pink (Bledsoe et al. 2004).
Fig. 1.10 General mechanisms of GCs and GR in anti-inflammatory action. From (Rhen et al. 2005). A. Dissociation of heat shock proteins (HSP); B. DNA dependent regulation, by binding glucocorticoid responsive element (GRE) within the target gene; C. The ligand-bound GR interference with other transcription factors (e.g., NF-κB, of which the heterodimers of p65 and p50 subunits is crucial for immune function); D. Non-genomic activation.
Interactions between the GC-GR complex and other transcription factors (e.g. NF-κB) generate further modulation of other glucocorticoid-responsive genes (Fig. 1.10C). Additionally, GCs may exert their action due to the non-genomic mechanism, which is accomplished through membrane-associated receptors and second messengers (Fig 1.10D).

1.4.1 The GRα complex

In the absence of ligands, GRα primarily resides in the cytoplasm as part of a multisubunit complex, including Hsp90, Hsp70, Hsp40, immunophilins, CyP40, and P23 (Kumar et al. 2005). Hsp90 is the fundamental protein in this complex and combines with the LBD of GRα (Orbak 2006) to stabilize the optimal and high affinity structure of the ligand binding pocket within the receptor (Kumar et al. 2005). In response to GCs, the GRα complex rapidly undergoes a conformational change and subsequently dissociates from the heat shock proteins. This results in exposure of the nuclear localisation signal adjacent to the DBD. After replacing immunophilin FBK51 with FBK52, ligand-bound GR is able to produce rapid non-genomic actions through interactions with signaling pathways via cytosolic kinases (Matthews et al. 2008). Subsequently, the ligand bound GRα translocates into the nucleus through the nuclear pore (a 125MDa protein complex), driven by the dynein motor protein (Harrell et al. 2004).

1.4.2 GR effects on gene transcription

Gene transcription is the first stage leading to gene expression. This is the process of creating an RNA copy from the complementary DNA sequence. The initiation of mRNA transcription by RNA pol II plays a key role in regulating gene transcripts of protein encoding genes (Roeder 2005). In eukaryotes, RNA polymerase II (Pol II) catalyzes the synthesis of mRNA, together with a series of protein factors that known as General Transcription Factors (GTFs).
In accordance with order of discovery and chromatographic elution profiles, these transcription factors (TF) have been named with TFIIA, TFIIB, TFIID, TFIE, TFII, and TFIIH (Orphanides et al. 1996).

They comprise the pre-initiation complex (PIC) to properly position the pol II at the start site of gene transcription via either a stepwise assembly or a preassembled pol II holoenzyme pathway (Fig. 1.11). It has been shown that either pathway may be recruited in responding to specific signaling molecules and the promoter context (Thomas et al. 2006).

The GR is a steroid activated transcription factor. It has been hypothesized for the past decade that GC anti-inflammatory effects are mainly attributed to GR transrepression, whereas GC side effects are predominantly mediated by GR transactivation. However, this viewpoint is challenged by raising numerous studies, which present that GR is able to form concerted multimers independent of the DBD-dimer interface (Adams et al. 2003; Beck et al. 2009). Actually mutation hampering homodimerization of the GR, which prevents the stimulation of GRE-driven genes, still resulted in some GC side effects (Kleiman et al. 2007). Not all the side effects of GCs are mediated by GR transcriptional activation, and also, some GC side effects are attributed to GR transrepression, e.g., HPA axis suppression (Beck et al. 2009; Schacke et al. 2002). Besides GC stimulation, the expression of anti-inflammatory genes has been shown to undergo mediation via an atypical GR transactivation pathway (Clark 2007). Therefore, a selective modulation of GR remains fundamental to develop GR-mediated regulation.

**Direct GR action**

Within target gene promoters, lie binding sites for ligand-bound GRs, including GREs, half GREs (GRE1/2) and negative GREs (nGRE).
Fig. 1.11 Model for the initiation of transcription by RNA pol II. In the stepwise assembly model, TFIID first binds the TATA element of the promoter. TFIIA and TFIIIB are next to bind. TFIIF then escorts pol II to the promoter. The preinitiation complex is completed by the binding of TFIIE and TFIIH. Upon the addition of ATP, the DNA strands at the start site of transcription separate (promoter melting), a process that involves TFIIE and TFIIH. Finally, in the presence of appropriate nucleotide triphosphates (NTPs), pol II begins transcription and subsequently breaks its contacts with the promoter (promoter clearance). The holoenzyme model represents an abbreviated form of the stepwise model in which a preassembled subset of GTFs (TFIIE, TFIIF, and TFIIH in the figure) and pol II bind the promoter in a single step (Orphanides et al. 1996).
Liganded GRs are able to bind both GRE and GRE1/2 to transactivate the target gene, whereas interaction between GR and nGRE transrepresses gene production (Hayashi et al. 2004). After importation into the nucleus, the ligand-bound GR may also bind to other transcription factors.

Following GR: DBD binding to a GRE, the fold of GR AF-1 significantly increases AF-1: TBP interaction, in the meantime, the added TBP leads to induced-fit folding of AF-1 (Kumar et al. 2004). As a result, the GR directly affect the gene transcription. GRs are also reported to interrupt the serine 2 phosphorylation of pol II at its carboxy-terminal domain, accordingly inhibits the transcription of gene targets (Nissen et al. 2000). The DNA-bound GR recruits proteins involving in chromatin remodeling and stimulates gene transcription through the opening of the chromatin structure (Kleiman et al. 2007).

GC-induced transactivation is the underlying mechanism for regulating metabolism and glucose homeostasis in liver (Hudson et al. 2007); however, it’s also responsible for most of GC-induced side effects, e.g. osteoporosis (Kleiman et al. 2007).

**Tethering GR action**

GR is capable of interfering with transcription factors that are already bound to DNA, such as STAT5, AP1 and NF-κB. This mechanism is known as tethering (Orbak 2006). GCs can extensively achieve their anti-inflammatory effects owing to the GR inhibition of AP1 and NF-κB. AP1 is pro-inflammatory transcriptions factor that response to a variety of stimuli, including cytokines and phorbol ester. This factor acts as a heterodimeric protein including Fos and Jun subunits (Hess et al. 2004). The ligand bound GR can bind phosphorylated Jun and prevent it association with Fos. Subsequently, the expression of AP1 target gene (e.g. IL2) is repressed (Paliogianni et al. 1993).
Concomitant with binding to AP1, the GC-GR complex can antagonize the action of NF-κB, which is able to stimulate transcription of genes involve in inflammatory response, host defense as well as lymphocyte development (Orbak 2006).

1.4.3 Transcriptional co-modulators of GR

Many coregulatory proteins can interact with the ligand-bound GR to facilitate transcriptional activation (known as coactivators) or participate in transrepression function (known as corepressors) of target genes (Horwitz et al. 1996). The mechanism of co-modulator binding to the GR AF-2 domain is well-understood. This is through the nuclear receptor box, a LXXLL (L=leucine, X=any amino acid) motif (Heery et al. 1997). In addition AF-1 can recruit co-modulators but the mechanism remains unclear. AF-1 recruited co-modulators include AdA2, CBP and SRC-1 (Ford et al. 1997; Warnmark et al. 2000).

Co-modulators translate GR binding to alteration in gene transcripts by chromatin remodeling, as well as recruitment of RNA Pol II (Yang et al. 2012). Two main classes of chromatin remodeling co-modulators are identified. One drives post-translational modulation of histone proteins, such as SRC-1.

The other drives remodeling of chromatin by enigmatic action that does not leave a modulated protein, such as the SW1/SNF complex.

1.4.4 Non-genomic mechanisms of GC action

Besides genomic regulation which may take hours to be manifest, GCs also rapidly interfere with cellular processes within minutes, including second-messenger cascades (e.g. actin structures), intracellular Ca2+ mobilization and phosphorylation events (Falkenstein et al. 2000a; Rhen et al. 2005).
Short-term GC treatment impairs phosphorylation of proinflammatory molecules responsible for MAPK signal transduction, and phospholipase A2 (PLA-2) (Rider et al. 1996). Non-genomic GC action also stimulates rapid phosphorylation and translocation of anti-inflammatory proteins, such as annexin-1 (Solito et al. 2003).

Clinical observations, such as the observed cardiovascular protective effects of GCs, have identified the existence of additional, rapid GC effects (Hafezi-Moghadam et al. 2002). These rapid GC effects suggest that GR plays an important role in the cytosol, as well as in the nucleus (Lowenberg et al. 2007).

Non-genomic GC actions are regulated by either cytosolic or membrane-bound GRs. Short-term GC treatment can interact with specific membrane receptors, e.g. G-protein-coupled receptors (Maier et al. 2005; Tasker et al. 2006). A putative membrane-bound GR was identified in peripheral blood mononuclear cells (PBMCs) and B cells, and may have important effects on the pathogenesis of inflammatory disease (Bartholome et al. 2004). Rapid GC action can also be mediated by nonspecific physicochemical interactions with cell membranes at very high GC concentrations (Falkenstein et al. 2000b). However, the mechanism underlying rapid GC action induced immunosuppression is still to be defined.

1.5- Modulation of GR action

GR signaling is determined by a combinatorial mechanism involving ligand accessibility, GR expression, subcellular trafficking and posttranslational modifications (PTM). Posttranslational modifications of GR play a key role in its activation, although the mechanisms remain poorly defined (Beck et al. 2009). These modifications include phosphorylation, ubiquitination, SUMOylation and acetylation, which are summarized in Fig. 1.12).
Fig. 1.12 The posttranslational modification sites within GR. A. Reported human GR phosphorylation sites are demonstrated in terms of location within the receptor. B The posttranslational modification sites located in GR are shown, except for its phosphorylation sites. NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; AF-1, activation function-1 domain; aa, amino acids; P, phosphorylation site; S, SUMOylation site; U, ubiquitination site; A, acetylation site; K, lysine.
1.5.1 Phosphorylation

Phosphorylation is a covalent binding of phosphate to the GR. In human GR six serine residues show ligand regulated phosphorylation, including S113, S141, S203, S211, S226 and S404 (Beck et al. 2009). In addition, serine and threonine residues, T8, S45, S134, S234, and S267, can be phosphorylated in a cell cycle dependent manner. All of these phosphorylation sites are located in the N-terminal domain of GR, which contains the transcriptional activation function-1 (AF-1) domain (Dephoure et al. 2008).

Phosphorylation of GR is regulated by various targeted kinases, for instance mitogen activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs). The cyclin-dependant kinases, Cdk2/cyclin A kinase complexes phosphorylate S203 and S211, whilst Cdk2/cyclin E only targets S203 (Beck et al. 2009). Depending on which site is phosphorylated, the GR transcriptional effect is altered. Generally, the transactivation of GRE-regulated promoters is increased in response to S203 and S211 phosphorylation (Krsticevic et al. 1997). Without GC treatment, both S203 and S211 phosphorylation of GR mainly appear in the cytoplasm, whereas ligand- induced S211 phosphorylated GR was found in the nucleus (Beck et al. 2009).

Phosphorylation is a reversible post-translational modification and is regulated by both phosphorylating kinases and dephosphorylating phosphatases. This modification induces a conformational change in target proteins including the GR. Consequently, GR function is activated or inhibited (Ciesla et al. 2011). Phosphorylation has been reported to affect GR subcellular localisation and interactions with coregulators, and also to alter DNA binding capability (Beck et al. 2009). GR phosphorylation at S211 is proposed to drive an alpha helical conformation, which generates a coregulator-binding surface, and therefore enhances GR transactivation on particular target genes. These findings suggest a potential mechanism whereby phosphorylation can regulate specific target gene regulation, a so-called “phosphorylation code” (Chen et al. 2008).
For GR dephosphorylation, S211 phosphatase has not been identified till now. Meanwhile protein phosphatase 5 was highlighted in the regulation of GR dephosphorylation at both S203 and S226 (Wang et al. 2007).

S211 phosphorylation is regarded as a hallmark for the GR transactivation. The peak of GR transactivation was found when the relative phosphorylation of S211 exceeds that of S226 (Chen et al. 2008). In lymphoid cells, the p38 MAPK may be partly responsible for S211 phosphorylation (Miller et al. 2005). The GR S404 phosphorylation, which is regulated by glycogen synthase kinase 3β (GSK3β), may be important in GR nuclear export (Beck et al. 2009; Galliher-Beckley et al. 2008). Since phospho- S404 expedites the nucleocytoplasmic transport of GR, this is capable of down-regulating either the transactivation of GRE-containing gene promoters, or transrepression of promoters regulated by NF-κB (Galliher-Beckley et al. 2008). Additionally the GR S226 phosphorylation, which is mediated by c-Jun N-terminal kinases (JNKs), acts to inhibit GR activation function. Accordingly, it was observed that UV attenuation of GR function was lost along with accelerated nuclear export in cells with a GR S226A mutant (Itoh et al. 2002).

1.5.2 Acetylation

Acetylation is characterised by addition of an acetyl group at the side chain of a lysine residue. This process is catalysed by enzymes with histone acetyltransferase (HAT) activity. The acetylation of GR cofactor (e.g. Hsp90) is crucial for the regulation of GR activity, suggesting the indirect acetylation effect of GR (Murphy et al. 2005). The GR is also a target for acetylation, at a motif identified at aa 492-495 within the human GR hinge region showing homology to the consensus K-X-K-K/R-X-K-K (Fig 1.9 B) (Ito et al. 2006).
GR acetylation is ligand-dependant and regulates GR function. Recent studies on the acetylation of GR have shown different outcomes of GR function, dependent on target gene and cell type. Ito et al. showed human GR acetylation at K494 and K495 and revealed the key role of histone deacetylase (HDAC) 2 induced deacetylation for the transcriptional repression of NF-κB p65 and its downstream target genes (Ito et al. 2006). On the contrary, Nader et al. reported that the acetylation of GR by the circadian rhythm transcription factor CLOCK, which has intrinsic acetyltransferase activity, enhanced the GR repression of NF-κB (Nader et al. 2009). These findings suggest the complex effect of acetylation on GR, and further research also requires distinguishing acetylation of GR from its cofactors.

1.5.3 SUMOylation

SUMOylation is characterised by the covalent addition of a small ubiquitin-related modifier (SUMO) motif to a lysine site of protein. This process is catalyzed by the concerted effects of a series of enzymes, including E1-activating enzyme, E2 conjugation enzyme (Ubc 9) and E3 ligase (Kaul et al. 2002). There are three SUMO-target sites within GR, K277 and K293 in the N-terminal domain and K703 in the ligand-binding domain (Fig. 1.11B). The N-terminal domain SUMOylation inhibits the GR-mediated activation. It is also reported that GR undergoes SUMOylation in a ligand-independent manner. However, the intact DNA-binding domain of GR is still essential to the SUMO-dependent transcriptional inhibition (Beck et al. 2009).

SUMOylation of GR results in various functional outcomes of its transcriptional activity. Le et al. reported that overexpression of SUMO-1 diminishes the stability of GR protein (Le et al. 2002). It is also found that mutation of the potential SUMOylation sites enhances GR transcriptional activity in a gene-selective manner (Tian et al. 2002). Furthermore, overexpression of SUMO-2 reduces expression of endogenous gene which regulated by GR.
GR SUMOylation is stimulated by S226 phosphorylation, suggesting phosphorylation perhaps directs GR SUMOylation (Davies et al. 2008), and further suggest that JNK induced GC resistance is mediated by a cascade of GR modifications.

1.5.4 Ubiquitination

The ligand-bound GR is a target for ubiquitin-dependent degradation by the proteasome. This process is characterised by the poly covalent attachment of 76 aa protein ubiquitin at lysine residue K419 (Fig. 1.9A), which located in a PEST motif (Proline [P], Glutamine [E], Serine [S] and Threonine [T]) (Rogers et al. 1986). The ubiquitination consists of three steps, including activation of ubiquitin, transfer and ligation, and subsequently each catalyzed by E1, E2 and E3 enzymes (Wallace et al. 2001). Since GR becomes hyperphosphorylated in response to GC binding, phosphoregulation of GR plays a key role in modulating this ubiquitin-dependent proteasomal degradation. It appears that GR phosphorylation is coupled to ubiquitination, as phsho mutant GR molecules are not targeted (Webster et al. 1997). Ubiquitination drives GR degradation, but also appears to be important in regulating GR transcriptional function. The PEST degradation motif within phosphorylatable GR S404 potentially decreases GR-mediated transactivation and enhances intranuclear GR mobility (Garside et al. 2006).

1.6- Glucocorticoid therapy

1.6.1 Clinical outcomes of standard ligands

1.6.1.1 Positive effects

In low doses, about 6-12 mg/m²/day, GCs efficiently provide physiologic replacement in patients with adrenal insufficiency. In higher doses, GC are widely used to repress a variety of allergic, inflammatory and autoimmune disorders in clinic, including allergies, eye
diseases, infections, inflammation of bones and joints, respiratory disorders, skin diseases, transplant, vascular lesions, and recognized as predominantly anti-inflammatory and immunosuppressant agents (Lu et al. 2006).

For instance, inhaled glucocorticoid (e.g. beclomethasone dipropionate, budesonide), are the most effective anti-inflammatory medications, and regarded as first-line treatment for adult and children asthmatics. Inhaled GCs effectively reduce asthma symptoms and release airway hyperresponsiveness. In addition, it has been observed that inhaled GCs are capable of improving lung function as well as decreasing the frequency and severity of exacerbations in patients with asthma (Bateman et al. 2008).

1.6.1.2 Limitations

High-dose or long-term GC therapy may cause serious systemic side effects, which have been summarized in Table 1-1. Potential mechanisms related to these side effects are varied and complex. For example, as discussed in early section, osteoporosis is a prevalent complication in patients with long-term GC therapy. GCs are capable of stimulating nuclear factor-kB ligand (RANKL), the receptor activator which activates osteoclastogenesis and mature osteoclasts, as well as repressing osteoprotegerin (OPG) which inhibits the osteoclast maturation and activation. In addition, it is shown that GC may directly influence osteoclast cytoskeletal rearrangements, leading to the repression of the whole bone-remodeling process (Mancini et al. 2007).

Recent studies also reported adverse effects of GC therapy may be partly reduced by locally-targeting therapy (Hadoke et al. 2009), with the lowest dosage compatible with therapeutic efficacy, and considering intermittent and localized routes of administration (Buttgereit et al. 2005). Therefore, it logically follows that determining or predicting disease, and patient sensitivity to GC would be of use in improving the therapeutic application of GC.
Table 1-1 Tissue-specific side effects of high-dose or prolonged GC therapy

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>Adrenal atrophy, Cushing’s syndrome</td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>Dyslipidemia, hypertension, thrombosis, vasculitis</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Changes in behavior, cognition, memory, and mood (i.e. GC-induced psychoses), cerebral atrophy</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>Gastrointestinal bleeding, pancreatitis, peptic ulcer</td>
</tr>
<tr>
<td>Immune system</td>
<td>Broad immunosuppression, activation of latent viruses</td>
</tr>
<tr>
<td>Integument</td>
<td>Atrophy, delayed wound healing, erythema, hypertrichosis, perioral dermatitis, petechiae, GC-induced acne, striae rubrae distensae, telangiectasia</td>
</tr>
<tr>
<td>Musculoskeletal system</td>
<td>Bone necrosis, muscle atrophy, osteoporosis, retardation of longitudinal bone growth</td>
</tr>
<tr>
<td>Eyes</td>
<td>Cataracts, glaucoma</td>
</tr>
<tr>
<td>Kidney</td>
<td>Increased sodium retention and potassium excretion</td>
</tr>
<tr>
<td>Reproductive system</td>
<td>Delayed puberty, fetal growth retardation, hypogonadism</td>
</tr>
</tbody>
</table>

Adapted from (Rhen et al. 2005)
1.6.2 Novel ligands

A number of studies have sought to enhance GC sensitivity, particularly in the context of inflammatory disease. Co-treatment with inhibitors of the MAPKs, especially p38, may prove useful in GC resistant asthma (Irusen et al. 2002), and there is some evidence that theophylline, by inhibition of PI3Kδ, is able to reverse GC resistance in COPD patients (Cosio et al. 2004; Marwick et al. 2009). In addition, a number of approaches to target P-glycoprotein and MIF are now being developed (Hoi et al. 2007; Nobili et al. 2006). In this context selective GR modulators (SGRMs) are an attractive pharmacological approach to treat inflammatory disease. These agents would ideally preserve useful anti-inflammatory activity but lack the side-effect profile of conventional GCs. Indeed, a number of compounds have been proposed and tested in-vivo, but so far none completely fulfil this criterion (McMaster et al. 2008a). However, if such agents could be identified their therapeutic use would still be improved by other approaches to target GC sensitivity.

1.7- Determinants of glucocorticoid sensitivity

People exhibit different responses to GC treatment regimens. For instance, some patients do not respond well to typically used, therapeutic doses of GC. A number of subjects have an initial response but develop a secondary resistance after an interval. As discussed before, GC treatment may lead to serious side effects for some patients, however, there are some individuals receiving high dose, high potency GC therapy to fulfill clinical effect without suffer from any adverse effect. An estimate of the size of this effect and its implications is that 10% of patients with rheumatoid arthritis show an inadequate response to typically used doses of GC.
1.7.1 Genetic variation in GR structure and function

1.7.1.1 Familial GC resistance syndrome

Inactivating mutations of GR gene are the primary cause of familial GC resistance. This syndrome is characterised by hypercortisolism without features of Cushing’s syndrome and was firstly explained as a GR mediated disorder in 1976 (Orbak 2006). High adrenocorticotrophin levels stimulate an over-secretion of non-corticosteroid adrenal steroids, such as aldosterone and androgens, such as DHEA and androstendione. Therefore clinical manifestations of this syndrome are hypertension, hypokalaemia and/or symptoms of androgen excess which occur as menstrual abnormalities and hirsutism in females (Barnes 2010). Familial GC resistance is very rare, in all cases due to mutations in the GRα gene, most of which affect the function of either LBD or DBD. Therefore GR function of nuclear translocation, ligand affinity and DNA binding are decreased (Charmandari et al. 2008). Patients have presented with infertility and hypertension (Karl et al. 1996; Kino et al. 2001).

All the reported locations of GR gene mutations, their manifestations and molecular defects are summarized and shown in Table 1-2. Of note, these patients are obviously different from those suffer from glucocorticoid-resistant inflammatory disease where GR appears to have no structure abnormality (Lane et al. 1994).

1.7.1.2 GR polymorphisms

Several single nucleotide polymorphisms (SNPs) within the GR gene locus are associated with altered GC sensitivity (Fig. 1.13). The ER22/EK23 mutation within GR exon 2 induces an arginine to lysine alteration of the NTD, which is associated with relative resistance to GC therapy (Russcher et al. 2005a). There is an elevated ratio of GRα-A to GRα-B with this GR polymorphism.
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
<th>Molecular mechanisms</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>D641V</td>
<td>Homozygous</td>
<td>Ligand affinity↓(×3); Transactivation↓; Delayed nuclear translocation</td>
<td>Hypertension; Hypokalemic alkalosis</td>
</tr>
<tr>
<td>4-bp deletion at exon/intro 6</td>
<td>Heterozygous</td>
<td>GRα number↓; Inactivation of the affected allele</td>
<td>Hirsutism; Male-pattern hair loss; Menstrual irregularities</td>
</tr>
<tr>
<td>V729I</td>
<td>Homozygous</td>
<td>Ligand affinity↓(×2); Transactivation↓; Delayed nuclear translocation</td>
<td>Precocious puberty; Hyperandrogenism</td>
</tr>
<tr>
<td>I559N</td>
<td>Heterozygous</td>
<td>GR number↓; Transactivation↓; Delayed nuclear translocation; Dominant negative activity</td>
<td>Hypertension; Hypokalemia; Infertility</td>
</tr>
<tr>
<td>R477H</td>
<td>Heterozygous</td>
<td>Transactivation↓; Complete lack of DNA binding; Delayed nuclear translocation</td>
<td>Hirsutism; Fatigue; Hypertension</td>
</tr>
<tr>
<td>G679S</td>
<td>Heterozygous</td>
<td>Ligand affinity↓(×2); Transactivation↓; Delayed nuclear translocation</td>
<td>Hirsutism; Fatigue; Hypertension</td>
</tr>
<tr>
<td>I747M</td>
<td>Heterozygous</td>
<td>Ligand affinity↓(×2); Transactivation↓; Dominant negative activity</td>
<td>Cystic acne; Hirsutism; Oligoamenorrhea</td>
</tr>
<tr>
<td>V571A</td>
<td>Homozygous</td>
<td>Ligand affinity↓(×6); Transactivation↓; Delayed nuclear translocation</td>
<td>Ambiguous genitalia; Hypertension; Hypokalemia; Hyperandrogenism</td>
</tr>
<tr>
<td>L773P</td>
<td>Heterozygous</td>
<td>Ligand affinity↓(×2.6); Transactivation↓; Delayed nuclear translocation</td>
<td>Anxiety; Acne; Hirsutism; Fatigue; Hypertension</td>
</tr>
<tr>
<td>F737L</td>
<td>Heterozygous</td>
<td>Ligand affinity↓(×1.5); Transactivation↓; Delayed nuclear translocation</td>
<td>Hypertension; Hypokalemia</td>
</tr>
<tr>
<td>2-bp deletion at exon 9</td>
<td>Homozygous</td>
<td>Complete lack of ligand binding; Null GR mutation</td>
<td>No endocrine abnormality</td>
</tr>
<tr>
<td>Single base deletion at exon 6</td>
<td>Heterozygous</td>
<td>Transactivation↓; Dominant negative activity</td>
<td>Fatigue; Hirsutism</td>
</tr>
</tbody>
</table>

Modified from (Charmandari et al. 2008)
**Fig. 1.13 Genetic polymorphisms of the GR.** Several GR polymorphisms known are related to GC sensitivity. GR ER22/23EK mutation located in exon 2 and results in GR resistance. GR N363S mutation located in exon 2. GR BclI polymorphism was observed in intron 2. Both of these two induce GC hypersensitivity.
Since GRα-A is less transcriptionally active than GRα-B this may explain the observed effect on GC insensitivity (Russcher et al. 2005b). Further downstream in exon 2 is a polymorphism which results in a switch of asparagine to serine substitution, N363S. This is associated with surrogate measures of increased GC sensitivity, including increased probability of low bone density and metabolic disorders, together with increased risk of cardiovascular disease (van Rossum et al. 2004). The N to S substitution apparently alters interaction between GR and transcriptional coregulators by enhancing phosphorylation in response to GC (Jewell et al. 2007). Within intron 2 there is a high-frequency polymorphism which gives rise to a BclI restriction fragment length polymorphism (RFLP). This polymorphism is also associated with increased sensitivity to GCs in some individuals. However, neither the BclI RFLP, nor N363S was associated with therapeutic advantage in acute lymphoblastic leukemia (Tissing et al. 2005c).

1.7.2 Environmental factors and extracellular signals

In chronic inflammatory diseases, many patients develop resistance to GC treatment. Clinically, this condition is termed as GC resistant inflammatory disease (Barnes et al. 2009). There are several mechanisms proposed for such acquired GC resistance. These are thought to be the result of significant changes in the cellular microenvironment, which occur over time as the disease progresses. These include alterations in GR translocation, and P-glycoprotein regulation of cellular ligand accumulation (De et al. 2011) (Fig. 1.14).

1.7.2.1 P-glycoprotein activation

As one of the ATP-binding cassette (ABC) transporters, the drug efflux pump P-glycoprotein 170 is responsible for transporting structurally and functionally unrelated drugs out of cells (De et al. 2011).
Fig. 1.14 Mechanisms of GC resistance induced by cytokines, oxidative stress and P-glycoprotein activation. CBP: CREB-binding protein; FKBP5: FK506-binding protein 5; GC: Glucocorticoid; GR: Glucocorticoid receptor; GRE: Glucocorticoid responsive elements; HAT: Histone acetyltransferase; HDAC2: Histone deacetylase 2; Hsp: Heat-shock protein; Hop: Hsp70/Hsp90 organization protein; KEAP1: Kelch-like ECH-associated protein 1; NF-κB: Nuclear factor κB; NO: Nitric oxide; Nrf2: Nuclear factor erythroid 2-related factor 2; P-gp: P-glycoprotein; SNO: S-nitrosylation.
This protein is encoded by the multidrug resistance gene MDR1 (ABCB1) (Callen et al. 1987). Recent studies on blood lymphocytes reported the high expression level of MDR1 in GC resistant inflammatory diseases (Farrell et al. 2000; Tsujimura et al. 2008). Meanwhile, it has been shown that certain single nucleotide polymorphisms within MDR1 are associated with GC resistance (Potocnik et al. 2004). However, to date this is only reported in GC resistant inflammatory bowel disease and rheumatoid arthritis. Therefore future research in other diseases, e.g. GC resistant pulmonary inflammation is needed (Barnes 2010).

1.7.2.2 Cytokines

In GC resistant asthma, there is augmented production of IL-2 and IL-4 in the airways (Leung et al. 1995). The combination of IL-2 and IL-4 diminishes GR translocation and binding affinity in target lymphocytes. In monocytes, IL-13 alone was also found to reduce GR activity (Irusen et al. 2002; Matthews et al. 2004; Sher et al. 1994). The impaired GR activity was rescued by inhibiting p38 MAPK activity, suggesting p38 MAPK mediated GR phosphorylation is a mechanism explaining such cytokine induced resistance (Irusen et al. 2002). Investigations in alveolar macrophages revealed a greater degree of p38 MAPK activation in GC resistant asthma, further supporting a role for p38 MAPK in regulating GR function, and GC sensitivity (Bhavsar et al. 2008).

In GC resistant ulcerative colitis, there are increased mucosal levels of tumor necrosis factor (TNF)-α, IL-6 and IL-8, which together downregulate GR expression. This decrease is proposed to explain the lack of clinical response (Ishiguro 1999). Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine and is genetically associated with several inflammatory diseases (Flaster et al. 2007). This cytokine is proposed to cause GC resistance by blocking GC induction of the DUSP1 phosphatase which limits action of the MAPK family (Roger et al. 2005).
Increased MIF expression has been reported in numerous GC resistant inflammatory diseases, including asthma, ulcerative colitis, rheumatoid arthritis and systemic lupus erythematosus (Barnes 2010). Therefore, MIF expression is proposed as a mechanism for acquired GC resistance in inflammation, although the findings are not always replicated (Kudrin et al. 2006).

Finally, animal experiments have identified inhibition of GR activity resulting from the actions of IL-1 (De et al. 2011).

1.7.2.3 Oxidative stress

Oxidative stress is proposed to limit GC response, possibly by modification of HDAC2 (Barnes et al. 2004). Oxidative stress significantly attenuates HDAC2 activity and expression, thereby potentially limiting recruitment by GR to sites of action in the genome. In GC refractory asthma, there is a markedly reduced expression of HDAC2 both in peripheral blood mononuclear cells (PBMC) or alveolar macrophages (Hew et al. 2006). A reduction in HDAC2 expression was also reported in the airways of smoking asthma patients, a group difficult to treat due to lack of GC efficacy (Adcock et al. 2005).

The role of oxidative stress is well studied in patients with chronic obstructive pulmonary disease (COPD). Among patients with COPD, there is a dramatically decreased HDAC2 activity in alveolar macrophages, airways and peripheral lung (Ito et al. 2005). Furthermore, it has been shown in vitro that the GC resistance of bronchoalveolar macrophages can be reversed by overexpression of HDAC2 (Ito et al. 2006). Recent studies suggest the HDAC2 reduction in COPD is due to oxidative stress activation of peroxynitrite and phosphoinositide-3-kinase (PI3K)-δ. Since both of these factors are crucial for inactivation and degradation of HDAC2, as a result, HDAC2 activity is reduced under oxidative stress (Ito et al. 2004; To et al. 2010).
1.7.2.4 Circulating factors

Acute phase proteins (APP) are a class of serum protein whose concentration is primarily varied in inflammatory condition. Thus it has been speculated that these proteins may affect GC responsiveness at cellular levels. Subsequently, many researchers have studied the association between circulating factors and GC sensitivity. For example, it has been observed in patients with COPD that the combination of inhaled corticosteroids and long-acting beta (2)-adrenergic agonist significantly decrease serum surfactant protein D levels (Sin et al. 2008). For paediatric patients, circulating adiponectin has been proposed to be acceptable possible endogenous biomarker of acute glucocorticoid-related side effects (Vihinen et al. 2009). In animal experiments, it is suggested that serum amyloid A protein may also affect GC action and also that a feedback loop involving GC regulation of leptin may be operational in inflammation (Glojnaric et al. 2007; Gualillo et al. 2000).

Most recently it was reported that serum contains an activity, as yet undefined, capable of attenuating GC sensitivity in target cells (Perogamvros et al. 2011; Rider et al. 2011). This is of interest as it may be regulated in disease, and plays a role in explaining the “missing” GC explanation.

1.7.2.5 Hypoxia

Recent studies have reported conflicting results for the cross-talk between GC action and hypoxia. GCs are widely used in treatment of many inflammatory diseases which themselves result in tissue hypoxia. Kodama et al showed that the expression of a GRE containing reporter gene was not activated in response to GC treatment under low oxygen tension (Kodama et al. 2003). Similar impaired GR transactivation was observed in hepatic cells, cultured in a hypoxic environment of 3% O2 (Wagner et al. 2008).
On the contrary, there are several studies reporting increased GR activity in hypoxia (Kitagawa et al. 2007; Leonard et al. 2005). These conflicting results may be due to specific cell lines and techniques in different experiments.

Hypoxia is observed at many sites of inflammation due to increasing numbers of activated inflammatory cells and subsequently increased oxygen demand (Bodamyali et al. 1998; Karhausen et al. 2005). Hypoxia-inducible factor 1 (HIF-1) is an important regulator of the cellular response to low oxygen. HIF-1α deletion induced a dramatically impaired the function of myeloid cells, inhibiting motility, invasiveness and bacterial killing (Cramer et al. 2003). Recent studies revealed HIF-1α is also able to enhance inflammation by hyperphosphorylation of IκB results in NF-κB activation (Scortegagna et al. 2008). Furthermore, hypoxia is capable of stimulating expression of cytokines, including IL-8. Jeong et al reported increased expression of the pro-inflammatory cytokines TNF, IL-6 and IL-8 in mast cells after culture with medium containing the hypoxia mimetic deferroxamine (DFX) (Jeong et al. 2003).

As a functional suppressor of p53, overexpressed MIF may further increase HIF-1α expression under hypoxia in a p53-dependent manner (Oda et al. 2008). Until now, the underlying mechanism explaining the cross-talk between hypoxia and cytokines remains to be determined.

1.7.2.6 Transcription factor cross-talk

As the most abundant AP1 complex in activated cells, the c-Fos:c-Jun heterodimer has a key role in the transcriptional regulation of asthma-relevant cytokines (de Groot et al. 1997; Halazonetis et al. 1988; Wang et al. 1994). The transcriptional activation of c-Fos is a marker of AP1 activity (Lane et al. 1998a). In GC resistant asthma there was increased c-Fos production, with resulting loss of GC suppression of JNK activity (Lane et al. 1998b).
The c-Jun component of AP1 is capable of binding to GR, and thereby inhibiting its actions, and may explain why cell sensitivity to GC is attenuated in cells with activated AP-1 (Adcock et al. 1995; Loke et al. 2006).

This interaction between GR and AP1 was originally reported to result in mutual antagonism (Diamond et al. 1990; Loke et al. 2006; Yamauchi et al. 1996; Yang-Yen et al. 1990) (Fig. 1.15). However, surprisingly AP1 has emerged a key partner protein for GR regulated transcription of endogenous target genes. AP1 regulates basal chromatin structure, and accessibility, and so enhances GR binding to specific sites in the genome (Biddie et al. 2011b). This suggests that the physiological interactions between GR and AP1 are both more extensive than previously realised, but also more complex, with evidence for enhancement, and diminution of GR activity (Biddie et al. 2011a).

In addition, proteins capable of regulating the cytoskeleton (e.g. coflin-1) are reported to decrease the transcriptional activation of GR (Barnes 2010). Indeed, gene array studies revealed that overexpression of coflin-1 was associated with GC resistance in T cells, although the precise mechanism, and the physiological significance remains unclear (Vasavda et al. 2006).

1.7.3 Chaperones

The GR heterocomplex is crucial to maintain the GR in a ligand-binding capable conformation before activation by binding to steroid. In addition to the GR, this heterocomplex consists of a number of chaperone proteins, including Hsp90, Hsp70, Hsp40, Hop, immunophilins, CyP40 and P23 (Kumar et al. 2005; Pratt et al. 2006). In an ATP-dependent process, Hsp90 and Hsp70 act as essential chaperones interacting with GR in a macromolecular complex, whereas Hsp40, Hop and P23 are nonessential co-chaperones.
**Fig. 1.15** Cross-talk between GR and transcription factors. Increased AP1 and NF-κB are capable of directly interacting with GR, subsequently induce GC resistance. AP1: Activator protein 1; GRE: Glucocorticoid responsive elements; NF-κB: Nuclear factor-kappa B.
Accordingly the LBD of GR and Hsp 90 are assembled, and the ligand binding cleft is opened to GC (Pratt et al. 2006). A large number of studies highlight the role of the GR heterocomplex, as a key regulator in GR transformation, stabilization, translocation, and DNA binding capability, as well as governing GR turnover in the cytoplasm (Chen et al. 1998; Dittmar et al. 1996; Dittmar et al. 1998; Hawkins et al. 2009; Johnson et al. 1998; Pratt et al. 2006).

Abnormal chaperone and co-chaperone function may alter how the mature GR heterocomplex works, which then may alter GR-mediated transcription. Therefore these changes could potentially contribute to decreased GC sensitivity, as discussed below (Gross et al. 2009b). Studies on certain New World primates have identified GC resistance due to increased expression levels of the GR chaperone complex protein FKBP51 together with decreased levels of FKB52 (Scammell et al. 2001). In vitro, high level of FKBP51 has been shown to inhibit transcriptional activity of GR and this could be overcome by co-expression of FKB52 (Wochnik et al. 2005).

The role of other chaperone proteins, Hsp90 and Hsp70, in GC resistance is controversial. GC resistance related to mutated Hsp90 protein and low expression levels of Hsp70 protein (Kojika et al. 1996). Subsequently, other research groups also detected abnormal expression levels of Hsp90 protein in certain GC-resistant cell lines (Gross et al. 2009b). Conversely, Tissing et al showed there is no significant correlation between expression levels of Hsp90/ Hsp70 protein and GC sensitivity when compared with control subjects (Tissing et al. 2005b).

1.7.4 Glucocorticoid resistance in cancer

GCs are widely used in the initial induction phase of anti-leukaemia therapy, due to their profound pro-apoptotic effects on T lymphoblasts.
However, chronic treatment frequently provokes development of resistant clones. In-vitro models suggest that the strong selection pressure causes either GR mutations, or deletions. In contrast, in-vivo resistance to GC therapy is usually accompanied by down-stream changes in the expression and/or function of apoptosis regulating proteins of the BCL2 family. These proteins are the physiological target of GCs in lymphoblasts, and regulate the pro/anti apoptosis status of host cells. This subject has been extensively reviewed recently in (Schlossmacher et al. 2011).

In non-lymphoid malignancy low expression of GR in human small cell lung carcinoma cell lines suggests a possible role in regulating the malignant phenotype (Ray et al. 1996). Accordingly, studies were performed to show that both in-vitro, and in-vivo restoration of GR expression in these cells powerfully induced apoptosis (Sommer et al. 2007; Sommer et al. 2010). This suggests a broader role for GR and GC signalling in regulating cell fate decisions, but in a cell type specific manner.

1.8- Hypotheses and Aims

A number of environmental factors can affect GC sensitivity. These factors can influence GR signaling directly through an alteration in GR function itself, or indirectly by altering the accessibility of GR target sites in the genome. Changes to the cellular environment that occur during periods of chronic inflammation limit the success of current therapies. Understanding why and how GCs are less effective in chronic inflammation is essential to designing more effective drugs.

**Project 1: Serum factors and GC sensitivity**

It is reported that factors present in normal human serum dose dependently affect GC transactivation (Perogamvros et al. 2011).
However, the identity of this factor(s) and the mechanism by which it modulates GR function is unknown. I predict that serum factors influence GR function through the activation of mitogen regulated protein kinases.

**Aims of the project:**

- To characterise the inhibitory effect of serum on GC action
- To identify the inhibitory factors present in serum
- To define the mechanism underlying serum induced GC resistance

**Project 2: Hypoxia and GC sensitivity**

Hypoxia is an important environmental change present at sites of inflammation (Eltzschig *et al.* 2011). Hypoxia is reported to influence GC sensitivity, but the mechanism by which this occurs remains controversial. I predict that changes in oxygen concentration affect GR interaction with unidentified cellular proteins and that these interactions determine GR access to target DNA binding sites.

**Aims of the project:**

- To characterise alterations of GR function in hypoxia
- To determine the genome-wide characteristics of DNA binding by GR
- To identify the GR co-modulators active under hypoxia
Chapter 2 - Materials and Methods
2.1- Cell culture

HeLa cells, a human cervical carcinoma cell line, derived from human cervix epitheloid carcinoma, were obtained from ECACC (No 93021013). HeLa cells became cancerous because of infection with human papilloma virus 18. Compared with normal cells, cancer cells produce telomerase, which is an enzyme that elongates the telomeres after chromosomes have been copied. Therefore HeLa cells are able to multiply continuously. HeLa cells require the medium containing inositol and cholesterol to grow optimally. They are characterised by GR expression and respond well to steroids in culture. This cell line also appears to be capable of tolerating hypoxia and/or acidic pH. When compared with other adherent cell lines, e.g. A549 cells, they have higher transfection efficiency.

2.1.1 Retrieving cells from Liquid Nitrogen

Cells were removed from liquid nitrogen storage, washed with 5mls of medium (to remove the residual DMSO in freezing medium), and resuspended in 5ml full growth medium and transferred to a T25 flask (Corning). The following day, culture medium was changed.

2.1.2 Routine cell maintenance

All cell lines were maintained in an incubator at 37 C with 5% CO2 and humid atmosphere. HeLa cells were cultured in 12mls of Dulbecos Modified Eagles Medium, containing stable low glutamine (1g/l), supplemented with 10% Fetal Bovine Serum (Invitrogen, Paisley, UK) in T75 vented flasks (Corning).

2.1.3 Cell passage

2.1.3.1 Adherent cells

Once cells had grown to 80% confluent, culture medium was removed and the cell sheet
was covered with 1ml of 10 × trypsin/EDTA (PAA, The cell culture company). After incubation at 37 C for 5mins, the trypsin was deactivated using DMEM containing 10% FBS. Cells were pelleted by centrifugation at 1000g for 5 mins and then resuspended in 10ml fresh growth medium. Subsequently, the cell suspension was split 1:5 into fresh T75 flasks, with liquid volume adjusted to 12mls.

2.1.3.2 Suspension cells
Suspension cells were split by removing 90% of the cell suspension when reached a growth density of 1×10⁶ cells/ml. Followed by replacing with equal volume of fresh growth medium to maintain cell density between 1-2 × 10⁵ cells/ml.

2.1.4 Cell counting
Following trypsinisation, 10 µl of the cell suspension was added to each side of a Neubauer Haemocytometer. Cells in each of the four corner squares were counted using 10 × objective of a cell culture inverted light microscope. The number of cells per ml was calculated by following:

Cells/ml = Mean Cell count ×10⁴

TC20 Automated Cell Counter (BIO-RAD) was also used to count mammalian cells, by loading 10µl of samples (with 5µl trypan blue staining for suspension cells or primary cells) onto a slide. Following insert the slide into the cell counter, the number of cells was automatically counted, and present a total cell count or total and live cell counts (with trypan blue) on the screen in 30 seconds.

2.1.5 Cryogenic freezing
Cell lines were routinely frozen and stored in liquid nitrogen at -196 C. The cells with low passage number were frozen down in medium containing 50% FBS and 10% dimethyl
sulfoxide (DMSO), in order to reduce the size of potential ice crystals under cryopreservation. For example, when adherent cells reached 80% confluent, they were trypsinised and pelleted, then resuspended in 3ml freezing medium. The cell suspension was aliquoted with 3 × 1ml into cryotubes (Nunc). Following held in the nitrogen phase for 24 hours, these cryotubes were transferred into liquid nitrogen tanks for long-term storage.

2.2- Special culture conditions

2.2.1 Hypoxic cell culture

HeLa cells were prepared in a hypoxic chamber with 1% oxygen for experiments under hypoxic condition. DMEM medium was transferred into the hypoxic chamber for 24 hours before use. HeLa cells were cultured in the hypoxic chamber after replacement with the hypoxic medium. The next day cells were treated with 100nM Dex or control for up to 4 hours prior to different experiments.

2.2.2 Steroid free cell culture

To prepare for ChIP, HeLa cells were cultured with DMEM medium in T225 flasks (Corning). In order to avoid residual steroids interference during experiments, cells were washed once with serum free medium, and then replaced with DMEM containing 10% charcoal stripped serum (Gibco, Life Technologies) 24 hours prior to the experiment.

2.3- DNA manipulation and purification

2.3.1 Plasmids

The construct of pMMTV-Luc reporter gene has been described before (Ray et al. 1996). TAT3-Luciferase (Luc), contains 3 copies of the glucocorticoid response element (GRE) from the tyrosine amino transferase plasmid, and was a kind gift from Professor Keith
Yamamoto (UCSF). Human wild-type GRα expression vector was a kind gift from Dr. R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The pSG5-SRC1 (steroid receptor coactivator 1) expression vector (CMV promoter driving SRC1 cDNA) was the kind gift of Dr. Michael Stallcup. IL-6 series reporter vectors were obtained from the BCCM plasmid collection (Zwijnaarde, Belgium). Expression vectors for M KK7 activated JNK and ERK were kind gifts from Professor Andrew Sharrocks (University of Manchester). The construct pcDNA5 was used as an empty control vector (Invitrogen). Where appropriate, renilla (Sea Pansy) luciferase plasmid was used to correct for transfections efficiency (Promega, Southampton, UK).

2.3.2 Transformation of competent E. COLI.

Prior to transformation with plasmid DNA, XL-10 GOLD ultracompetent E.COLI bacteria (45ul per transformation, Invitrogen), were activated by the addition of β mercaptoethanol and incubated on ice for 10 mins. 500ng DNA plasmid (or pUC18 as a positive control) was added to the bacteria mixture, and incubated on ice for a further 30 mins and then heat shocked at 42 C for 30 seconds. The mixture was then returned to ice for 2 mins. Samples were supplemented with 500 µl pre-warmed sterile LB Broth solution (Sigma), and the bacteria mixtures were cultured in shaking incubator at 37 C, 220rpm for 1 hour. For prepare streak and spread plates on LB/ampicillin/agar plates (Tryptone 1%, Yeast extract 0.5%, Agar 1.5%, NaCl 8.6 mM, Glucose 20 mM), 20 µl and 200 µl aliquots of the bacterial mixture were used respectively. Plates were cultured upright in the incubator at 37 C for 30mins, then inverted and incubated for 16-18 hours (overnight).

2.3.3 Plasmid preparation

Plasmid DNA was purified using commercial kits (Qiagen). Single colonies were selected from agar plates and used to inoculate 5ml of LB/ampicillin. Following growth in a
shaking incubator for 16 hours, plasmid was purified from 5ml culture using a Qiagen spin miniprep kit (max 20 μg DNA in 50 μl eluate). Large amount of plasmid was obtained from inoculating 500ml culture with a 5ml starter culture and plasmid purified with a Qiagen maxiprep kit (max 500 μg DNA in 1 ml eluate) following manufacturers instructions. The concentration of plasmid was measured with ND-1000 spectrophotometer (NanoDrop) by loading 1 μl DNA sample.

2.3.4 Glycerol stock

400μl of sterile glycerol was added to 800μl of overnight culture in a 1.5 ml microcentrifuge tube, mixed by inversion and then transferred -80 C for long-term storage.

2.3.5 Restriction digest

Restriction endonucleases are found in bacteria and archaea and able to cut double stranded DNA at specific DNA sequences called restriction sites. Generally restriction enzymes that recognise palindromic 6 base pair sequences are used, which leave a ‘sticky’ end with dNTP overhanging at the 3’ or 5’ end of DNA fragment, although some leave ‘blunt’ ends without overhangs. Various factors determine the efficiency of enzyme restriction, including location of restriction sites, type of DNA template, characteristics of enzyme, temperature and salt concentration. For example, in order to cleave DNA sequences, supercoiled DNA requires more enzymes, while the end of linearised DNA is more difficult to digest by certain enzymes.

2.3.5.1 Digestion of plasmid DNA

Typical restriction digests were set up as follows:

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

When multiple enzymes were required, the most appropriate buffer was selected and the amount of water was adjusted accordingly. To achieve greatest enzyme activity, the digest reaction was allowed to proceed at the optional temperature e.g. 25, 30 or 37 °C for between 1 and 4 hours.

If multiple digestions were set up using incompatible enzymes, DNA was digested with one enzyme for 4 hours, followed by isolation of the DNA using Qiagen PCR cleanup kit. Then, clean DNA is cut with the second enzyme in appropriate buffer.

2.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA molecules by size. The underlying mechanism is to move negatively charged nucleic acid through an agarose matrix with an electric field (electrophoresis). Several factors influence the speed of migration, including length of DNA molecule, conformation of DNA, agarose concentration of gel and voltage. Among these factors, length of DNA fragment is most important, and shorter molecules move quicker than longer ones.

Increasing the agarose concentration of the gel reduces the migration speed, and increases resolution of small DNA molecules. Agarose gel electrophoresis can be used to separate DNA fragments ranging from 50 base pair to several megabases. Conventionally, 1% agarose of the gel is used to separate a range of 100bp to 20kbp. Although higher voltage results in faster speed of DNA movement, high voltage is limited, as the current heats and melts the gel.

Ethidium bromide (EtBr) is traditionally the most commonly used fluorescent dye to stain nucleic acids and makes the bands of DNA or RNA visible under UV light. However, due to its extremely toxic nature, it is less frequently used and sometimes replaced by Safeview
nucleic acid stain. Bromophenol blue and Xylene cyanol are typical loading dyes, which run at about 300bp and 5000bp respectively. Loading dyes make tracking electrophoresis easier. Orange G and Cresol Red are also used as markers with about 50bp and 125bp respectively.

**Protocol**

1-2% w/v agarose (Melford labs, UK) was dissolved in 1×TBE buffer (89mM Tris Base; 89mM Boric acid and 2mM EDTA.2H2O) by boiling in the microwave for 2mins. The gel mixture was cooled briefly with water, and supplemented with ethidium bromide to reach a final concentration of 50 ng/ml. The gel was pour into an agarose tray with comb and left at room temperature for 1 hour to set, and then submerged into a horizontal electrophoresis tank filled with 1×TBE buffer. It was ensured that the level of the TBE buffer was above gel surface, to avoid melting during running.

DNA samples were mixed with 5× loading dye (30% Glycerol; 2.5% Bromphenol Blue) and pipetted into the sample wells. In order to detect DNA fragments, calibrated DNA markers Hyperladder I or IV (Bioline) were loaded alongside samples on the gel. Generally electrophoresis was carried out at 90 volts, room temperature for 1 hour, until the DNA loading dye front reached 5mm distance from edge of the gel. Finally, the gel was placed on a UV light box, and images of ethidium bromide stained DNA were captured using a gel-doc (BioRad).

**2.3.7 DNA quantification**

DNA was quantified using an ND-1000 spectrophotometer (NanoDrop), which uses ultra violet light to measure nucleic acid concentration for 2-3700ng/µl by detecting the absorbance of the light at 260nm and 280nm. Loading 1µl sample onto the measuring surface, a 260/280 ratio of ~1.8 is generally considered as ‘pure’ for DNA.
2.4- Transfection

2.4.1 Principle

Transfection is the process of introducing foreign DNA nucleic acids into eukaryotic cells. There are several methods to carry out this process.

One of the earliest methods developed was to combine DNA with calcium phosphate, in a precipitate which is taken by endocytosis. Electroporation is an alternative method of transfection, which uses a brief pulse of electric current to disrupt cell membrane, to allow DNA enters into cells (Weaver 1995).

For adherent cell lines, the most common and high efficiency method of transfection is using cationic lipids, which are able to bind the negative charge of DNA with their positive charge, so that they form micelles (500-700nm) around DNA (Fig. 2.1). The excess positive charge of lipids is capable of binding to sialic acid residues on cell surface, and results in the passage of DNA into cells through an endocytosis pathway (Friend et al. 1996; Wrobel et al. 1995; Xu et al. 1996; Zabner et al. 1995).

The efficiency of transfection is varied depending on the number of cells undergoing mitosis, cell passage number, growth kinetics, and batch to batch variation of cationic lipids. The nuclear envelope is the major barrier to efficient transfection, and therefore, cells rapidly undergoing mitosis present much higher reporter gene expression, due to their breakdown of the nuclear membrane (Mortimer et al. 1999).

2.4.2 Transient transfection

Transient transfection offers required introduction of genes into cells. However, as the introduced DNA is not integrated into chromatin, transgenes are expelled during subsequent rounds of cell division.
**Fig. 2.1 Principle of cationic lipid based transfection.** Following binding to lipid based reagent, the negative charge of DNA is neutralized by the positive charge of lipid, and then added to the cells. Subsequently the DNA complex binds to cell surface receptors, triggers endocytosis, and traffics inwards, towards the nucleus. The complexed DNA within the endosome is released intracellularly and then the DNA enters the nucleus. Finally the exogenous DNA can be transcribed and translated.
Protocol

$5 \times 10^5$ cells were seeded into 10cm$^2$ plates and cultured in DMEM (10% FBS) overnight. As shown in manufacturer’s instructions, the following day cells were transfected with FuGENE 6 at a ratio of 3:1v/w. For example, 150µl Opti-MEM I reduced serum medium (Invitrogen) was incubated with 10.5µl FuGENE 6 (Roche), 2µg of reporter gene construct, 1µg GR or pcDNA5, and 0.5µg of renilla vector were mixed in 150µl Opti-MEM I. Both mixtures were left to equilibrate at room temperature for 5 mins and then combined. After 30mins incubation at room temperature, the transfection mixture was added dropwise to the cell monolayer and left overnight.

2.5- Assay of GR-dependent gene regulation

2.5.1 Principle

In cell biology, firefly luciferase is a commonly used reporter gene to measure promoter activity, due to its incredible sensitivity and rapid translation process, which does not require any posttranslational modification for activity. The firefly luciferase enzyme (Photinus pyralis) catalyses light production by oxidation of its substrate D-Luciferin to oxyluciferin. Following transfection with plasmids containing luciferase gene, it’s possible to measure expression of luciferase gene by incubation of whole cell lysates with D-Luciferin and ATP.

The sea pansy (Renilla reniformis) luciferase enzyme, a monomeric 36kDa protein, has a dissimilar enzyme structure and substrate to the firefly luciferase, which catalyzes coelenterate-luciferin (coelenterazine) to produce light. This results in distinct measurement of respective bioluminescent reactions. The amount of light production is measured by a luminometer, which is directly proportional to the number of Luciferase molecules, and is regarded as a tool of direct measurement of gene activity.
2.5.2 Firefly luciferase assay

Following transfection with the appropriate luciferase reporters, cells were trypsinised and transferred to 24-well plates and left to adhere overnight. Cells were incubated with appropriate treatments (specified in results) overnight. To process samples, cells were washed twice with 2ml PBS, any residual PBS aspirated and then 100µl 1× Passive Lysis Buffer (Promega) added to each well.

Cells were incubated for 30mins at room temp and 50µl of the cell lysates transferred into a 96-well luminometer plate (Greiner). To prepare luciferase reagent, 10ml of the supplied Luciferase Assay Buffer II was added to the lyophilized Luciferase Assay Substrate. Fresh Stop & Glo Renilla Luciferase reagents were prepared before analysis, by 1:50 diluted 50× Stop & Glo Substrate in Stop & Glo reagent. 25µl of luciferase reagent and renilla Stop & Glo reagent were added into 50µl cell lysates, and the cumulative bioluminescence was quantified over a period of 3 seconds and 0.1 second respectively, using Orion L Microplate Luminometer (Berthold Detection Systems, Germany). Following analysis of the luciferase expression, firefly units were divided by renilla units to normalize for transfection efficiency.

2.6- Protein detection

2.6.1 Antibodies

Anti-hGR (mouse, clone 41) (1:1000 dilution) was purchased from BD Biosciences (Oxford, UK). Anti-phospho-(Ser211)-GR (1:1000 dilution), anti-phospho-JNK (1:1000 dilution), anti-phospho-p38 (1:1000 dilution) and anti-phospho-ERK (1:2000 dilution) were obtained from Cell Signalling Technology (MA, USA). Anti-α-tubulin (1:2000 dilution) and anti GR was from Sigma-Aldrich (Dorset, UK). Horseradish peroxidase conjugated anti-mouse (1:5000) and anti-rabbit (1:5000) were obtained from GE Healthcare (Buckinghamshire, UK). Fluorophore conjugated (Alexaflour 546 and 488)
anti-mouse (1:500) and anti-rabbit (1:500) were from Invitrogen molecular probes (Paisley, UK)

2.6.2 Protein quantification

2.6.2.1 Bradford assay

This is a colorimetric protein assay, which is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 from 465 nm to 595 nm in response to binding to proteins. The anionic form of the dye binds to both ionic and hydrophobic residues within proteins, results in the red form of the dye under acidic conditions being converted into its blue form.

Protocol

For protein analysis, 2μl samples were added into 1ml of 1× Bradford Reagent (Bradford protein assay, BioRad) followed by incubation at room temp for 15 mins. 200 μl of each sample was transferred into a flat-bottomed clear 96-well plate in duplicate alongside BSA standard dilution series from 0-0.8 mg/ml (the linear range for this assay) and the absorbance at 595 nm recorded. Protein concentrations were estimated using a standard curve.

2.6.2.2 Immunoblot analysis

Cells were plated into 10 cm dishes overnight, treated as specified in the results then lysed in 1× RIPA buffer (50 mM TrisCl pH7.4, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing 50 μl protease (1:100 dilution, Calbiochem) and 50 μl phosphatase inhibitors (1:100 dilution, Sigma). Cells were scraped and insoluble debris pelleted by centrifugation at 13,000 rpm for 30 min. The concentration of protein in the supernatant was measured with BIO-RAD protein assay (Bio-Rad Laboratories). Cell lysates containing 25μg proteins were electrophoresed on Tris/Glycine 4-12% gels (Invitrogen) at 150V for 45min and then transferred to 0.2 micron nitrocellulose membranes (BioRad Laboratories, Hertfordshire, UK) overnight at 10V, 4°C. Membranes
were blocked for 4 hours (0.15M NaCl, 1% dried milk, 0.1% Tween 20) and incubated with primary antibodies (diluted in blocking buffer) overnight at 4°C. After three 10 minute washes (88mM Tris pH 7.8, 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 three times, each for 10 minutes. Immunoreactive proteins were visualised on Biomax MRI film using enhanced chemiluminescence (ECL Advance, GE Healthcare).

2.6.3 Localisation and trafficking

2.6.3.1 Immunofluorescence

Cells were seeded on glass coverslips in 12 well plates and cultured overnight. Cells were treated as specified in the results then fixed with 4% paraformaldehyde (PFA) for 30 minutes at 4°C, then permeabilised (0.02% Triton X-100 in PBS) for 30 minutes at room temp. Fixed cells were blocked (1% FBS in PBS) for 4 hours at RT with agitation, then cultured in primary antibody (1:250, diluted in blocking buffer) overnight at 4°C. After three 10 minute washes in PBS, cells were incubated in secondary antibody (1:500, diluted in PBS) for 2 hours. A Hoechst nuclear stain (Sigma) was added for 5 min. Following three further 10 minute washes, coverslips were mounted using vectashield hard set mountant (Vector Laboratories, Peterborough, UK). Images were acquired on a Delta Vision RT (Applied Precision) restoration microscope using a 60x/ 1.42 Plan Apo objective and the Sedat filter set (Chroma 89000). Images for DAPI, Alexa488 and Alexa546 were excited with the 405-, 488-, and 543-nm laser lines. The images were collected using a Coolsnap HQ (Photometrics) camera with a Z optical spacing of 0.5 μm. Raw images were deconvolved using the Softworx software. Average intensity projections of these deconvolved images were processed using Image J.
2.6.3.2 Live cell imaging

Cells were transfected with 5µg hGR-GFP and transferred to a glass bottomed 24 well plate. Cells were maintained at 37°C and 5% CO₂ for the duration of data collection. Images were acquired every minute on a Nikon TE2000 PFS microscope using a [60x/1.40 Plan Apo or 40x/1.25 Plan Apo] objective and the [Sedat] filter set (Chroma [89000]). The images were collected using a Cascade II EMCCD camera (Photometrics) with a Z optical spacing of 0.2µm. Raw images were then processed using Image J.

2.6.3.3 Fluorescence recovery after photo bleaching assay

Cells were transfected with 5 µg hGR-GFP and transferred to a glass bottomed 24 well plate. Cells were maintained at 37°C and 5% CO₂ for the duration of data collection. The 488- and 514-nm laser lines were used for bleaching, with a bleach pulse of 0.25s at 100% laser power. Images were collected at 0.6-s intervals, and selected with the same rectangular region of fixed size in the nucleus. Fluorescence was measured in terms of 5 frames pre-bleaching, 5 frames bleaching and 40 frames post-bleaching. Results were quantified at every time point (every 0.661 second) and evaluated in terms of the bleached region compared to the level before bleaching. Images were collected on a Leica TCS SP5 AOBS inverted confocal using a [63x/0.50 Plan Fluotar] objective [and 7x confocal zoom]. The confocal settings were as follows, pinhole 1 airy unit, scan speed 1000Hz unidirectional, and format 1024 x 1024. Images were collected using the following detection mirror settings; FITC 494-530nm using the 488nm (13%).

2.7- RNA measurement

2.7.1 Principle of qRT-PCR

Reverse transcription polymerase chain reaction (qRT-PCR), uses the enzyme reverse transcriptase to reverse transcribe RNA into its DNA complement (complementary DNA,
or cDNA). The complementary DNA is monitored by accumulation of amplicons after each amplification cycle, to calculate the initial quantity of DNA. Typically, a fluorescent dye called SYBR Green is used to quantify DNA after each round of amplification. SYBR Green is able to intercalate with double stranded DNA, and emits a vastly stronger fluorescent signal once bound. PCR products are also corrected with a melting curve, in order to eliminate non-specific amplification.

Three single steps construct a cycle of qRT-PCR:

**Denaturation**: Double stranded DNA is heated to 94 to 98°C for 10 mins, and melts into two single stranded templates during this step.

**Annealing**: The reaction is cooled down to 45-65°C. Then single stranded primers anneal to single stranded DNA template. DNA polymerase attaches to the primed template, and starts to incorporate complementary nucleotides.

**Extension**: The temperature is increased to 65-75°C, where the optional temperature for Taq polymerase is 72°C. DNA polymerase extends primed sequence during this cycle, with the nucleotides complementary to the DNA template yielding a double stranded DNA. Subsequently, SYBR Green binds to the synthesised DNA double helix and fluoresces. The amount of fluorescence is measured at end of each cycle, and also the intensity above background level is calculated, in order to quantify newly generated DNA.

Above three steps are repeated approximately 35-40 cycles.

**Protocol**

Cells were plated in 6cm dishes and treated as specified in results. RNA was extracted using RNeasy mini kit with DNase 1 digestion (Qiagen) following the manufacturers recommended protocol. Reverse transcription of mRNA (1 μg) was completed using SuperScript III Platinum Two-Step qRT-PCR kit. qRT-PCR was performed in triplicate wells using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in a 7500 real-time PCR system (Applied Biosystems). Gene expression was analysed using the 2KDDCt method relative to GAPDH expression (Livak et al. 2001).
QuantiTect qRT-PCR primers were from Qiagen. Non-QuantiTect primers were designed using Roche Applied Sciences Assay design center, and are shown in Table 2-1.

### Table 2-1 Primer sequences used in the SYBR-Green based qRT-PCR assay

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-GCC AGC CGA GCC ACA TC-3’  5’-GTG ACC AGG CGC CCA AT-3’</td>
</tr>
<tr>
<td>FKBP5</td>
<td>5’-TGT CTC CCA CGT GTG TAT TAT G-3’  5’-TTT GCT CAG AAC CAC TCA CAC-3’</td>
</tr>
<tr>
<td>GILZ</td>
<td>5’-TGT GGA TGA GGG ATG AAC AA-3’  5’-ACC CGC TAC AGA CAA GCT TT-3’</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>5’-CCA TGT CAC CAA CAT CAA AAA-3’  5’-CCT TGG CTA AAC TCT CTA CGA CTC-3’</td>
</tr>
<tr>
<td>MT1X</td>
<td>5’-CAG CTG TGC TCT CAG ATG TAA A-3’  5’-TGT AGC AAA CGG GTC AGG-3’</td>
</tr>
<tr>
<td>PER1</td>
<td>5’-CTG CAC CAG CTA GAC TCC ATT-3’  5’-GGA GAA GAA AGC CTC TCA TGG-3’</td>
</tr>
</tbody>
</table>
The primers closest to a specific gene probe set (Affymetrix HG U133A chip) on the mRNA sequence were selected for the qRT-PCR experiment. The primer sequences were also compared against the whole genome on the NCBI BLAST search engine to ensure the specificity of the primer sequences.

2.7.2 RNA purification and quantification

To ensure an RNase-free environment, all the benches, glassware and pipettes were sprayed with RNaseZap or 70% ethanol. Cells were washed twice with PBS, then lysed in RLT and total RNA purified 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) ensured the removal of any genomic DNA contamination.

To measure the concentration and quality of RNA, applied Nanodrop untra-low volume spectrophotometer (Nanodrop Technologies) at an absorbance of 260nm, by loading 1µl of RNA sample on the top of probe. RNA purification is determined by the A260/A280 ratio which indicates the presence of protein or other contaminants if it’s smaller than 2.0. The 260/230 ration is also an indicator of RNA quality. The definition of purified RNA is 260/230 greater than 2.0.

2.7.3 Reverse transcription (RT)

A high Capacity cDNA Reverse Transcription Kit (Invitrogen) was used to generate first strand cDNA (10µl of 2×RT buffer, 1µl of RT Enzyme Mix, 820ng RNA and Nuclease-free water to 20µl). For –ve RT samples, RT Enzyme was substituted with DPEC-treated water. The reaction was completed on a DNA Engine tetrad 2 Peltier Thermal cycler (BIO-RAD) (37 °C for 60mins, 95 °C for 5 mins, and then incubation at 4 °C for 20mins (or overnight)). cDNA samples were stored at -20 °C (or -80 °C for long term storage) before qRT-PCR.
2.7.4 Running the qRT-PCR

Samples were run in triplicates, with each reaction of 10–25µl per well, in 96-well MicroAmp optical reaction plates (Applied Biosystems, USA). Either quantitect or non-quantitect primers were used; therefore qRT-PCR was carried out following different protocols. In each case, preparation of master mix is crucial for reduction of pipetting errors.

qRT-PCR mix for non-quantitect primers (e.g. 12.5 µl each reaction)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>×2 SYBR Mix (Invitrogen)</td>
<td>6.25</td>
</tr>
<tr>
<td>H2O</td>
<td>5.375</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.25</td>
</tr>
<tr>
<td>Primer F/R</td>
<td>0.25</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.125</td>
</tr>
<tr>
<td>Total</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Final concentration of primers is 200nM.

qRT-PCR mix for quantitect primers (e.g. 12.5 µl each reaction)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>×2 SYBR Mix (Invitrogen)</td>
<td>6.25</td>
</tr>
<tr>
<td>H2O</td>
<td>4.25</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.25</td>
</tr>
<tr>
<td>Primer</td>
<td>1.25</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>12.5</td>
</tr>
</tbody>
</table>

The qRT-PCR plate was sealed with an ABI prism optical adhesive cover (Applied Biosystems), and then centrifuged for 1min 30secs at 3600rpm. Applied Biosystems StepOnePlus Real Time PCR Systems (Applied Biosystems) was used.
For non-QuantiTect primers:

Holding stage: 95°C for 10mins

Cycling stage: 40 cycles of 95°C; 15 seconds
60°C; 1 minute

Melt Curve Stage: 95°C; 15 seconds
60°C; 1 minute
95°C; 15 seconds

For QuantiTect primers:

Holding stage: 95°C for 15mins

Cycling stage: 40 cycles of 95°C; 15 seconds
55°C; 30 seconds
72°C; 30 seconds

Melt Curve Stage: 95°C; 15 seconds
60°C; 1 minute
95°C; 15 seconds

The melting curve analysis ensures the production of a single qRT-PCR amplification product.

2.7.5 qRT-PCR data analysis

The amplification plot was produced after a qRT-PCR reaction, and the data is analysed using the comparative Ct method, by comparing the Ct values of test samples to that of a control. Ct value is a cycle number to measure the level of the fluorescence produced by the amplified PCR product is higher than the fluorescence produced by background. The more PCR product is detected, the smaller Ct value is.
Since there’s no cDNA template in negative control samples; therefore no signal could be detected after 40 cycles of amplification. The background fluorescence is known as ‘threshold level’ and corresponds to an exponential increase of the amplified PCR product. Ct values were calculated by ABI 7300 system and then exported to Microsoft Excel spreadsheets. The mean ΔCt value was calculated as follow. Normally use GAPDH as housekeeping (control) gene.

\[ \Delta\text{Ct} = \text{Mean target gene Ct} - \text{Mean control gene Ct} \]

\[ \Delta\Delta\text{Ct} = \Delta\text{Ct} \text{ (difference compared, e.g. treatment)} - \Delta\text{Ct} \text{ (Calibrator or control, e.g. no treatment)} \]

Fold Induction = Power \(2, -\Delta\Delta\text{Ct}\)

If the result of fold induction < 1, then could present in the way of:

Fold reduction = 1 / Fold induction
2.8- Chromatin immunoprecipitation (ChIP) assay

2.8.1 Principle of ChIP

Chromatin immunoprecipitation (ChIP) assay is used to study the interaction between protein and the genome. This technique is a powerful tool to investigate how proteins physically interact with DNA in living cells. Generally, protein-DNA interactions are first captured by treated cells or tissues with a small and reversible crosslinker, e.g., formaldehyde, which is able to rapidly diffuse into cells (Fig. 2.2). After cell lysis and nuclear isolation, DNA is fragmented by sonication or enzyme (between 100 - 500bp), and then protein-DNA complexes are enriched using immunoaffinity capture for the protein of interest. An aliquot of input DNA is used as control for immunoprecipitation. The enriched protein-DNA complexes are isolated, and crosslinks for both input and the enriched DNA are reversed, and then protein digested leaving purified DNA. Subsequently the enriched DNA relative to control sample can be measured by different techniques, e.g. qRT-PCR or comparative sequencing analysis.

2.8.2 Protocol

Reagents

1M Tris-HCl (pH6.5): 7.88g in 50ml (157.6mg/ml)
1M Tris-HCl (pH8): 121.14g in 1L (121.14mg/ml)
0.5M CaCl2: 1.47mg in 20ml (147.02mg/ml) (1 in 100 diluted)
100mM PMSF: 87.1mg in 5ml isopropanol (174.2mg/ml) (1 in 100 diluted)
1M HEPES (pH6.5): 11.915g in 50ml (238.3mg/ml)
50mM EGTA: 190.175mg in 10ml (19mg/ml)
100mM EDTA: 1.6812g in 50ml (33.6mg/ml)
1M NaCl: 2.922g in 50ml (58.44mg/ml)
5M NaCl: 14.61g in 50ml (58.44mg/ml)
**Fig 2.2 Principle of ChIP assay.** The ChIP assay is a useful tool to identify protein-DNA interaction. This assay requires several steps and molecular biology methods, including DNA-protein cross-linking, cell lysis, shearing, antibody-based immunoprecipitation and DNA purification. The enriched DNA can be measured by different techniques, e.g. PCR, qRT-PCR, DNA microarray, or comparative sequencing analysis (Collas *et al.* 2008).
10% SDS: 5g in 50ml (288.8mg/ml; 1% = 1g in 100ml)
1M LiCl: 2.12g in 50ml (42.4mg/ml)
10% sodium deoxycholate: 5g in 50ml (100mg/ml)
1M NaHCO3: 4.2g in 50ml (84.01mg/ml) (Make fresh)
100×Protease Inhibitor cocktail set I (Cat no 539131): 1ml water per vial (1 in 100 diluted; keep on ice)
1.25M Glycine: 1.41g in 15ml PBS (Make fresh)

**Procedure**

To get a good signal-to-noise ratio, use one 150mm dish of cells for one ChIP experiment, i.e., prepare one 150mm dish of HeLa cells, after lysis buffer wash and sonication; equally divide the total nuclei lysate into two aliquots, one for YFP IP, one for GR IP.

**Table 2-2 Antibodies used for ChIP assay**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-NR3C1(GR)</td>
<td>Sigma-Aldrich; HPA004248</td>
</tr>
<tr>
<td>GR Antibody (H-300)</td>
<td>Santa Cruz technology; sc-8992X</td>
</tr>
<tr>
<td>GR Antibody (M-20)</td>
<td>Santa Cruz technology; sc-1004</td>
</tr>
<tr>
<td>YFP Antibody (FL)</td>
<td>Santa Cruz technology; sc-32897</td>
</tr>
</tbody>
</table>

Cells were fixed by 1% formaldehyde (diluted in DMEM) and rocked at 20 rpm for 10mins at room temperature. The crosslink reaction was stopped by addition of 0.125M glycine with rocking at 20rpm, room temp, for 5mins. Cells were then washed twice (PBS) and scraped into ice-cold PBS buffer, containing protease inhibitors, and cells collected by centrifugation at 720rcf, 4 °C for 5 minutes.

Cells were then lysed in 1.5ml cell lysis buffer 10mM HEPES pH 6.5, 0.5mM EGTA, 10mM EDTA, 0.25% Triton X-100) and incubated on ice for 5 minutes.
Following centrifugation at 700 rcf, 4°C for 10 mins, the nuclei were washed in 1.2ml nuclear wash buffer (10mM HEPES pH 6.5, 0.5mM EGTA, 1mM EDTA, 200Mm NaCl) and collected by centrifugation. The nuclei were lysed in 0.5ml nuclear lysis buffer (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS) and sheared by twice probe sonication (SONICS VCX130) with energy more than 50% as 12 secs on/ 40secs off, followed by water sonication by bio-rupter (Next Generation) for 40 mins (6×83µl aliquote of nuclear lysates per round), at high level, 30secs on/ 30 secs off, 4 °C. Cell debris was removed by centrifugation at 13200 rpm, 4 °C, for 10 mins.

The sheared chromatin was taken 25µl aliquot as input and stored in -80°C freezer. Then the left was 1 in 10 diluted in IP dilution buffer, and incubated with antibodies or YFP as 1.5 µg Ab per 1ml IP, rotating in cold room at 20rpm overnight.

Added 10µl Dynabeads A/G (Invitrogen) after wash once in 0.5ml IP dilution buffer (16.7mM Tris-HCl pH 8.1, 1.2mM EDTA, 167mM NaCl, 0.01% SDS, 1.1% Triton X-100), into 1µg antibody IP, and rotated the samples in cold room at 20 rpm for 4 hours. Washed IPs as twice 1ml wash buffer 1 (20mM Tris-HCl pH 8.1, 2mM EDTA, 50mM NaCl, 0.1% SDS, 1% Triton X-100), once 1ml wash buffer 2 (10mM Tris-HCl pH 8.1, 1mM EDTA, 250mM LiCl, 1% NP40, 1% sodium deoxycholate) and twice 1ml TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0), by rotating in cold room 15mins per wash. The protein-DNA complexes were pulled down by adding beads with 100µl elution buffer and shaking at 14,000rpm, 65 °C for 30mins. Also 3× elution buffer (1% SDS, 100mM NaHCO3) (75µl) was added into input sample (25µl)). Crosslink of both protein-DNA complexes and input were reversed by adding 4µl 5M NaCl and incubation at 65°C at least 6 hours (less than 18 hours).

Each sample was treated with 0.7µl proteinase K (10mg/ml), 2 µl 0.5M EDTA (100 µM) and 4 µl 1M Tris-HCl pH 6.5 (40mM), and incubated at 600rpm, 55°C for 1 hour. DNA was cleaned up using MinElute Qiagen kit (Cat. 28006) and eluted in 10 μl EB buffer (provided in MinElute Qiagen kit).
DNA samples were quantified using Qubit fluorometer (Invitrogen). Diluted input samples 1 in 50 in EB buffer, and then quantified both diluted input and ChIP DNA by gel-PCR and qRT-PCR.

ΔCt value was calculated for each sample to account for the DNA quantity, 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 of treatment with Dex was compared with vehicle and then present as fold change.

Recipe for gel-PCR:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10×NH4 buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer F/R</td>
<td>0.5</td>
</tr>
<tr>
<td>BIOTAQ</td>
<td>0.5</td>
</tr>
<tr>
<td>DNA</td>
<td>1</td>
</tr>
<tr>
<td>H₂O</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>

Recipe for qRT-PCR:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>×2 SYBR Mix</td>
<td>12.5</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.4</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer F/R</td>
<td>0.3</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>
### Table 2-3 Primer sequences used for ChIP assay

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GILZ1</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-AGT TGG TAC AAG AAA GTG C-3’ (19)</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CTC GTA TGT CAC AAA CTC C-3’ (19)</td>
</tr>
<tr>
<td><strong>GILZ3</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-GGG AAT TCT GAT ACC AGT TAA GC-3’ (23)</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GGG AGA CAA TAA TGA TCT CAG GA-3’ (23)</td>
</tr>
<tr>
<td><strong>FKBP5</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-AAG TCT GAG TCC CTG GTT TAC-3’ (21)</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-TTC ACG CCT GTG TGC TTT TA-3’ (20)</td>
</tr>
<tr>
<td><strong>MT1X</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-GCA GGT GCT CTT TGT GAT GA-3’ (20)</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CCC ATT TGA TCC CTA CAT GG-3’ (20)</td>
</tr>
</tbody>
</table>
2.8.3 Optimisations

Sonication

Optimisation of sonication is a critical step (Fig. 2.3). The duration of crosslink by formaldehyde is a direct factor in determining sonication efficiency. Some protocols suggest longer duration for crosslinking when using suspension cell lines and tissues compared with adherent cell lines. However, longer crosslinking typically requires increasing the intensity and/or duration of sonication to get the optimal size of DNA fragment (<300bp), and conversely, over-sonication can result in poor DNA enrichment. The sonication efficiency can vary dependent on cell type, cell culture condition and treatment. Therefore, optimisation of sonication is a key step to achieve a small DNA fragment while maintaining a good enrichment.

Fig. 2.3 The optimal size of DNA fragments for ChIP-sequencing. For ChIP-sequencing assay, the size of sheared DNA fragments is between 100 bp to 500 bp, and the size of most fragments should be about 200 bp.
Antibody

More antibodies will pull down more enriched DNA. Generally to detect a transcription factor requires 6µg antibody for about $2 \times 10^7$ cells, to get 5ng DNA for analysis. As more histone exists in nuclei, therefore we usually use ~2 µg of applied ChIP grade histone antibody. Some antibodies can have varied affinity for protein modifications and so it is best, where possible to use an antibody cocktail. This gives an unbiased pull down.

2.8.4 Primer design

ChIP primers were designed through Primer3 website as below.

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

Selected species of Mispriming Library (repeat library), e.g. Human, and defined the size of product between 100-200bp. Once got the primers, pasted the sequences in silico PCR option on the UCSC genome browser, to check whether the primers could produce a single PCR product. The designed primers are not qualified to be used if they could construct more than one PCR product.

2.8.5 Qubit fluorometer

Qubit® 2.0 Fluorometer (Invitrogen) is a more accurate way to measure DNA concentration, which is based on detection of fluorescence dye that binds specifically to DNA, to quantify DNA in samples with concentrations as low as 10 pg/µL. It’s able to measure both DNA and RNA in the same sample. Since the concentration is detected based on the specific fluorescence, effects of contamination e.g. proteins is reduced. Qubit™ dsDNA HS Assay Kit (Q32854) was used for DNA quantification and the workflow is below (Fig. 2.4).
**Fig. 2.4 Workflow of the Quant-iT™ assay by Qubit® Fluorometer.** The workflow applied a simple mix-and-read format. Added 1μl of DNA samples to Quant-iT™ mixture and analysis of the concentration was completed by incubation times of only 2 minutes (www.invitrogen.com/bp62).
2.9- ChIP-sequencing assay

ChIP combining with high-throughput sequencing technology (ChIP-Seq) identifies binding sites of DNA-associated proteins efficiently and accurately, widely applied to study histone modification and transcription factors regulation.

2.9.1 Workflow of ChIP-sequencing

To carry out ChIP-seq assay, a minimum of 10 ng ChIP-ed DNA (Qubit result) was needed for a single library construction. GC content should be between 35%-65%. The size of DNA fragments is between 100 bp to 500 bp, and the size of most fragments should be about 250 bp.

![Workflow of ChIP-sequencing](image)

Fig. 2.5 Workflow of ChIP-sequencing. Several steps involved in ChIP-seq assay. After collected qualified samples, target DNA was enriched and amplified. The qualified DNA library was sequenced on Illumina platform and subsequent raw reads results were analysed. Generally the whole project requires ~40days to complete.
2.9.2 Library construction

The workflow of library construction is present in Fig. 2.6.

![Pipeline of library construction](image)

**Fig. 2.6 Pipeline of library construction.** The library construction was completed following the manufacturer’s protocol. DNA-end repair, 3’-dA overhang and ligation of methylated sequencing adaptors. Amplified PCR and selected size usually between 100 and 300bp including adaptor sequence. And the qualified DNA library is used for further deep sequencing on Illumina HiSeq 2000 platform.
2.9.3 Illumina Sequencing

The DNA library was sequenced by the strategy of 50 SE, Illumina’s HiSeq 2000 (NGS Platform). One lane is able to sequence maximum 8 ChIP-ed samples and generate 80M raw reads per lane (about 70% clean reads after removing adaptor sequences, contamination and low-quality reads).

2.9.4 Bioinformatics analysis

The workflow of computational methods for ChIP-seq data analysis is shown in Fig. 2.7.

![Pipeline of bioinformatics analysis](image)

**Fig. 2.7 Pipeline of bioinformatics analysis.** Sequencing data were mapped to the reference genome. Only the unique mapped reads were used for standard analysis and personalized bioinformatics analysis.
2.9.4.1 Peak identification

Filtered raw reads were aligned to Build 19 of the human genome (hg19) from February 2009. Only the alignments within 2 mismatches were considered in peak calling. Whole genome peak scanning was based on a defined analysis model. The results included peak location, peak sequence, etc. The candidate peak region was extended to be long enough for modeling. Dynamic Possion Distribution was used to calculated p-value of the specific region based on the unique mapped reads. The region would be defined as a peak when p-value<1e-5.

Software: MACS-1.4.0

2.9.4.2 Peak distribution

The analysis of peak distribution was completed on Galaxy/Cistrome platform, by ‘CEAS: Enrichment on chromosome and annotation’ under option of ‘Integrative analysis’. The link is: http://cistrome.org/ap/root#

CEAS is able to annotate the given intervals and scores with genome features such as gene body, and determine genomic distribution of peaks from the transcription start site (TSS) and downstream from the transcription termination site (TTS), so that to calculate coverage of promoters and downstream regions. CEAS could also be used for generating Venn diagram of intersections by giving 2 or 3 intervals.

2.9.4.3 UCSC genome browser

Peaks files in .wig format were uploaded to UCSC Genome Browser which is developed and maintained by the Genome Bioinformatics Group at the University of California Santa Cruz (UCSC). On “genome browser” webpage selected human genome assembly hg19 and submitted. Clicked the option ‘add custom tracks’ and then uploaded ChIP-seq data files in .wig format. The Peak distribution across the genome will be shown on the following page.
2.9.4.4 Overlap analysis

The overlap analysis was completed in Galaxy which is a web-based platform for data intensive biomedical research, with a minimum of 1 bp overlap accepted. The link is https://main.g2.bx.psu.edu/ For example, the peak files (e.g. Bed format) can be uploaded to Galaxy and performed comparison between different treatment following option ‘Operate on Genomic Intervals’---‘Join the intervals of two datasets side-by-side’. Also genome coordinates can be converted between assemblies and genomes, e.g. from ‘hg18’ to ‘hg19’ under option ‘Lift-Over’.

2.9.4.5 Gene ontology (GO) analysis

GO function analysis of peak-related genes was completed by a web-based Genomic Regions Enrichment of Annotations Tool (GREAT version 2.0.2) at link as below. http://great.stanford.edu/public/html/splash.php. Four-column BED file, including chromosome number, summit, summit and peak number, for peaks with false discovery rate (FDR) less than 0.1 was used. Each ChIP-seq region is assigned to all genes whose domains it overlaps. Significantly enriched terms from the Biological Process, Cellular Component and MSigDB Pathway were reported.

2.9.4.6 Network construction

STRING is a web-based tool to predict protein-protein interactions. Multiple names of genes assigned to ChIP-seq regions were uploaded, and the image of protein network was saved as PNG file with evidence view. Different line colors represent the types of evidence for the association. The link of STRING is: http://string.embl.de/

2.9.4.7 Motif analysis

Motif discovery was completed by web-based Regulatory Sequence Analysis Tools (RSAT) at: http://rsat.ulb.ac.be/rsat/
Peak files were analysed with summit -100bp/+100bp (200bp for transcription factor), and sequences of the peaks were fetched from UCSC following option ‘NGS-ChIP-seq’---‘fetch-sequences from UCSC’.

The fetched peak sequences were reported in FASTA format. The peak motifs was discovered following option ‘NGS-ChIP-seq’---‘peak-motifs (ChIP-seq analysis)’ and the email output is preferred.

Motif discovery parameters were included:

### Motif discovery parameters

<table>
<thead>
<tr>
<th>Continuous words</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Discover over-represented words [oligo-analysis]</td>
</tr>
<tr>
<td>- Discover words with a positional bias [position-analysis]</td>
</tr>
<tr>
<td>- Discover words with local over-representation [local-word-analysis]</td>
</tr>
</tbody>
</table>

**Oligomer length** for the three programs above 06 07

*Note: motifs can be larger than word sizes (words are used as seed for building matrices)*

**Markov order (m) of the background model for oligo-analysis (k-mers)** (only for single-dataset analysis, will be ignored if control set is provided)

- [Automatic (adapted to sequence length)]

**Spaced word pairs (dyads)**

- [Discover over-represented spaced word pairs [dyad-analysis]]

**Number of motifs per algorithm** 10

**Search on** both strands

### 2.9.4.8 Difference analysis of multi-samples

Difference analysis of peaks was completed using seqMINER_1.3.3e software. Peaks data was ordered in terms of ‘Nd_specific’, ‘commen_genes’ and ‘Hd_specific’, with genes of FDR less than 0.1. Mapped reads files, e.g. in bed/sam/bam format, was matched to the peaks. Set ‘Tools’-‘Options’-‘General’-‘Clustering options’-‘Wiggle step’ at 50.
Select ‘Clustering Normalization’ as KMeans linear. Set ‘Expected Number of Clusters’ as 1. Heat map was adjusted to background with commonly either white or black colour. The tag density curve was merged and used as well. For analysis of histone marks, the heatmap is shown as a blank line in the middle of background. Since there was no obvious peak in control or vehicle treated samples, only positive control data of ChIP-seq was shown in the heat map. I also saved the Mean density figure under ‘Merged dataset profile’ option.

2.10- Statistical analysis

Data were analysed using Graphpad Prism version 5.01 (Graphpad Software Inc., San Diego, CA, USA), and present in terms of average ± SEM as error bars. Analyses were run using SPSS software version 16.0. Comparisons between groups were performed using Student’s t-test, two tailed for independent samples. Significant results were defined as p value <0.05.
Chapter 3 - Results
3.1- Serum shock and GC sensitivity

3.1.1 Serum impairs GC action

After nuclear translocation, ligand-bound GR dimerizes and binds directly to DNA sequences to activate target genes expression. To determine serum regulation of GC action, luciferase assays were carried out using three GR responsive reporter genes, pHH-Luc, TAT3-Luc and MMTV-Luc. Serum selectively impaired GR transactivation of MMTV-Luc, but not pHH-Luc or TAT3-Luc, in response to treatment with either dexamethasone (Dex) or hydrocortisone (HC) (Fig. 3.1.1). Culture in 50% serum induced a markedly right-shifted response on MMTV-Luc (decreased potency) as well as decreased maximum efficacy.

3.1.2 A reversible model of GC resistance in 50% serum

Steroid receptor coactivator (SRC-1) is known to bind GR-LBD to increase GR transactivation. To evaluate whether the impaired GR transactivation in 50% serum is reversible, a reporter gene assay was completed with a pSG5-SRC1 expression vector cotransfected with the MMTV reporter gene and GR. After treatment with GCs, SRC-1 robustly induced the expression of luciferase from the MMTV reporter gene (Fig. 3.1.2). Compared with transfection of pcDNA5 FRT empty vector, SRC-1 plasmid transfection resulted in approximately three fold greater response after treatment with either Dex or HC. SRC-1 prevented the loss of GC transactivation observed in high serum. This implies the impaired GR transactivation in 50% serum can be recovered, suggesting that serum induces a reversible GC resistance model to study.
Fig. 3.1.1 Serum selectively impairs MMTV-Luc response to GCs.

HeLa cells were transiently transfected with MMTV-Luc (a, b), pHH-Luc (c) or TAT3-Luc (d) reporter gene, together with GR and Renilla control plasmid overnight. Following cultured in 10% or 50% serum cells were treated with dexamethasone (Dex) or hydrocortisone (HC) (0-100nM) for 16 hours before luciferase assay. Cells transfected with MMTV-Luc, but not TAT3-Luc or pHH-Luc, show impaired dose response in 50% serum when compared with 10% serum. Graphs show mean ± SEM of triplicate wells. Experiments were repeated on three occasions. RLU, relative light unit. Comparison made by independent sample T test, * P<0.05.
Fig. 3.1.2 Recruitment of cofactors to GR restores GR transactivation in 50% serum.

Hela cells were transfected with MMTV-Luc and GR combined with either GR co-activator SRC-1 or empty vector pcDNA5 FRT as control. Cells were subsequently cultured in either 10% or 50% serum overnight and treated with 100 nM dexamethasone (Dex) (A) or hydrocortisone (HC) (B) for 16 hours before reporter gene assay. The serum impaired GC action was overcome by transiently cotransfection with a GR coactivator pSG5-SRC1 expression vector. Graphs show mean ± SEM of triplicate wells. Experiments were repeated on three occasions. RLU, relative light unit. Comparison made by independent sample T test, * P<0.05, SPSS.
3.1.3 Serum selectively impairs GR transactivation of endogenous gene expression

To explore the role of serum in GR transactivation of endogenous target genes, qRT-PCR was used to test four well characterised GR regulated genes, including metallothionein 1X (MT1X), FK506 binding protein 5 (FKBP5), glucocorticoid leucine zipper (GILZ) and period circadian protein homolog 1 (PER1). Followed four hours Dex treatment, all four endogenous genes were robustly induced (Fig. 3.1.3A). Expression of GILZ and PER1 were comparable in 10% and 50% serum, with approximately six fold induction. However, 50% serum impaired transactivation of both FKBP5 and MT1X. There was no baseline difference in the expression of the two genes between 10% and 50% serum (Fig. 3.1.3B). These findings suggest that serum selectively impairs GR transactivation of endogenous gene expression, as well as synthetic reporter genes.

3.1.4 Impaired GR transactivation of endogenous gene expression is reversible

Further qRT-PCR assays were completed to investigate whether the impairment of GR transactivation is recovered by return to culture in 10% serum. HeLa cells were cultured in either 10% or 50% serum overnight. Some of cells cultured in 50% serum were transferred to culture in 10% serum four hours before treatment with Dex (8hrs before harvest), or when Dex was added (4hrs before harvest) (Fig. 3.1.4A). As above, after the four hours treatment with Dex, the robust GR transactivation of either MT1X or FKBP5 seen in 10% serum was impaired by 50% serum (Fig. 3.1.4B).

Impairment of MT1X was rapidly reversed by culture in 10% serum for four hours prior to Dex treatment, whereas FKBP5 regulation was not recovered by culture in 10% serum for eight hours. This suggests the existence of different mechanisms to explain variation in GC response, acting in a template dependent manner.
Fig. 3.1.3 Serum selectively impairs GR transactivation of MT1X and FKBP5.
HeLa cells were cultured in either 10% or 50% serum overnight. Cells were treated with 100 nM Dex for 4 hours before RNA purification (A). HeLa cells were cultured in either 10% or 50% serum overnight before RNA purification (B). Four well-characterised GR transactivated endogenous genes were tested by qRT-PCR, including FK506 binding protein5 (FKBP5), metallothionine 1X (MT1X), glucocorticoid leucine zipper (GILZ) and period circadian protein homolog 1 (PER1). Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test, * P<0.01, ** P<0.001.
Fig. 3.1.4 A reversible model of impaired GR transactivation.

HeLa cells were either consistently cultured in 10% or 50% serum, or transferred to 10% serum as shown in (A) before treatment with 100nM Dex for 4 hours (B). Expressions of GR transactivated endogenous genes, including MT1X, FKBP5, GILZ and PER1, were analysed by qRT-PCR. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test, * P<0.01, NS: Not significant.
3.1.5 Serum regulates GR transrepression partly in an AP1 dependent manner.

To investigate GC repression of pro-inflammatory stimuli, further reporter assays were completed using a series of luciferase constructs based on the promoter of wild-type interleukin 6 (IL-6). In contrast to transactivation, GC repression of IL6-Luc is increased in 50% serum (Fig. 3.1.5A). PMA is a phorbol ester (Phorbol 12-myristate 13-acetate, Sigma-Aldrich), which serves as a physiological activator of protein kinase C. Interestingly, this augmented GC repression is lost when the IL-6 promoter 5’AP1 site is deleted (Fig. 3.1.5A). qRT-PCR assay was also used to detect serum regulation of endogenous IL-6 and interleukin 8 (IL-8) genes transcript. In response to four hours Dex treatment, the ligand-bound GR robustly represses the expression of IL-6 and IL-8 in both 10% and 50% serum (Fig. 3.1.5B). Increased serum concentration therefore augments induction of the cytokine genes, but permits a greater repressive effect by GR (Fig. 3.1.5B).

3.1.6 Serum does not alter GR expression, Ser^{211} phosphorylation or localisation

Ser^{211} phosphorylation is a marker of GR activation. To determine serum action on GR expression and phosphorylation, immunoblot analysis was performed using global and phospho state-specific antibodies. In response to Dex treatment, steady state GR expression decreases (Fig. 3.1.6A), a result of targeting for degradation by the proteosome. GR S^{211} phosphorylation was increased after treatment with GCs, which was evident by one hour. Serum did not affect the expression or phosphorylation of the GR.

Without GC treatment, the GR localizes mainly to the cytosol, with evidence of some nuclear GR accumulation (Fig. 3.1.6B). Immunofluorescence assay was applied to investigate serum action on the GR localisation.
Fig. 3.1.5 Serum increases GR transrepression of IL-6 through 5’AP1 domain.

(A) HeLa cells were transiently transfected with different IL-6 plasmids and then cultured in 10% or 50% serum. Following culture in medium containing 100nM PMA (Phorbol 12-myristate 13-acetate, Sigma-Aldrich) overnight to activate IL-6 expression, cells were treated with 100nM Dex for 16 hours and then lysed for luciferase assay. (B) HeLa cells were cultured in 10% or 50% serum medium containing 5ng/ml TNF overnight. Cells were treated with 100nM Dex for 4 hours prior to lysis and RNA extraction. Serum effect on GC repression of interleukin 6 (IL-6) and interleukin 8 (IL-8) was analysed by qRT-PCR. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.05, ** P<0.001.
Fig. 3.1.6 Serum does not alter GR expression, Ser^{211} phosphorylation or localisation. (A) HeLa cells were cultured in either 10% or 50% serum, treated with 100nM Dex for up to 24 hours before lysed protein and analysed by immunoblotting. Images demonstrate endogenous GR expression and Ser^{211} phosphorylation in response to the treatment. Tubulin expression illustrates equivalent loading of protein in each condition. (B) HeLa cells were grown on coverslips and cultured in either 10% or 50% serum overnight, and then fixed for analysis of immunofluorescent microscopy. Cells were tested using specific GR antibody (green). DAPI (blue) illustrates cell nucleus. The unliganded GR localisation in 10% and 50% serum are shown as above. Scale bar equals 25µm. (C) Expression of either GR or phosphoS211 GR protein was quantified by Image J, and data presented as mean and standard error. Both immunoblot and immunofluorescent experiments were repeated on three occasions.
There was a similar subcellular distribution of unliganded GR in either 10% or 50% serum, suggesting serum does not alter GR localisation.

### 3.1.7 GR subcellular trafficking is not altered in 50% serum

To investigate the rate of GR subcellular trafficking in response to treatment with GCs, live cell imaging was completed. GR underwent a rapidly nuclear translocation and mostly localized within nucleus after treatment with Dex after 30mins (Fig. 3.1.7). Serum had no effect on the rate of GR subcellular trafficking.

Further fluorescence microscopy was applied to investigate GR subcellular distribution in response to GC treatment. Similarly, the ligand-bound GR rapidly underwent Ser211 phosphorylation and translocation into the nucleus (Fig. 3.1.8). There was no difference in GR subcellular localisation between 10% and 50% serum, suggesting GR compartmentalization was not affected by serum.

### 3.1.8 Serum does not affect GR intranuclear mobility

To investigate the serum effect on GR intranuclear mobility, fluorescent recovery after photobleaching (FRAP) experiment was completed. The fluorescence intensity of the bleaching region was measured to estimate GR intranuclear mobility (Fig. 3.1.9A). Recovery of the bleached fluorescence was slower in response to treatment with Dex, indicating reduced mobility and therefore increased GR-DNA binding compared to the vehicle treated control (Fig. 3.1.9B). Culture in either 10% or 50% serum, resulted in no change in fluorescent recovery, suggesting that unliganded or liganded GR intranuclear mobility is not altered by serum concentration.
Fig. 3.1.7 Serum does not change GR subcellular trafficking.

HeLa cells were transfected with 5µg hGR-GFP for 16 hours and then cultured in either 10% or 50% serum overnight. Cells were treated with 100nM Dex for one hour before live cell imaging. Images were taken every 5 mins. Scale bar equals 25µm (A). Average time for GR nuclear translocation in either 10% or 50% serum was calculated and presented in (B). Experiments were repeated on three occasions.
Fig. 3.1.8 Serum action on GC dependent subcellular distribution of GR.
HeLa cells were cultured in either 10% or 50% serum overnight and treated with 100nM Dex for 0, 10, 30, 60 mins. Cells were then fixed and analysed using specific antibodies for GR (green) and nuclei counterstained with DAPI (blue). Scale bar equals 25µm. Experiments were repeated three times.
Fig. 3.1.9 Serum does not affect GR intranuclear mobility.
Fluorescence recovery after photobleaching (FRAP) was applied to investigate serum effect on GR intranuclear mobility. HeLa cells were transfected with 7.5 μg of green fluorescent protein (GFP)-tagged human GR overnight and treated with 100nM Dex or DMSO (Veh) as control for 4 hours before analysis. (A) Three steps were involved in this analysis. In nucleus, a representative GR binding region was selected. After bleaching with the laser, fluorescent recovery was quantified to identify GR intranuclear mobility. (B) Fluorescent results were measured with the level before photo bleaching and shown as percentage of fluorescent intensity. Experiments were repeated on three occasions.
3.1.9 Serum effects on endogenous gene expression are not GR specific

As expression of MT1X is transactivated by Zn ions as well as GC, the serum effect of Zn transactivation on MT1X was determined. HeLa cells were treated with either 100 nM Dex or 100 µM ZnSO4 for 4 hours before RNA extraction. Cells cultured in 10% serum presented an 8-fold induction of MT1X after treatment with Dex, whereas a 17-fold induction in response to ZnSO4 treatment (Fig. 3.1.10A). However, these robust transcriptional effects were both dramatically decreased following culture with 50% serum, with only 3 and 2 fold induction of MT1X respectively.

Serum regulates a number of target genes; therefore three serum-activated endogenous genes were investigated. Serum had no effect on expression of SRF. However, cells cultured in 50% serum showed increased expression of early immediate genes c-Jun and c-Fos when compared with 10% serum (Fig. 3.1.10B).

3.1.10 Cyclic changes in GC action

GCs are secreted under strong circadian control, and the action of the GR is dependent on phospho interaction with cryptochrome, a core cellular clock protein (Lamia et al. 2011). Serum shock is used as a synchronizing agent to coordinate the core clock. To investigate circadian effects of serum regulation on GC action, cells were synchronised with 50% serum and then GC induced MMTV response tracked longitudinally (Fig. 3.1.11). The cellular response to GC varied depending on the duration of serum supplement (Fig. 3.1.12B).

Importantly, there was a strict time dependent variation in cellular GC sensitivity following serum shock, with a nadir at +12 hours, and subsequent recovery. This is compatible with serum shock synchronizing cells, and driving cryptochrome expression (Lamia et al. 2011).
Fig. 3.1.10 Serum increases expressions of c-Fos and c-Jun.

(A) HeLa cells were cultured in either 10% or 50% serum overnight, and then treated with either 100nM Dex or 100μM ZnSO4 for 4 hours before RNA purification. (B) HeLa cells were cultured in either 10% or 50% serum overnight. Following RNA extraction three known serum-regulated endogenous genes, including serum response factor (SRF), c-Fos and c-Jun were analysed by qRT-PCR. Graphs show mean ± SEM. Experiments were repeated on three occasions. Comparison made by independent sample T test. * P<0.05, ** P<0.01, *** P<0.001.
Fig. 3.1.11 Experimental design to investigate circadian clock effects on GC action.

Luciferase reporter assay, qRT-PCR and western blot were carried out to investigate cyclic effect on GC action. HeLa cells were specifically seeded in DMEM with 10% fetal bovine serum (FBS). Following day the medium was changed to DMEM with either 10% FBS (Group 1, 2), or 50% FBS for synchronisation (Group 3), one hour before culture in either 50% or 10% charcoal stripped serum (CSS) medium for 20 hours. The cells were treated with 100nM Dex for 4 hours, or DMSO as control (Veh), at defined time points before harvest.
Fig. 3.1.12 Cyclic changes in GC action.

HeLa cells transiently transfected with MMTV-luc reporter were synchronised by culture in 50% FBS for 1 hour, and then treated with 100nM Dex for 4 hours at defined times following synchronisation prior to harvesting. Individual doses (A) and dose response (B) are shown. Graphs show mean ± SEM. Experiments were performed in triplicates, RLU, relative light unit.
Culture in 50% serum blunted the oscillatory response, suggesting that synchronisation was not the mechanism.

3.1.11 Response of GR expression to cell synchronisation.

To investigate the possible role of the cellular circadian clock or its components on GR expression I used a serum shock to entrain the cells. This intervention induces a single oscillation in the expression of the core clock genes, with attendant changes in clock downstream targets. I analysed GR protein expression in the presence and absence of Dex 100nM at different time points. Steady state GR expression was decreased following treatment with 100nM Dex (Fig. 3.1.13). There was no cyclic change in GR expression, and serum had no effect on GR degradation, suggesting a post GR mechanism of action, possibly through cryptochromes expression.

3.1.12 Does serum shock induce circadian clock gene synchronisation?

To investigate whether serum can induce cycling of the core molecular clock, qRT-PCR assays were completed to measure expression of GR regulated circadian genes, including PER1 and PER2. HeLa cells were seeded in 6-well plates culture in DMEM with 10% FBS overnight. Following synchronisation as culture in 50% FBS for one hour, the cells were cultured in 10% charcoal stripped serum for 20 hours, and then collected at indicated times (Fig. 3.1.14). There was no apparent rhythm for the expression of either PER1 or PER2; suggesting serum synchronisation does not induce clock gene oscillation in HeLa cells. In fact most immortalized cell lines demonstrate defects in core circadian clock function, and do not show sustained circadian oscillations when synchronised with serum, or other factors.
Fig. 3.1.13 Cyclic changes in GR expression.

HeLa cells were cultured in 50% serum, or 10% serum, or were synchronised by culture in 50% FBS for one hour, before return to 10% serum containing medium. Cells from the three groups were then treated Dex 100nM for 4 hours prior to harvesting as indicated. Times refer to the time elapsed since synchronisation. (A) Immunoblot of GR expression is shown. (B) GC induced degradation of GR protein in (A) was quantified by Image J software. GC induced reduction is expressed as the vehicle density divided by the GC treated density. Mean and standard error are presented, n=3.
Fig. 3.1.14 Serum synchronisation does not induce clock gene oscillation.

HeLa cells were synchronised by culture in 50% FBS for one hour prior to RNA extraction at the indicated times. Expression of two circadian genes, PER1 and PER2, were tested by qRT-PCR assays. Experiments were repeated three times and present as mean ± SEM.
3.1.13 Serum regulates GC action through MKK7 activated JNK

Serum significantly increased expressions of both c-Fos and c-Jun, as shown before (Fig. 3.1.10). Therefore, further experiments were completed to investigate serum regulation of MAP kinases. Firstly, GC activated expression of MMTV reporter gene was measured in response to treatment with Latrunculin-B, a drug that sequesters G-actin and inhibits SRF activation (Gineitis et al. 2001). There was no change in the serum induction of GC resistance (Fig. 3.1.15A). To explore the role of MAP kinases in mediating repression of GR transactivation, cells were treated with three MAPK inhibitors. Inhibition of JNK prevented the inhibitory effect serum, but inhibition of either ERK or p38i had no impact (Fig. 3.1.15B). To further specify the role of JNK, cells were transduced with either constitutive JNK (MKK7+JNK) or constitutively activated ERK expression vector (as a control). The combined JNK plasmid attenuated GR transactivation, and blocked the serum effect (Fig. 3.1.15C). Overexpression of ERK impaired GC action but did not impair the serum effect (Fig. 3.1.15D).

3.1.14 Activation of JNK by MKK7 diminishes serum impaired GR transactivation

To investigate the role of JNK on endogenous gene regulation again cells were transduced with JNK/MKK7. As predicted both MT1X and FKBP5 were affected by serum concentration, in a JNK dependent manner (Fig. 3.1.16). In contrast serum did not affect PER1 or GILZ. These data support a role for JNK in mediating the selective modulation of GR action by serum.

Further immunoblot analyses were completed to measure serum regulation of the expression and activation of MAP kinases. Serum induced striking activation of JNK (Fig. 3.1.17), but no significant difference was found in neither ERK nor p38 activity.
Fig. 3.1.15 MKK7 activated JNK regulates GC action.

HeLa cells were transiently transfected with MMTV-Luc and GR overnight, followed by treatment with 1µM or 10µM Latrunculin B and 100nM Dex for 16 hours prior to luciferase assay (A). Cells were transiently transfected with MMTV-luc and GR, and then treated with different MAP kinase inhibitors together with 100nM Dex for 6 hours before measurement of luciferase (B). Cells were transiently transfected with MMTV-Luc and GR, together with MKK7 activated JNK plasmid or ERK plasmid overnight. Cells were treated with 100nM Dex, and 10µM JNK inhibitor or ERK inhibitor for 6 hours (C, D).

MAP kinase inhibitors were from Calbiochem, including SB 202190 (p38 inhibitor), PD 98059 (ERK inhibitor) and JNK Inhibitor II (Cat # 420128). Graphs show mean ± SEM. Experiments were performed in triplicates, RLU, relative light unit. Comparison made by independent sample T test. * P<0.05, NS: Not significant.
Fig. 3.1.16 MKK7 activated JNK diminishes serum impaired GR transactivation.

HeLa cells were overexpressed with MKK7 activated JNK, or ERK, and were cultured in either 10% or 50% serum. Cells were treated with 100nM Dex for 4 hours before RNA extraction. Expressions of four GR transactivated endogenous genes were tested, including metallothionein 1X (MT1X), FK506 binding protein5 (FKBP5), glucocorticoid leucine zipper (GILZ) and period circadian protein homolog 1 (PER1). Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.05, NS: Not significant.
Fig. 3.1.17 Serum activates specific JNK isoform.

HeLa cells were cultured in either 10% or 50% serum overnight and then lysed and protein extracts were analysed by immunoblot for expression and phosphorylation of JNK, ERK and p38 MAP kinases. Tubulin expression illustrates equivalent loading of protein in each condition. The activation of the three MAP kinases was expressed as phosphor density divided by total density. Data is presented as mean ± standard error, n=3. * P<0.05
3.2- Cholesterol and GC sensitivity

3.2.1 Impaired GC action is not due to proteins

To characterise the serum component(s) regulating cellular GC sensitivity, further reporter assays were performed. The most abundant protein in serum, albumin, and dose dependently potentiated MMTV-Luc in response to treatment with Dex (Fig. 3.2.1A). This excludes albumin as the mediator of the serum effect.

Serum contains many growth factors that signal through tyrosine kinase pathways. Treatment with genistein, a general inhibitor of tyrosine kinases, inhibited the cellular response to Dex, but had no effect on serum induction of GC resistance (Fig. 3.2.1B). This result excludes growth factor pathways as the mechanism of resistance. Further investigations were carried out to indentify the role of protein(s) in mediating the serum inhibitory effect on GC action. As a serine protease, trypsin is able to digest multiple proteins, and so trypsin was used to degrade the protein components of serum (Nagoshi et al. 2004). There was no difference in GC action between culture in serum with or without trypsin treatment, suggesting that a protein was not the mediator (Fig. 3.2.1C). Serum proteins can also be heat inactivated and therefore the impact of heat treatment of both regular serum and charcoal stripped serum was tested. The serum effect was not abolished by heat inactivation, but interestingly, charcoal dextran removed the serum inhibitor of GC action suggesting involvement of a small molecule (Fig. 3.2.1D).

3.2.2 Lipid potentially impairs GC action

Lipid is an important component of serum, and will be removed by activated charcoal dextran. Reporter gene assays again were applied to characterise the lipid effect on GC action.
A.

![Graph](image1.png)

B.

![Graph](image2.png)

C.

![Bar Graph](image3.png)

D.

![Graph](image4.png)

**Fig. 3.2.1 Impaired GC action is not due to proteins.** HeLa cells were transiently transfected with MMTV-Luc reporter gene and GR overnight, followed by cultured in specific medium as indicated, prior to treatment with Dex for 16 hours before luciferase assay. (A) The culture medium was containing 10% serum, and 0, 4 mg/ml or 40mg/ml bovine serum albumin (BSA). (B) Cells were cultured in medium containing 10nM-100µM Genistein for 4 hours, and then treated with 100 nM Dex for 4 hours before lysis. (C) Serum was treated with trypsin or trypsin inhibitor 2 hours before diluted into 10% or 50% serum for cell culture. (D) Both fetal bovine serum (FBS) and charcoal stripped serum (CSS) were heated at 40, 56, 60 or 70 degree for 1 hour to inactivate proteins. Following centrifugation at 3000 rpm for 1 hour, supernatant fluid was collected and diluted to either 10% or 50% final serum concentration. Cells were treated with 100 nM Dex O/N. Experiments were performed in triplicates, RLU, relative light unit.
As predicted serum concentration impaired GC action, and cell culture in charcoal stripped serum showed significant increase of Dex response (Fig. 3.2.2A). To analyse the possible role of serum lipids in regulating GC action, lipoprotein deficient serum was used, which contains cholesterol 1 mmol/l and triglycerides 0.64 mmol/l (quantified by supplier, Sigma). This compares to average cholesterol concentration in human serum of 5.1 mmol/l, and triglycerides 1.7 mmol/l. The reduced lipoprotein serum further increased GC response (Fig. 3.2.2B).

Simvastatin inhibits cholesterol synthesis, but can not block the cellular uptake of exogenous cholesterol. Cellular response to Dex was intensified with increasing amount of simvastatin, i.e reducing cholesterol production (Fig. 3.2.2C). Importantly, Simvastatin effects were comparable regardless of whether cells were cultured in 10% or 50% serum. This is important as it further implicates cellular cholesterol as a regulator of GC sensitivity.

Cholesterol is the principal sterol synthesized in mammalian cells and plays a key role in maintaining the structure of cell membranes. To investigate the role of cholesterol in GC action, cells were transfected with MMTV-Luc and supplemented with exogenous cholesterol, which was delivered to cells using 10 µg/ml water-soluble cholesterol (molar ratio, 1:6 cholesterol/methyl-β-cyclodextrin) from Sigma (catalog no. C4951) (Rong et al. 2003). Alternatively, cells were treated with methyl-β-cyclodextrin, a cholesterol binding compound which is able to extract cholesterol from cell membranes. As predicted, addition of exogenous cholesterol dramatically reduced Dex response (Fig. 3.2.3). Two concentrations of methyl-β-cyclodextrin were used: a lower concentration (0.5%) matched the amount of methyl-β-cyclodextrin used as the carrier for exogenous cholesterol. The higher concentration (5mM) was used in excess to bind cholesterol in the serum. 0.5% methyl-β-cyclodextrin reversed the impairment of GC action (Fig. 3.2.3A).
**Fig. 3.2.2 Cholesterol deficient serum retains GC action.**

HeLa cells were transiently transfected with MMTV-Luc reporter gene and GR overnight. (A) Cells were cultured in either regular serum or charcoal stripped serum as indicated, and treated with 100nM Dex for 16 hours before lysis. (B) Cells were cultured in regular serum or serum with lipoprotein deficient (Sigma) or charcoal stripped, and then treated with 100nM Dex for 16 hours. (C) Cells were cultured in medium containing 1nM-10µM simvastatin overnight, followed by treatment with 100nM Dex for 16 hours prior to luciferase assay. Graphs show mean ± SEM of triplicate wells. Experiments were repeated on three occasions. RLU, relative light unit. Comparison made by independent sample T test. **P<0.01, * P<0.05
Fig. 3.2.3 Characterise cholesterol effect on GC action

HeLa cells were transiently transfected with MMTV–Luc and GR overnight. (A) Cells were cultured in 10% serum with 10 µg/ml cholesterol (CHOL), and 0.5% methyl-β-cyclodextrin (MCD), respectively. (B) HeLa cells were cultured in 50% serum, together with cholesterol or methyl-β-cyclodextrin as indicated concentration overnight. Then cells were treated with 100nM Dex for 16 hours followed by measurement of luciferase. Graphs (mean ± SEM) show the fold change in luciferase readings from three representative experiments performed in triplicate. RLU, relative light unit. Comparison made by independent sample T test. ** P<0.01, * P<0.05
Meanwhile, cells cultured in 50% serum required treatment with more methyl-β-cyclodextrin compared with 10% serum (Fig. 3.2.3B). Importantly, the higher amount of methyl-β-cyclodextrin was toxic only in 10% culture, likely a result of extraction of the membrane cholesterol necessary for plasma membrane integrity.

3.2.3 Cholesterol selectively impairs GR transactivation of endogenous genes

To investigate the cholesterol effect on GR transactivation, the previous selected endogenous GR transactivated genes were measured, including MT1X, FKBP5, GILZ and PER1. In response to four hours treatment with Dex, exogenous cholesterol selectively impaired expression of MT1X and FKBP5, but had no effect on GILZ or PER1 (Fig. 3.2.4). Similarly, treatment with methyl-β-cyclodextrin abolished the 50% serum impact on GR transactivation of MT1X and FKBP5 (Fig. 3.2.5). There was no cholesterol effect on the baseline expression of these endogenous genes.

3.2.4 Cholesterol increases endogenous gene expression

To investigate cholesterol regulation of endogenous genes, qRT-PCR assays were conducted to measure the expression of SRF, c-Fos and c-Jun in response to treatment with either cholesterol or methyl-β-cyclodextrin (Fig. 3.2.6). Consistent with previous findings, serum concentration regulated expressions of these endogenous genes. Adding exogenous cholesterol increased the expression of both c-Fos and c-Jun in 10% serum, whilst removing cholesterol by methyl-β-cyclodextrin abolished the stimulation of all these genes following culture in 50% serum.
Fig. 3.2.4 Cholesterol selectively impairs GR transactivation
HeLa cells were cultured in 10% serum or 10% serum with 10 µg/ml cholesterol overnight. Cells were harvested after treated with either 100 nM Dex or DMSO as control for 4 hours, and analysed by qRT-PCR. Expressions of four GR transactivated endogenous genes were tested, including MT1X, FKBP5, GILZ and PER1. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.001, NS: Not significant.
Fig. 3.2.5 Cyclodextrin reverses the serum effect on GR transactivation.

HeLa cells were cultured in 50% serum or 50% serum with 5mM methyl-β-cyclodextrin overnight. Cells were harvested after treated with either 100 nM Dex or DMSO as control for 4 hours, and analysed by qRT-PCR. Expressions of MT1X, FKBP5, GILZ and PER1 were tested. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.001, NS: Not significant.
Fig. 3.2.6 Cholesterol increases expressions of serum-regulated endogenous genes.

Cells cultured in 10% or 50% serum were treated with 10 µg/ml cholesterol or 5mM methyl-β-cyclodextrin overnight. Expressions of serum-regulated endogenous genes, including SRF, c-Fos and c-Jun were analysed by qRT-PCR. Graphs show mean ± SEM. Experiments were repeated on three occasions. Comparison made by independent sample T test. * P<0.05, ** P<0.01, *** P<0.001.
3.2.5 Cholesterol stimulates JNK activation

Further immunoblot analyses were performed to detect the cholesterol impact on JNK activation. Treatment with cholesterol stimulated activation of JNK (Fig. 3.2.7). This selective regulation by cholesterol was also observed in response to treatment with methyl-β-cyclodextrin, with a dramatic decrease in the phosphorylation of JNK. In addition, cholesterol had no effect on expression of total JNK.

Membrane cholesterol rafts are often found in caveolae; therefore the effect on the caveolae forming protein caveolin-1 was measured as control. Expression of caveolin-1 was not altered by serum concentration or cholesterol effect.

3.2.6 Cholesterol selectively impairs GR transactivation

To further identify JNK role in GR transactivation, the expression of endogenous GR target genes, including MT1X and FKBP5, was measured by qRT-PCR, after treatment with cholesterol in the presence of a JNK inhibitor. As predicted, cholesterol selectively impaired GR transactivation of MT1X and FKBP5 in response to four hours Dex treatment (Fig. 3.2.8). The impaired transactivation was rescued by supplement with JNK inhibitor, which identifies the mechanism of cholesterol action as being through JNK activation.
Fig. 3.2.7 Cholesterol stimulates JNK activation.

HeLa cells cultured in 10% or 50% serum were treated with 10 µg/ml cholesterol or 5mM methyl-β-cyclodextrin overnight, and then protein lysates were analysed by immunoblot for expressions of JNK and caveolin 1, and phosphorylation of JNK. Tubulin expression illustrates equivalent loading of protein in each condition. Protein expression was quantified using Image J software. Activation was expressed as phospho kinase density divided by total protein density, and plotted as mean and standard error, n=3. * P<0.05
Fig. 3.2.8 Cholesterol impaired GR transactivation is rescued by JNK inhibitor.

HeLa cells were cultured in 10% serum and treated with 10µg/ml cholesterol, together with 10µM JNK inhibitor (Cat # 420128, Calbiochem) for 6 hours. Following treatment with 100 nM Dex for 4 hours, expressions of GR transactivation of either MT1X or FKBP5 were tested by qRT-PCR. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.05, ** P<0.001.
3.3- Hypoxia and GC sensitivity

3.3.1 Hypoxia impairs GR transactivation

Hypoxia is an important microenvironmental factor in inflammatory disease. To investigate the hypoxia effect on GC action, qRT-PCR analysis was carried out to measure transcripts of the GR regulated endogenous genes GILZ, FKBP5, MT1X, PER1, and IGFBP1. HeLa cells were cultured in either normoxia or hypoxia overnight, and then treated with 100nM Dex for four hours again under normoxic or hypoxic conditions before harvest. Transcript levels of these five genes were measured, and GC induced expression of all these genes were dramatically impaired in hypoxia (Fig. 3.3.1).

3.3.2 Hypoxia increases GR transrepression of IL-8

To investigate GR regulation of pro-inflammatory factors, expression of IL-6, IL-8, c-Fos and c-Jun were also measured by qRT-PCR assays. In response to four-hour treatment with Dex, GR transrepression of IL-8 was increased in hypoxia compared with normoxia (Fig. 3.3.2A). GC activated expressions of both c-Fos and c-Jun in normoxia (Fig. 3.3.2B). Meanwhile, hypoxia induced a markedly increase in the baseline expression of either c-Fos or c-Jun.

3.3.3 Hypoxia does not alter GR expression or Ser\(^{211}\) phosphorylation

To determine the effect of hypoxia on GR expression and phosphorylation, immunoblot analysis was performed using global and phospho state-specific antibodies. In response to Dex treatment, steady state GR expression decreases (Fig. 3.3.3). GR phosphorylation was increased after treatment with GCs, which was evident by one hour. Hypoxia did not affect the expression or Ser\(^{211}\) phosphorylation of the GR.
Fig. 3.3.1 Hypoxia impairs GR transactivation.

HeLa cells were cultured in either normoxia or hypoxia overnight. Cells were treated with 100nM Dex for 4 hours prior to lysis and RNA extraction. Expressions of GR transactivation of GILZ, FKBP5, MT1X, PER1 and IGFBP1 were tested. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P<0.05
Fig. 3.3.2 Hypoxia increases GR transrepression of IL-8.
HeLa cells were cultured in either normoxia or hypoxia overnight. Then cells were treated with 100nM Dex for 4 hours before RNA purification. Expressions of IL-6, IL-8, c-Fos and c-Jun were tested. Graphs show mean ± SEM. Experiments were repeated on three occasions. Comparison made by independent sample T test. * P<0.05
Fig. 3.3.3 Hypoxia does not alter GR expression or Ser$^{211}$ phosphorylation.

HeLa cells were cultured in either normoxia or hypoxia, treated with 100nM Dex for 4 hours before lysed protein and analysed by immunoblot. Images demonstrate endogenous GR expression and Ser$^{211}$ phosphorylation in response to the treatment. Tubulin expression illustrates equivalent loading of protein in each condition. Samples from two independent experiments are shown. Expression of either GR or phosphoS211 GR protein was quantified by Image J software, and is presented relative to tubulin protein expression. Data is presented as mean and standard error, n=3.
3.3.4 Hypoxia does not alter GR subcellular trafficking

Fluorescence microscopy was applied to investigate GR subcellular distribution in response to GC treatment. The ligand-bound GR rapidly underwent Ser\textsuperscript{211} phosphorylation and translocation into the nucleus (Fig. 3.3.4). There was no difference in GR localisation between culture under normoxia and hypoxia, suggesting hypoxia has no effect on GR subcellular trafficking.

3.3.5 GR binds to specific regions of DNA in either normoxia or hypoxia

To prepare ChIP-ed samples for Illumina sequencing, several experiments were carried out to validate the approach, and the resulting quality of the samples. Firstly, agarose gel electrophoresis was completed to optimize the size of DNA fragment. The size was less than 500bp, but most was around 200bp (Fig. 3.3.5A). Secondly, using western blot to optimize the efficiency of the GR antibody, used to pull down the GR-DNA complexes (Fig. 3.3.5B). Furthermore, gel electrophoresis was also completed to investigate antibody enrichment of GR bound to the GILZ gene compared with a control antibody to YFP. Treatment with 100nM Dex stimulated the GR enrichment on GILZ (Fig. 3.3.5C). However, hypoxia markedly impaired GR enrichment with or without treatment. Meanwhile, GR enrichment of the ChIP-ed samples was investigated by qRT-PCR assays. Here, GILZ3 was used as positive control for GR enrichment as GR binds strongly to this element in response to GC, whereas GILZ1 was used as a negative control with no GRE. GR enrichment on GILZ3 was increased in response to treatment with 100nM Dex, as expected (Fig. 3.3.5D). This GR enrichment was reduced by hypoxia.
Fig. 3.3.4 Hypoxia does not alter GR subcellular trafficking.

HeLa cells were cultured in normoxia or hypoxia overnight and treated with 100nM Dex for 0, 10, 30, 60 minutes. Cells were then fixed and analyzed using specific antibodies for GR (green) and nuclei counterstained with DAPI (blue). Scale bar equals 25µm. Experiments were repeated on three occasions.
**Fig 3.3.5 Optimisation of ChIP-sequencing samples.**

(A) Gel electrophoresis shows the size of DNA fragment is below 500bp, using HyperLadder IV as marker. (B) Western blot presents most of GR-DNA complexes can be pulled down by GR antibody. YFP, one purified IgG Ab, was used as control. (C) Gel electrophoresis shows enrichment of GR was increased in response to treatment with Dex. This GR enrichment was impaired by hypoxia. (D) qRT-PCR assays present GR enrichment on the GILZ gene was increased in response to 100nM Dex. Similarly, hypoxia reduced GR enrichment on GILZ. GILZ3 was used as positive control for GR enrichment as GR binds strongly to this element in response to GC, whereas GILZ1 was used as a negative control with no GRE.
3.3.5.1 Genome-wide binding profile of GR

To investigate GR binding regions in human genome, HeLa cells were cultured in hypoxia or normoxia overnight and then treated with 100nM Dex or DMSO as vehicle for one hour. Cells were crosslinked, and the genomic occupancy of GR was identified by chromatin immunoprecipitation and then deep sequencing on the Illumina’s HiSeq 2000 analyser. The experiment used purified DNA of GR-immunoprecipitated in hypoxia or normoxia, and input samples as control. Reads were mapped to hg19 and visualized in the UCSC Genome Browser (Kent et al. 2002). Efficiency of the deep sequencing was shown in Table 3-1. Here the comparison between column 2 and 3 shows that, the amount of DNA has no correlation with the number of reads sequenced from each reaction.

Enriched tag regions were identified using MACS 1.4.0 software, in pair wise comparisons between GR-immunoprecipitated and input samples (Zhang et al. 2008). Significant enriched GR peaks were selected using a False Discovery Rate (FDR) filter. The FDR control is a statistics method, and indicates the percent chance of false positive in a given region. Here GR peaks with FDR more than 0.1 were discarded. This led to the identification of GC induced GR binding regions as 435 in hypoxia and 335 in normoxia (Fig. 3.3.6). Only 4 peaks were identified in vehicle samples. The mean peak length in hypoxia was 475 bp, and the median was 477 bp. Under normoxia, regions bound by GR had a mean length and median length of 581 bp and 593 bp, respectively (Fig. 3.3.7).

In order to validate the regions bound by GR from ChIP-seq dataset, ChIP-qPCR experiments were carried out to investigate the enrichment of immunoprecipitated GR in either normoxia or hypoxia (Fig. 3.3.8). Cells were fixed following treatment with either 100nM Dex or DMSO as vehicle for one hour. Input DNA was sheared and immunoprecipitated with antibodies against either GR or YFP (non-specific control). Three positive controls of GR binding regions were tested, which includes FKBP5, GILZ and MT1X, by primers enclosing the summits of each region (Table 2-3).
Table 3-1 Efficiency of ChIP sequencing.

1. Sequencing samples name. 2. DNA amount of either immunoprecipitated or input samples used for library generation. 3. Total number of reads obtained from the sequencing. 4. Number of reads that can be aligned to reference genome. 5. Number of reads which align to the genome after elimination of redundant reads. 6. Ratio of unique mapped reads to total reads.

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<th>1. Sample</th>
<th>2: Amount of DNA [ng]</th>
<th>3: Total number of reads</th>
<th>4: Mapped reads</th>
<th>5: Unique mapped reads</th>
<th>6: Unique mapped reads as % of total</th>
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<td>9573500</td>
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Figure 3.3.6 Identification of GR binding regions in both hypoxia and normoxia.

Peaks called by MACS at p=1e-5 using GR vs. Input datasets as source files. Stringent selection of peaks was carried out with False Discovery Rate (FDR), where data with FDR more or equal to 0.1 were served as the basis for subsequent analyses. Hd, Hypoxic Dex; Hv, Hypoxic Vehicle; Nd, Normoxic Dex; Nv, Normoxic Vehicle.
Fig. 3.3.7 Peak length distribution.

The X axis shows the primary length of peaks in either hypoxia (A) or normoxia (B), in response to treatment with Dex. The Y axis shows the number of peaks.
Fig. 3.3.8 Validation of the ChIP-sequencing dataset.

GR-bound regions were validated in ChIP-qPCR experiments. Peak profiles for the GR samples in either normoxia or hypoxia are shown. FKBP5 (A), GILZ (B) and MT1X (C) were used as positive controls of GR binding. These RefSeq annotated genes are present as the gene direction termed by an arrowhead. Here the Y-axis indicates signal strength of these regions with 0 to 50 reads per million. Bar charts on the right present fold enrichment of immunoprecipitated GR in response to treatment with Dex. Graphs showed three biological repeats of ChIP experiment. * P<0.05, NS: not significant.
All tested regions showed enriched GR signals in response to treatment with Dex, however, the values were in some cases altered in hypoxia (Fig. 3.3.8, bar charts). Similarly, hypoxia induced changes of GR binding regions were also observed in ChIP-seq dataset. Therefore, these results confirm our selection of GR binding regions for further bioinformatics analysis.

### 3.3.5.2 Regions bound by GR are enriched near specific features of the genome

The occupancy of transcription factor within the genome contributes to availability of cofactors and the histone mark landscape, and therefore results in different functional outcome of binding events. To investigate the genomic distribution of GR binding regions, summits from ChIP-seq dataset were used for determining the localisations of the regulatory elements by CEAS (Ji et al. 2006; Shin et al. 2009). In comparison to the total genomic annotation, regions bound by GR were significantly enriched in promoters (up to 3000 bp upstream of the TSS) in both hypoxia and normoxia (Table 3-2). Nearly 50% of the GR binding events were located in distal intergenic regions. This stands in agreement with the findings of a widespread observation by the three-dimensional shortcuts of the genome in diverse mammalian systems (Hakim et al. 2010) (Fig. 3.3.9 and Table 3-2). Also, over 30% of the GR binding regions lay in introns and downstream of genes. However, there is no obvious difference between hypoxia and normoxia.

### 3.3.5.3 The GR binding pattern differs between normoxia and hypoxia

In order to investigate differential GR binding pattern between normoxia and hypoxia, the overlap of regions bound by GR was overlapped by Galaxy. There were 175 of GR peaks in hypoxia overlap intervals identified in normoxia (Fig. 3.3.10A).
Table 3-2 CEAS-derived genomic distribution of GC induced GR-bound regions in either hypoxia (A) or normoxia (B).

### A.

<table>
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<tr>
<th>Location</th>
<th>% GR ChIP-seq</th>
<th>% genome</th>
<th>P value</th>
</tr>
</thead>
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<td>N/S</td>
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<td>0.7</td>
<td>N/S</td>
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<tr>
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### B.

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<td>0.3</td>
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<td>51.5</td>
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Fig. 3.3.9 Genomic distribution of GR binding regions.

Venn diagrams represent the genomic distribution of GC induced GR-bound regions in either hypoxia (A) or normoxia (B); see Table 3-2 for details.
Fig. 3.3.10 Differential analysis of GR binding events between normoxia and hypoxia.

(A) The overlap of GR associated transcription ID between normoxia and hypoxia. (B-C) 595 GR binding sites were aligned according to their summits. Tags from either normoxia (Norm) Dex or hypoxia (Hyp) Dex calculated in every 50-bp bin. The middle point of each panel (indicated by small arrows below) represents the summit of the GR peak. 5 kb upstream and 5 kb downstream around the summit were plotted.
From ChIP-seq dataset in HeLa cells, 260 regions bound by GR were specifically identified in hypoxia, which dictate the specificity of gene transcription.

To further identify the effect of hypoxia on GR binding pattern, the summit dataset of core GR binding events was plotted together with 5 kb upstream and 5 kb downstream, and measured by every 50-bp bin (Fig. 3.3.10B, C). Collectively, most of the core GR binding events in normoxia (293 out of 405 regions) were overlapped with regions occurred in hypoxia. There were 160 GR binding events with significantly stronger binding affinity in normoxia and 260 GR binding events that were stronger in hypoxia.

### 3.3.5.4 GR binding regions associate with functionally distinct groups of genes

So far our findings clearly demonstrate changes in the GR binding pattern and sequence affinity under hypoxia. This discrimination may contribute to different sets of genes being regulated, and consequently induce different biological properties in hypoxia. To investigate the effect of hypoxia on biological function, gene ontology (GO) analysis was performed on the core GR peak dataset using GREAT (McLean et al. 2010). Several GO terms were significantly enriched in both hypoxia and normoxia. Hypoxia induced GR binding identified specific oxygen related biological process, such as the cellular response to oxygen levels, and also depleted several biological terms e.g. inflammatory response (Fig. 3.3.11). Further analysis was carried out on the specific biological process terms in hypoxia. The underlying GR target genes were listed in Table 3-3, which are associated with either cellular response in hypoxia, or inflammatory response in normoxia. This finding suggests hypoxia is associated with different biological processes and warrants further analysis of the role of hypoxia in inflammatory disease.
Fig. 3.3.11 Gene ontology analysis of GR target regions.
The analysis of GR binding regions related gene ontology was carried out using GREAT (McLean et al. 2010). Significantly enriched terms from the biological process are presented as bar chart in either hypoxia (A) or normoxia (B).
Table 3-3 Genetrack associated with cellular response to oxygen levels in hypoxia (A) or inflammatory response in normoxia (B).

**A.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
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</thead>
<tbody>
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<td>BBC3</td>
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<td>VEGFA</td>
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</table>

* Above is the list of genes related to significantly enriched GO terms of cellular response to oxygen levels in hypoxia. Analysis was carried out using GREAT version 2.0.2.

**B.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
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<td>CCR7</td>
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<td>CXCR4</td>
<td>EPHA3</td>
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<td>LY86</td>
<td>LY96</td>
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</tr>
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<td>THBS1</td>
<td>TLR3</td>
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</tr>
</tbody>
</table>

* Above is the list of genes underlying significantly enriched GO terms of inflammatory response in normoxia. Analysis was carried out using GREAT version 2.0.2.
Subsequent disease ontology analysis was performed on the core GR peak dataset in hypoxia, and identified several significant terms associated with inflammatory diseases, e.g. arthritis, using GREAT (Fig. 3.3.12). The associated target genes were summarized (Fig. 3.3.12B). These findings contribute to further analysis on possible mechanism induces arthritis in clinic.

3.3.5.5 Regions bound by GR show different occupancy of transcription factors in hypoxia

The specific locations of GR binding to DNA regulate gene expression. This is potentially due to the frequency and variance of GR binding sites, and the presence of co-modulators. To investigate this variation in hypoxia, the sequences of core GR peak regions were used for motif discovery experiments. Motifs were discovered by a word-based oligo-analysis program (Thomas-Chollier et al. 2012), and subsequent position-weight matrices (PWM) applied to scan sequences and predict binding sites were also compared with known frequency matrices from the JASPAR database (Bryne et al. 2008).

We analysed DNA motifs enrichment, and identified GRE and FOXC1 motifs in the hypoxic GR binding events, and GRE and KLF4 motifs in the normoxic GR binding events (Fig. 3.3.13). The presence of AP1 motif was observed in both hypoxia and normoxia, and as one of the three top discovered motifs in hypoxia. Also, NFIC motif was enriched in GR binding events retained during hypoxia process.
Fig. 3.3.12 Disease ontology analysis of GR target regions in hypoxia.
Disease ontology analysis was completed using GREAT, version 2.0.2. (A) Strongly enriched terms from the disease ontology are represented. (B) List of genes associated with arthritis, and found to be due to GR binding sites specifically identified in hypoxia.
Fig. 3.3.13 Motif discovery analysis.

Enriched motifs within the GR binding events that discriminate between normoxia (A) and hypoxia (B). Sequences of regions were subjected to motif discovery analysis without masking, and discovered motifs were compared with the JASPAR 2010 database (Portales-Casamar et al. 2010).
3.3.6 Hypoxia markedly impairs the expression of KLF4

To investigate the GR coregulators identified by motif analysis, qRT-PCR analysis was carried out to estimate expression of these factors at the transcriptional level. HeLa cells were cultured in either normoxia or hypoxia overnight, and then harvested. Total RNA was purified and converted to cDNA, and then the expression of these five endogenous genes transcript was measured, including KLF4, SP1, FOXC1, FOXL1 and NFIC. Hypoxia significantly decreased the expression of both KLF4 and SP1 transcripts. Also, there is no difference at the transcriptional level of FOXC1, FOXL1 or NFIC between hypoxia and normoxia (Fig. 3.3.14).

Furthermore, immunoblot analysis was performed to investigate hypoxia action on the expression of both SP1 and KLF4 using specific antibodies. Protein lysates from HeLa cells were collected following culture in either normoxic or hypoxic condition overnight. Steady state expression of KLF4 was dramatically decreased in response to hypoxia (Fig. 3.1.15).
Fig. 3.3.14 Hypoxia impairs transcription of both KLF4 and SP1.

HeLa cells were cultured in either normoxia or hypoxia overnight. RNA samples were purified and expressions of KLF4, SP1, FOXC1, FOXL1 and NFIC transcripts were measured. Graphs show mean ± SEM of experiments performed in triplicates and repeated three times. Comparison made by independent sample T test. * P≤0.001
Fig. 3.3.15 Hypoxia significantly decreases KLF4 expression.

HeLa cells were cultured in either normoxia or hypoxia overnight, followed by protein extraction and analysis using immunoblot. Images demonstrate the baseline expression of both KLF4 and SP1 performed in triplicate. Experiments were repeated three times. Data was quantified using Image J software, and normalised to tubulin level. Graphs show mean ± SEM. * P<0.01.
Chapter 4 - Discussion
4.1- Serum shock and GC sensitivity

Therapeutic application of GCs remains the first line of treatment in inflammatory disease. GCs are steroidal ligands for the GR, a well characterised ligand-activated transcription factor, which regulates expression of both anti-inflammatory and pro-inflammatory target genes. The GR is part of the nuclear receptor subfamily, which includes receptors for other steroid hormones, as well as thyroid hormones, vitamins, including vitamin A and D, and products of intermediary metabolism, such as PPAR and LXR.

Optimizing GC treatment is a challenge, a consequence of wide variation in host sensitivity and undesired effects attributed to long-term use. The GR is nearly ubiquitously expressed, and therefore obtaining selectivity of action is difficult to achieve. Developing selective GR modulators to improve the therapeutic index of GC may be a way to improve GC therapy in the clinic (Beck et al. 2009). This is analogous to the approach applied to the oestrogen receptor, where partial agonists show antagonist activity in the breast, and weak agonist activity in the uterus. However, as the precise mechanism by which GCs mediate their range of anti-inflammatory effects remains unclear attempts to identify new molecules with selective action has proved difficult. Notwithstanding this companies including AstraZeneca have partial agonists in clinical trial. The difficulties in applying therapeutic GC in inflammatory disease are only compounded by the observation that the inflammatory environment itself feeds back to modulate GR function.

Several lines of evidence have suggested the importance of understanding how circulating factors within serum affect the GC sensitivity of target cells and tissues. These include (i) cortisol binding globulin (CBG) which regulates the bioactivity of natural GC such as cortisol, but affects synthetic GC such as dexamethasone to a far lessor extent, (ii) circulating cytokines, which are present at high concentrations in inflamed tissues, but also present in the circulating serum and (iii) growth factors which may activate pathways that antagonise GR.
Recently, while developing a sensitive GC bioassay, we identified a striking and unexplained effect of serum on the ability of GR to transactivate a simple reporter gene (Perogamvros et al. 2011). Therefore, I set out to determine how serum regulates target cell GC sensitivity.

The mechanisms by which the activated GR regulates target gene transcription have been studied intensively, due to the therapeutic importance of this target. It remains unclear how a single factor can exert both positive and negative effects on gene expression. The DNA sequences to which the GR binds likely drive conformational changes to the GR, and thereby drive recruitment of a different repertoire of co-modulators. However, some studies have convincingly shown that a single co-modulator, GRIP1, can drive both gene induction, and repression. However, in many cases gene induction requires assembly of a GR homodimer on consensus glucocorticoid response elements (GREs). In contrast, gene repression typically requires monomeric GR, often bound to other transcription factors in a tethered mechanism of action. In this way the monomeric GR appears to impede transactivation by the other transcription factors, often NF-kB, or AP1. However, the apparent simplicity of this arrangement has been called into question with recent studies employing genome wide analysis in an agnostic approach. In this way AP1 and NF-kB binding sites have emerged as serving a pioneer role in enhancing GR recruitment to particular sites to regulate target genes in a cell-type, and context dependent manner (Biddie et al. 2011b; Uhlenhaut et al. 2013; Wiench et al. 2011).

Despite the difficulties of basing drug design on an incompletely understood mechanism of action advances have been made. Some molecules have been described with selective preservation of transrepression, but with loss of transactivation, so-called selective glucocorticoid modulators (SEGRAs). Some of these molecules, such as RU24858 were subsequently shown merely to be low efficacy agonists (Vayssiere et al. 1997), and others appear unlikely to act as ligands for the GR at all (De et al. 2005).
Companies have developed SEGRA programmes the point of clinical trial, and in time it is possible that these will emerge as new treatment options for patients with chronic inflammatory diseases (McMaster et al. 2008b).

In addition to identifying ligands with selective activity it may also be possible to find alternative approaches to enhance transrepression, or diminish transactivation to achieve the same result. Indeed, it remains possible that human disease states arise from such a switch in the potency of GC acting through the two pathways. Certainly, in-vitro transrepression requires significantly lower ligand concentrations than transactivation, but in inflammatory disease models, and patients supraphysiological concentrations of GC are required for effect, thereby exposing the patient to side-effects. For example IFI16 is a molecule activated by type I interferons, and has been shown to selectivity impair transactivation (Berry et al. 2010). Therefore, it is very important to establish a selective model of GC action. Interestingly, serum impaired GC action in a template specific manner, reducing GC activation of MMTV-Luc reporter gene, but not pHH-Luc or TAT-3-Luc. While high serum impaired GR transactivation, it paradoxically augmented GR tranrespression. Therefore, understanding how serum acts on target cells to selectively re-programme how they respond to GC may provide insights into designing rational drug screens for selective GC, or devising adjuvant treatments to improve the therapeutic index on administered GC. Furthermore, the mechanism of this serum effect is transient in action and readily reversible, as evidenced by studies showing that overexpressing the GR co-activator SRC-1, or applying JNK inhibition effectively reversed the inhibitory effect of serum on GC transactivation of MMTV-Luc.

A number of experiments were designed to delineate any direct effects of serum on GR expression, phosphorylation, and trafficking. Serum shock had no effect on steady state GR expression, ligand-modulated stability or phosphorylation on serine 211, a marker of GR activation.
A green fluorescent protein (GFP) tagged GR was used in transfection studies to measure serum effects on nuclear translocation, and intranuclear mobility (McNally et al. 2000; Muller et al. 2001; Rayasam et al. 2005). Here, the GR-GFP model was first applied in live cell imaging and these studies showed that serum had no effect on either steady state or ligand-regulated GR subcellular trafficking. Secondly, FRAP studies were used to track the intranuclear mobility of the GR. FRAP is a live-cell microscopy technique which relies on the susceptibility of GFP to undergo bleaching, or loss of fluorescence in response to high-energy laser light. This results in the emergence of a “spot” lacking fluorescence, where the laser impacts. In this way a spot is bleached in the cell nucleus, and the time taken for new GFP molecules to move into the bleached area, and to restore fluorescence is measured. In response to ligand binding the intranuclear mobility is reduced, and there appears to be a relationship between the affinity of GC ligands and further reductions in intranuclear mobility. This inverse relationship may reflect the stability of the GR bound, and so immobilised on DNA. My studies identified no impact of serum on GR nuclear mobility following GC treatment. All the above results suggest that the gene selective mechanism of action of serum results from parallel activation of other signalling cascades and convergence on target genes based on the presence of transcription factor binding sites for GR, and other factors lying in such a way as to permit cross-talk.

Therefore, my studies moved to analyse other signalling cascades induced by serum exposure to identify potential candidates for functional targeting. Now, serum induces numerous secondary mediators potentially capable of the selective impairment of GR transactivation. Several candidate effector signalling cascades and proteins were investigated, including serum- and glucocorticoid-induced kinase 1 (SGK1), serum response factor (SRF), c-Fos and c-Jun. SGK1 serves as an early regulated gene, and is transcriptionally activated by both serum and GCs (Itani et al. 2002). We observed no significant induction of SGK1 in response to either serum shock or Dex treatment.
This finding is supported by previous studies, which have shown a cell type specific manner of SGK1 regulation (Lee et al. 2001; Reiter et al. 2011). Recently, SRF has been highlighted as an important mediator of a number of diseases involving in cardiovascular system, cancer, and central nervous system ect (Miano 2010). SRF exerts its regulatory effects through binding to and transactivation of serum response elements (SREs) within target genes, e.g. c-Fos (Cen et al. 2004; Johansen et al. 1995; Wang et al. 2000). SRF activation of the c-Fos SRE requires the cofactor p62TCF, which contains transcriptional activation domain activated by MAPK. The c-Jun protein is post-translationally activated by JNK binding and subsequent phosphorylation of Ser-63 and Ser-73 with the N terminal domain (Adler et al. 1992; Hibi et al. 1993; Pulverer et al. 1991). JNK itself is activated by the upstream kinases MKK4 and MKK7 (Davis 2000). My studies suggest that activation of the JNK pathways mimics the serum-effect on gene transactivation (inhibition), and also prevents any further serum attenuation. In keeping with this emerging role for JNK, a JNKi completely abolished the serum effect on GC sensitivity. This implies JNK specific regulation of GC action during serum shock. Immunoblot analysis confirmed increased JNK activation (phosphorylation) after serum shock. These results are again supportive of emerging evidence for JNK regulation in modulating GR action, for example in the bronchial epithelium (Loke et al. 2006).

A number of papers have highlighted the complex cross-talk between GR and AP1, the dimeric transcription factor comprising JUN and FOS family members in a variety of homo and heterodimeric forms. Studies previously defined a mutually antagonistic mode of interaction (Schule et al. 1990), but more recently, AP1 has emerged as a necessary pioneer factor for GR access to target sites in chromatin (Biddie et al. 2011b; Uhlenhaut et al. 2013). Here, in order to address the issue of template selectivity, I investigated the impact of high serum on several GR transactivated genes.
Only the expression of MT1X and FKBP5 were attenuated by serum. The impairment of MT1X was reversed by 4 hours washout into low serum media, but FKBP5 remained insensitive even after washout. I analysed the sequence of the MT1X gene, and identified both AP1 and GRE binding sites in close proximity (Velazquez et al. 2002; Viarengo et al. 2000). In response to heat shock, which induces c-Fos production, and activation AP1, there was reduced GC transactivation of MT1X (Andrews et al. 1987). Interestingly, Olsson and co-workers analysed the sequence of the metallothionein A gene promoter in rainbow trout and also demonstrated a GRE adjacent to an AP1 site (Fig. 4.1) (Olsson et al. 1995). The binding of AP1 possibly blocks, or interferes with the function of the GR on its GRE. This may explain the reversible effect of serum.

In summary, my results from this portion of work show a selective model of reversible, and gene target selective GC resistance mediated through activation of the JNK pathway and possible cross-talk between AP1 and GR for template specific binding, or subsequent transcriptional regulatory function. This model may provide insight into how inflammatory pathways feed back to modify GC action.
Fig 4.1 Structure of metallothionein A promoter. Transcription binding sites are shown for IL-6 (NF-IL-6), glucocorticoid response element (GRE), activation protein-1 (AP1), and metal-regulatory transcription factor-1 [MTF1; a metal response element (MRE)].

Adapted from (Bury et al. 2008)
4.2- Cholesterol and GC sensitivity

Having identified the mechanism by which serum regulates target cell GC sensitivity, I next embarked on a series of studies to identify the serum factor(s) responsible for conferring the effect. Serum is a highly complex material, containing transport, nutrient, and signalling molecules of protein, lipid, and small molecule classes, some of which require complex carrier molecules and complexes to permit solubility in aqueous media.

Having identified a serum effect activating JNK as the final mechanism acting on GR signalling I started by considering likely serum components capable of activating JNK. Activation of JNK signalling as a generalized response to osmotic stress was a possibility. As albumin is the most abundant serum protein, I first characterised its effect on GC action in target cells using the MMTV luciferase assay that I had previously shown was serum-sensitive. My finding suggests that albumin is not an inhibitor, but rather acted as an activator of GC action, in target cells. These findings effectively exclude simple osmotic stress as a factor underlying the acquisition of GC resistance.

Another possible serum factor acting on target cells was the abundant presence of growth factors. These growth factors act through a broad family of transmembrane receptors, but share intracellular tyrosine kinase activation as a common signalling transducer. Genistein serves as a general tyrosine kinase antagonist, and therefore offers the opportunity to block the actions of all serum-contained growth factors (Akiyama et al. 1987). Increasing genistein concentration alone impaired GC transactivation of the MMTV reporter gene; however, the serum effect was not reversed. This result indicates that although serum contains numerous growth factors potentially capable of activating intracellular JNK, and AP1 in fact these factors do not appear capable of altering target cell GC sensitivity. More generalized protein effects were examined by heat inactivation (Ayache et al. 2006; Bruinink et al. 2004; Masuda et al. 2012; Matmati et al. 2013).
This approach relies on using heat treatment to denature, or disrupt the tertiary structure of serum proteins. In this way their biological function can be disrupted. The approach is generally applicable, and is agnostic of which protein(s) may be required for the serum effect on GC response. Heat-treated serum was just as effective as temperature-controlled serum in driving the selective impairment of GC action. This further supports the genistein studies, and further weakens the case for serum proteins as causative agents. However, as heat-treatment can have a differential effect on protein denaturation depending on the size, and complexity of the tertiary structures involved, I also adopted a further and different approach. I used trypsin to digest serum proteins. To avoid carry-over effects of trypsin on the target cells I followed the trypsin treatment with trypsin inhibitor, and as a further control treated aliquots of serum first with the trypsin inhibitor and then the trypsin (Nagoshi et al. 2004; Rivera-Burgos et al. 2012; Slysz et al. 2003). Again, the serum effect was not abolished in response to protein digestion. Overall, these results imply that serum shock impaired GC action was not mediated by a protein factor present in the serum.

Recently, an association between circulating cortisol concentrations and serum lipid components has been investigated. Fraser et al observed a positive correlation between cortisol secretion and body mass index (BMI) in healthy population, with circulating cortisol concentration being inversely associated with high-density lipoprotein (HDL)-cholesterol. This was proposed to be due to altered peripheral cholesterol metabolism (Fraser et al. 1999). However, the association may also suggest a causal or regulatory role of circulating lipid species on GC sensitivity. My experimental results suggest that serum lipid components are capable to regulating target cell GC sensitivity. I showed that the response to GC was augmented in cell cultured in charcoal dextran treated (delipidated) serum when compared with normal serum. Furthermore, lipoprotein deficient serum also increased GC action, to an even greater extent than the charcoal stripped serum.
Of note, GR polymorphisms have been shown to be associated with variation in the serum concentrations of various lipid components. I now propose that these changes in lipid composition may in turn affect GC action (van Rossum et al. 2004). Similarly, interventions to target cellular cholesterol metabolism also revealed interesting effects. Simvastatin, which inhibits cellular cholesterol synthesis from acetate, was found to affect GC action, greatly increasing the expression of the MMTV reporter genes in response to GC. This is of direct relevance to patient care, as statin therapy is widely used in medical care of patients with atherosclerosis, and at least 1% of the UK population hold a regular repeat prescription for GC medication, indicating the widespread co-prescription of these two drug classes.

My findings highlight the role serum cholesterol, acting through JNK activation to regulate GC sensitivity. I found that total expression of JNK was not changed by cholesterol; however, JNK activation was increased. The increase in JNK activation seen in response to high serum concentrations was rapidly reversed by co-incubation with methyl-β-cyclodextrin. The use of methyl-β-cyclodextrin to absorb cholesterol, and prevent access of the cholesterol to target cells is supportive of a direct role for free cholesterol in regulating target cell GC response.

Previous reports have identified a link between oxidised lipoprotein particles and activation of AKT in the vascular endothelium (Prieur et al. 2010). However, my studies were conducted with fetal calf serum, which contains minimal oxidised LDL, and also addition of free cholesterol, which had a similar effect to that seen with serum. Therefore there is no evidence of a role of oxidised LDL in mediating the serum effect on GC sensitivity.

Methyl-β-cyclodextrin is a cholesterol binding drug. It is widely used experimentally to remove cholesterol and disrupt lipid rafts within the plasma membrane (Brown et al. 2000; Simons et al. 2000).
Treatment with methyl-β-cyclodextrin increased GC transactivation in normal culture and rescued the impairment of GC action by high serum. Interestingly, culture in high serum required higher concentrations of methyl-β-cyclodextrin, suggesting that the increased concentration was required to bind the increased cholesterol content of 50% serum cell culture medium.

In line with this, I found that application of high concentrations of methyl-β-cyclodextrin to cultures in low serum was highly toxic, a likely consequence of removal of membrane cholesterol and loss of membrane integrity. Furthermore, treatment with methyl-β-cyclodextrin increased the expression of serum effector proteins, including SRF, c-Fos and c-Jun. In fact, the methyl-β-cyclodextrin effect on JNK activation is supported by investigation of cell responses using plitidepsin. This molecule is an extremely potent inducer of apoptosis, and acts through the sustained activation of JNK. Plitidepsin is a cyclic depsipeptide, and has been well characterised to against a variety of tumors in phase II clinical trials. In MDA-MB-231 cells, treatment with methyl-β-cyclodextrin inhibits plitidepsin induced activation of JNK, whilst less cholesterol was detected in plitidepsin-resistant HeLa cells (Gonzalez-Santiago et al. 2006; Suarez et al. 2006).

Emerging evidence has implicated LDL in the selective regulation of JNK isoforms. So far, ten JNK protein isoforms derived from three encoding genes have been identified (Gupta et al. 1996). Therefore, characterising the different JNK isoforms for their impact on GR action is critical to specify the activation of JNK pathway involving in cholesterol induced GC resistance. In hematopoietic cells, JNK1 knockout confers lower risk of insulin resistance associated with diet-induced obesity (Solinas et al. 2007). JNK2 is also involved in metabolic regulation, however, its function might be masked by regulatory interaction with JNK1 (Tuncman et al. 2006). JNK activation is potentiated with scaffolding proteins, e.g. JIP1 and JIP2 proteins in mice, which contain a PTB domain that is capable of interacting with the LDL receptor-related protein (Gotthardt et al. 2000).
LDL signalling involves in multiple kinase activities, such as JNK2/3, JNK–c-Jun and ERK1/2 (Prieur et al. 2010). LDL activation of ERK1/2 may contribute to partially regulation of ERKi, in rapid cholesterol impaired GR transactivation of MT1X within 6 hours (Zhu et al. 1998).

Taken together, my results present in this section illustrate an important role of cholesterol for JNK activation, indicate that GR signalling is secondary modulated by the membrane compartment, and stress that cholesterol is crucial to trigger GC resistance (Fig. 4.2).
**Fig. 4.2** Cholesterol results in a selective GC resistance model. Excess cholesterol induces JNK activation, and subsequently pro-inflammatory factor AP1 is activated. AP1 is able to directly interacting with GR and results in GC resistance. AP1: Activator protein 1; GRE: Glucocorticoid responsive elements.
4.3- Hypoxia and GC sensitivity

Recent advances in genomic technologies provide a broader opportunity to study multiple levels of control of gene expression, which is a highly intricate process. In this section, my results describe a global model of GR regulation of transcription in hypoxia. I have identified the complete map of GR binding to the genome, in a single cell type, in either hypoxia or normoxia. I used HeLa cells for these studies, a robust, widely used human epithelial cell type. Compared with normal cells, HeLa cells produce telomerase, which elongates the telomeres after chromosomes have been copied, and consequently these cells are able to multiply continuously. This cell line is not only characterised by GR expression and responds well to steroids in culture, but also appears to be capable of tolerating hypoxia and/or acidic pH.

I found that most GR binding sites were located in gene distal regions of the genome, and identified enrichment for the binding sites of other transcription factors under the identified peaks of GR recruitment. This analysis revealed KLF4 binding sites in normoxia, that were lost as cells were cultured in hypoxia. In contrast, I found that the binding sites favoured by GR in hypoxia were enriched for binding by FOXC1. This finding suggests that the differential activity of these two transcription factors may explain the change in GR cistrome regulated by cellular oxygen tension.

In this manner I propose that oxygen tension differentially regulates transcription factors, including KLF4 and FOXC1, which, in turn, serve as “pioneer” factors opening up a state-specific GR cistrome, and thereby permitting efficient integration at the cellular level of environmental oxygen sensing, and glucocorticoid action. As the GR cistrome appears to be very different between cell types this additional level of fine-tuning the spectrum of glucocorticoid action may explain why previous reports addressing the effects of hypoxia on glucocortioid function have yielded conflicting results. My findings suggest that the
issue is not a global change in the amplitude of glucocorticoid response, but rather a variation in the selection of GR regulated target genes.

The global binding of GR to DNA is well studied. Most GR binding events are found at great distances from gene promoters, but are not proximal to TSSs, with distribution of almost evenly between upstream and downstream sequences (So et al. 2007). The binding of transcription factors to distal regions is principally associated with the control of lineage- and tissue-specific gene expression (Kleinjan et al. 2005; Ong et al. 2011). This phenomenon stands in accord with my findings, which present nearly 50% GR binding sites located at distal regions. GR occupies only a small subset of potential binding sites, and controls the expression of genes which are associated with specific cellular functions in a given cell type.

Indeed, the overlapped GC regulated expression profile among observed cell lines is modest (John et al. 2009; Rogatsky et al. 2003; Wang et al. 2004). The factors regulating access of GR and other transcription factors to DNA remain unclear. It seems likely that epigenetic regulation is key, which includes nucleosome positioning, histone modification, and possibly DNA modification as well. For example, nucleosome positioning may contribute to recruitment of transcription factors to DNA binding sites. For active genes, several positioned nucleosomes are well characterised in the promoters. Also, when potential binding sites move to more accessible positions, this can activate the localisation of nucleosomes within the functional enhancers (Schones et al. 2008). One interesting possibility to explain the distribution of GR binding sites is that proximal promoters tend to be relatively more conserved, to maintain the assembly of the general transcription machinery, but more distal regions are possibly more prone to mutation, to exert their great biological plasticity.

Gene ontology (GO) analysis is a powerful tool, to analyse the integrated impact of multiple genetic regulatory events. Here, I used identified DNA binding events across the
entire genome, to assess the functional significance of cis-regulatory regions (McLean et al. 2010). My results from the GO term categories of GR regulated genes in either hypoxia or normoxia are discussed here. Genes encoding components of inflammatory response and positive regulation of apoptosis are generally controlled by the normoxic GR cistrome. On the other hand, the hypoxic GR cistrome is strongly associated with cellular response to oxygen levels and plasma membrane long-chain fatty acid transport. This suggests an alteration of GR function in hypoxia. Also, hypoxia is an important environmental factor in inflammatory disease. Therefore, I further annotated sequencing data with disease ontology, to establish hypoxia associated disease. Not surprisingly, genes annotated in hypoxia are dramatically associated with inflammatory diseases, e.g. arthritis.

Genome-wide GR regulatory elements are composed of multiple factor binding sites. These neighbouring sequences serve as an important factor to GR occupancy of a specific site (Bolton et al. 2007; So et al. 2007). In fact, most of the GR potential binding sites are never occupied, and some are recognized in a cell- and tissue-specific manner (Wiench et al. 2011). Development of deep DNA sequencing techniques revealed diverse GRE motifs. However, the individual loci, which feature the GRE sequences, remain evolutionarily conserved and are considered as a good predictor of GR occupancy (So et al. 2008). I used motif discovery analysis to identify peak sequences associated transcription factor binding motifs. As predicted GRE motifs were firstly discovered in either hypoxia or normoxia. HIF1α is a well studied transcription factor linked to hypoxia (Elsby et al. 2009). However, accordant to my analysis, HIF1α motif was not one of the Top 5 significant enriched motifs occurred in hypoxia. Results from this thesis exhibit that hypoxia induced a decrease of krueppel-like factor 4 (KLF4) occupancy, and instead increased recruitment of forkhead box C1 (FOXC1) adjacent to GR binding regions. This functional switch between KLF4 and FOXC1 serves as potential mechanism to explain impaired GR transactivation in hypoxia.
Accordingly, the interaction between GR and KLF4 has been observed in vivo, where corticosteroid treatment, and expression of KLF4 coordinately accelerated barrier acquisition, and it was observed that genes regulated by GR and KLF4 showed significant overlap (Patel et al. 2006). KLF4, as a zinc finger transcription factor, is expressed in several types of epithelial tissues, e.g. intestine (Shields et al. 1996). KLF4 has important impact in decreasing cell proliferation, migration or differentiation (Ghaleb et al. 2011).

Recent studies have characterised the tumour suppressive function of KLF4 in the gastrointestinal tract (Choi et al. 2006; Zhao et al. 2004). Also, KLF4 serves as a suppressor of hypoxia-stimulated tumour metastasis, which has been observed in pancreatic and colorectal cancer (Chen et al. 2012; Wei et al. 2008). One putative mechanism underlying KLF4 function is its interaction with CREB-binding protein. The CREB-binding protein, also known as CBP, displays histone acetyltransferase activity, and therefore is responsible for transcriptional stimulation (Korzus et al. 1998; Kouzarides 1999; Martinez-Balbas et al. 1998). So far plausible evidence has identified the role of CBP as a co-activator for many transcription factors, including p53, a well-known tumour suppressor, through its binding to the p53 transactivation domains 9aaTADs (Gu et al. 1997; Piskacek et al. 2007). Interestingly, it has been identified that cross-talk exists between p53 and GR under physiological stresses. A potential mechanism is the interaction of p53 with Hdm2 may contribute to the ubiquitylation, and so degradation of GR, although in my studies I observed no change in GR protein abundance (Korzus et al. 1998; Kouzarides 1999; Martinez-Balbas et al. 1998). In summary, the above findings potentially establish a KLF4 relevant model to explain altered GR function in hypoxia.

Forkhead box proteins are a group of transcription factors with a crucial impact during the progression and invasion of cancer. There is emerging evidence to reveal the role of forkhead box proteins as therapeutic targets in treatment of cancer, and also serve as biomarkers to prognoses patients’ survival (Myatt et al. 2007). Forkhead transcription
factors are also well-established pioneer factors for other steroid receptors, including perhaps most notably the estrogen receptor. Indeed, analysis of hormone sensitive and refractory breast cancer found the forkhead transcription factors were the major predictor of estrogen receptor cistrome, and indeed patient prognosis (Ross-Innes et al. 2012). Recent studies have established models specifically suggesting that FOXC1 regulates increase invasion and metastasis in progression of breast and endometrial cancer (Bloushtain-Qimron et al. 2008; Chung et al. 2012; Ray et al. 2010; Sizemore et al. 2012; Wang et al. 2012). However, there is little evidence linking GR to FOXC1. In addition, there is little to suggest a role for FOXC1 in regulating the cellular response to hypoxia. Therefore, results from this thesis point out an important acknowledge gap, and provide a novel sight for future research.

In accordance with all the available literature, my results as discussed in this section identify the existence of several determinants involved in the specific pattern of DNA binding by the GR in hypoxia. This distinct GR binding pattern results in direction of the GR to specific genes, opening up a hypoxia-specific GR cistrome. I found that there was no significant alteration in either the genomic-wide distribution of GR binding domains, or the sequence of GRE motif by hypoxia. Therefore, a large proportion of GR binding regions remain in hypoxia, which is responsible for regular biological process of cells, e.g. proliferation. Recruitment of GR to specific DNA sequences may be affected by post translational modification of the GR, perhaps to the non DNA binding domains. In fact, this change of DNA binding affinity has been observed in different transcription factors, e.g. the mouse Six3 factor (Jeong et al. 2008). The expression level of a given transcription factor is another well-characterised determinant affecting the selection of its DNA binding sites (Boros et al. 2009; Hsiao et al. 2002). However, this model is not applicable to explain the alteration in GC action by hypoxia, where I show no change in GR expression, or modification between hypoxia and normoxia using immunoblot.
Finally, the binding of GR to DNA is known to be regulated by the interaction between GR and other transcription factors, offering opportunities for signal cross-talk, and integration of multiple input signals at the genome level. Indeed several critical GR pioneer factors capable of directing the recruitment of GR to distinct binding regions (Biddie et al. 2011b; Collingwood et al. 1999; Wiench et al. 2011).

Also, results from this section highlight the role for both KLF4 and FOXC1 as novel GR pioneer factors, operating as a switch mechanism between normoxia and hypoxia. The switch between FOXC1 and KLF4 suggests an attractive molecular mechanism to explain how hypoxia regulates GR action (Fig. 4.3). Here, my findings further develop the viewpoint that the availability, and activation state of pioneer transcription factors, but not the specific DNA sequence preference, are more important for directing the GR cistrome in a state-dependent manner (Li et al. 2011).
Fig. 4.3 Hypoxia induces a functional switch between KLF4 and FOXC1. As shown above, less oxygen in hypoxia results in recruitment of FOXC1 (B), instead of KLF4 (A), which contributes to different regulation of gene targets.
4.4- Future directions

Serum model in altered glucocorticoid sensitivity

(1) As a primary determinant of GC action GR occupancy of regulatory sites in the genome is critical to understanding the cellular response. Therefore, to investigate further the mechanisms mediating serum shock regulation of GR function I would propose to apply genomic techniques, including ChIP or ChIP-seq. In this way I would seek to find evidence for pioneer factor activity specific to this cellular response. I anticipate, based on my data so far, that transcription factors activated by the MAPK signalling network, including AP1 would be natural candidates to explore at an early stage.

(2) Furthermore, it might be interesting to detect the function of scaffolding proteins, e.g. Islet-brain 1 (IB1), in cholesterol induced JNK activation. As scaffolding proteins are known to interact with lipoprotein receptors it is possible that cellular effects result from such interaction. It is also noteworthy that at sites of injury and inflammation cell death results in massive local release of lipid material, potentially serving as a locally acting signal to alter GC action.

(3) JNK is activated by either MKK4 or MKK7. Therefore, it would be of interest to study MKK7 activated JNK pathway in serum shock induced GC resistance. Meanwhile, further studies should investigate specific role of either LDL or HDL in GC action, to indentify clinical biomarker of GC resistance.

(4) Finally, in order to establish a model of serum shock activated GC resistance, more cell lines should be investigated. Also it would be valuable to establish an animal model of altered cholesterol homeostasis to investigate the implications for GC action, to pave the way for translational studies in humans.
Hypoxia model in altered glucocorticoid sensitivity

The results presented in this section point out the existence of a complex mechanism underlying GR transcriptional regulation, through interaction with partner proteins. However, there are several questions remain unanswered.

(1) ChIP-sequencing experiments for KLF4 and FOXC1 should be carried out, in order to better address their specific co-operation with GR in regulating expression of gene targets. Also, it’s of interest to establish a model with either KLF4 or FOXC1 knockout, to investigate how this manipulation regulates the efficiency of GR recruitment to DNA.

(2) Hypoxia decreases KLF4 transcription and expression; therefore it’s of interest to determine KLF4 association with inflammatory diseases in tissues by immunochemistry assays. Moreover, as its well-characterised role in hypoxia, HIF1α can be used as a marker for the preparation of hypoxic tissue. Finally, the FOXC1 transcript was not significantly increased in hypoxia, due to the complex pathway involved in the regulation of transcription. Therefore, investigation the expression of microRNA, e.g. miR-103/107, may provide a novel view to explain this complex mechanism.
In summary, this thesis describes the mechanisms of two models related to changes in environmental factors result in altered GC sensitivity. Firstly, I establish that serum shock induces a selective impairment of GC transactivation, through JNK activation. Recent studies found a possible role of cytokines or growth factors in regulating cellular GC response, but my studies identified serum lipid components, specifically cholesterol in mediating the effect. Secondly, I identify hypoxia impairs GC action due to the switch of GR coregulator KLF4 to FOXC1. This adds to the current understanding of GR regulation of target genes, where despite similar intrinsic binding motif preferences, the recruitment of coregulator determine GR ultimate DNA occupancy, and the resulting effect on regulation of functionally distinct groups of genes.
Chapter 5 - References


Chapter 6 - Appendices
Detailed description of genes listed in Table 3-3 and Figure 3.3.12.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
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<tbody>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette sub-family B member 1</td>
</tr>
<tr>
<td>ADAMTS7</td>
<td>A disintegrin and metalloproteinase with thrombospondin type 1 motif, 7</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Beta-2 adrenergic receptor</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>Arachidonate 5-lipoxygenase-activating protein</td>
</tr>
<tr>
<td>ANKH</td>
<td>Progressive ankylosis protein homolog</td>
</tr>
<tr>
<td>ANXA1</td>
<td>Annexin A1 (or lipocortin I)</td>
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<tr>
<td>B4GALT1</td>
<td>Beta-1,4-galactosyltransferase 1</td>
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<tr>
<td>BBC3</td>
<td>Bcl-2-binding component 3 (or p53 upregulated modulator of apoptosis (PUMA))</td>
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<td>BMPR1B</td>
<td>Bone morphogenetic protein receptor type-1B</td>
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<tr>
<td>CAMK1D</td>
<td>Calcium/calmodulin-dependent protein kinase ID</td>
</tr>
<tr>
<td>CAV1</td>
<td>Caveolin 1</td>
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<tr>
<td>CCR4</td>
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<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7 (or CD197)</td>
</tr>
<tr>
<td>CD180</td>
<td>CD180 antigen</td>
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<tr>
<td>CD2</td>
<td>Cluster of differentiation 2</td>
</tr>
<tr>
<td>CD34</td>
<td>CD34 molecule</td>
</tr>
<tr>
<td>CD83</td>
<td>Cluster of Differentiation 83</td>
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<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1</td>
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<tr>
<td>CEBPB</td>
<td>CCAAT/enhancer-binding protein beta</td>
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<tr>
<td>CIITA</td>
<td>Class II, major histocompatibility complex, transactivator</td>
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<td>CST3</td>
<td>Cystatin 3</td>
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<tr>
<td>CXCL13</td>
<td>C-X-C motif chemokine 13 (or B lymphocyte chemoattractant)</td>
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<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
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<tr>
<td>EBAG9</td>
<td>Receptor-binding cancer antigen expressed on SiSo cells</td>
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<td>EDN1</td>
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<td>EPAS1</td>
<td>Endothelial PAS domain-containing protein 1 (or hypoxia-inducible factor-2alpha)</td>
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<td>EPHA3</td>
<td>Ephrin type-A receptor 3</td>
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<td>FKBP5</td>
<td>FK506 binding protein 5</td>
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<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
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<tr>
<td>GADD45B</td>
<td>Growth arrest and DNA-damage-inducible, beta</td>
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<td>GDF5</td>
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<td>GGTL5</td>
<td>Gamma-glutamyltransferase 5</td>
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<td>HIF1A</td>
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<td>HIPK2</td>
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<td>IL1R1</td>
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<td>Integrin beta-6</td>
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<tr>
<td>JAG1</td>
<td>Jagged 1 (or CD339)</td>
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<td>JUN</td>
<td>Jun proto-oncogene</td>
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<tr>
<td>KCNIP3</td>
<td>Calcinilin (voltage-gated potassium (Kv) channel-interacting proteins)</td>
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<tr>
<td>LY86</td>
<td>Lymphocyte antigen 86</td>
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<tr>
<td>LY96</td>
<td>Lymphocyte antigen 96</td>
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<tr>
<td>MAF</td>
<td>V-maf musculoaponeurotic fibrosarcoma oncogene homolog</td>
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<tr>
<td>MECOM</td>
<td>MDS1 and EVI1 complex locus</td>
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<tr>
<td>NAMPT</td>
<td>Nicotinamide phosphoribosyltransferase</td>
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<tr>
<td>NFKBIA</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
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<tr>
<td>NRID1</td>
<td>Rev-ErbA alpha</td>
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<tr>
<td>OSM</td>
<td>Oncostatin M</td>
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<td>P4HA2</td>
<td>Prolyl 4-hydroxylase subunit alpha-2</td>
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<tr>
<td>PLA2G4A</td>
<td>Cytosolic phospholipase A2</td>
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<td>PRKCE</td>
<td>Protein kinase C epsilon type</td>
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<tr>
<td>PRKCH</td>
<td>Protein kinase C eta type</td>
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<tr>
<td>PROK2</td>
<td>Prokineticin 2</td>
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<tr>
<td>PTGER2</td>
<td>Prostaglandin E2 receptor</td>
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<tr>
<td>PTPN1</td>
<td>Tyrosine-protein phosphatase non-receptor type 1</td>
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<tr>
<td>RHOB</td>
<td>Ras homolog gene family, member B</td>
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<tr>
<td>RXRA</td>
<td>Retinoid X receptor alpha</td>
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<tr>
<td>SCG2</td>
<td>Secretogranin II (chromogranin C)</td>
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<tr>
<td>SEMA7A</td>
<td>Semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group) (or CD108)</td>
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<td>SLC22A5</td>
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<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
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<td>SOX9</td>
<td>SRY (sex determining region Y)-box 9</td>
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<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
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<td>TGFBR2</td>
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<td>THBS1</td>
<td>Thrombospondin 1</td>
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<tr>
<td>TLR3</td>
<td>Toll-like receptor 3</td>
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<tr>
<td>TRIL</td>
<td>TLR4 interactor with leucine rich repeats</td>
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<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VIPR2</td>
<td>Vasoactive intestinal peptide receptor 2</td>
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Publications

Work from this thesis has been published, submitted or prepared to submit to journals as below.

