

**The Transcription Factor ERG is a Gatekeeper of Endothelial Cell
Homeostasis**

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**This thesis is submitted to Imperial College London for the degree of
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**Imperial College London
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Declaration of originality

I, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Endothelial cells (EC) maintain homeostasis through the tightly controlled balance between expression of protective genes and repression of pro-inflammatory genes, and loss of this balance can cause endothelial dysfunction, leading to inflammatory diseases including atherosclerosis. We have previously shown that the ETS transcription factor Erg is involved in maintaining EC homeostasis through transactivation of genes involved in key functions including angiogenesis, migration and survival. In addition to the role for Erg as a transcriptional activator, recent genome wide gene expression analysis has also highlighted a role for Erg in the repression of multiple pro-inflammatory genes. In this Thesis I describe a novel mechanism for Erg-mediated repression of these pro-inflammatory genes using ICAM-1 as a model. We identify two ETS binding sites (EBS) within the ICAM-1 promoter (EBS-118 and -181) which are required for Erg mediated repression. One of these EBS is within a functional NF- κ B binding site. We show that the increase in ICAM-1 expression upon Erg inhibition is NF- κ B dependent, and that Erg prevents NF- κ B p65 from binding to the ICAM-1 promoter, suggesting a direct mechanism of interference. Gene Set Enrichment Analysis (GSEA) of transcriptome profiles of Erg and NF- κ B dependent genes, together with chromatin immunoprecipitation (ChIP) studies, reveals that this mechanism is common to other pro-inflammatory genes, including cIAP2 and IL8. We investigate the role of chromatin modifying enzymes and histone modifications in Erg-mediated repression and show that in quiescent EC the ICAM-1 promoter is also bound by the histone methyltransferase ESET, and by HDAC1, both indicators of a repressed chromatin structure. Moreover, *in silico* data on histone modifications suggest that in quiescent EC the ICAM-1 promoter is in a repressed conformation. The results from this Thesis suggest that Erg acts as a gatekeeper to inhibit transactivation of pro-inflammatory genes in quiescent EC, providing an important barrier to protect against inappropriate endothelial activation.

For My Family

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Publications arising from this Thesis

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Sperone A, *Dryden NH*, Birdsey GM, Madden L, Johns M, Evans PC, Mason JC, Haskard DO, Boyle JJ, Paleolog EM, Randi AM. The transcription factor Erg inhibits vascular inflammation by repressing NF-kappaB activation and proinflammatory gene expression in endothelial cells. *Arterioscler.Thromb.Vasc.Biol.* 2011;31:142-150

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Chapter One

Introduction

1.1. Inflammation

Inflammation is the protective response of the immune system to infection or injury, and results in the characteristic symptoms first identified by Celsus around 40AD of rubor, tumor, calor, and dolor; or redness, swelling, heat, and pain. Inflammation is in the most part beneficial and enables the elimination of harmful substances followed by resolution of inflammation (Medzhitov, 2008).

Inflammation is triggered by stimuli released from pathogens, broken cells and signals from neurons (Ley et al., 2010). Sentinel macrophages and mast cells present in the affected tissue recognise and phagocytose pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) (Minnicozzi et al., 2011). PAMPs and DAMPs have common molecular structures which are non-evolving, therefore can be recognized by cells of the innate immune system such as granulocytes and macrophages. PAMPs include lipopolysaccharide (LPS), and low density lipoproteins (LDL) modified by oxidation or glycosylation, while DAMPs include heat shock proteins and uric acid. They are recognized by cell surface receptors on neutrophils and macrophages such as scavenging receptors which trigger endocytosis, or toll like receptors (TLR) which trigger signalling pathways, for example the nuclear factor kappa B (NF- κ B) and mitogen activated protein kinase (MAPK) pathways (Takeda et al., 2003). Ligation of these receptors results in expression of paracoids and autocoids including cytokines such as tumour necrosis factor (TNF) α , products of the complement cascade, reactive oxygen species (ROS) and chemokines. This leads to endothelial cell (EC) activation and recruitment of leukocytes from the blood stream. Leukocytes remove the inflammatory stimuli before they apoptose and the affected tissue is healed (Serhan and Savill, 2005). Chronic signalling causes the receptors that trigger the innate immune response to be desensitised; therefore, the acute inflammatory response is finite and relatively short lived. However, if the inflammatory stimulus persists and is not cleared by the innate immune response, T and B lymphocytes of the adaptive immune response are recruited to the site of injury (Libby, 2007). T-lymphocytes recognize antigens presented on the surface of leukocytes and this induces the expression of cytokines which have a pleiotropic effect on B-lymphocyte maturation, and induce leukocytes to express more ROS, pro-inflammatory cytokines and lipids. Resolution of inflammation occurs through the cessation and destruction of the inflammatory signal, and the apoptosis and clearance of activated inflammatory cells (Maskrey et al., 2011; Pober and Sessa, 2007). However, if

inflammatory signals persist or resolution mechanisms fail, the result is chronic inflammation. The pathological effects of chronic inflammation are characteristic of many diseases including atherosclerosis, diabetes, pulmonary arterial hypertension (PAH), some cancers such as breast and pancreatic cancer, multiple sclerosis and rheumatoid arthritis (Libby et al., 2011; Gregor and Hotamisligil, 2011; Crosswhite and Sun, 2010; Ben-Neriah and Karin, 2011; Lassmann, 2008; Mantovani et al., 2008; Firestein, 2005).

1.1.1. Inflammation in atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial wall, which causes stenosis of arteries and can lead to ischemia such as myocardial infarction or peripheral vascular disease (Ross, 1999). Atherosclerosis is thought to be a result of the chronic inflammatory response to stimuli associated with dyslipidemia, hypertension, diabetes or insulin resistance, and pro-inflammatory mediators triggered by environmental factors such as smoking (Libby et al., 2010). The haemodynamics of the artery are also an important factor in determining sites prone to atherosclerosis, with protection in regions of high flow and susceptibility in regions of disturbed flow such as the branches and curvatures of the medium sized muscular arteries of the arterial tree (Gimbrone, Jr. et al., 2000).

1.1.2. Mechanisms of inflammation in atherosclerosis

The study of the mechanisms involved in atherosclerotic plaque formation is an evolving field with much knowledge gained from the analysis of *in vitro* and *in vivo* models; however, many mechanisms are still to be translated clinically. EC are thought to be activated by oxidised LDL and inflammatory cytokines which trigger the expression cell surface adhesion molecules such as intercellular adhesion molecules, selectins and vascular-cell adhesion molecules (Pober and Sessa, 2007). These receptors bind ligands expressed on leukocytes which facilitate their extravasation to the intima. Leukocytes, consisting mostly of monocytes, migrate into the arterial intima attracted by monocytes chemoattractant protein (MCP)-1, while monocyte-colony stimulating factor (M-CSF) promotes their maturation to macrophages. These macrophages then take up oxidised LDL and become lipid laden foam cells. This forms the first sign of the atheroma formation, the fatty streak (Ross, 1999). Cytokines expressed by leukocytes within the plaque, including interferon (IFN)- γ and TNF- α , amplify the inflammatory signal to activate EC and smooth muscle cells (SMC) resulting in the recruitment of larger numbers of leukocytes. Further steps towards lesion formation include the migration of SMC from the tunica media to join resident SMC in the intima. In

response to stimuli including platelet derived growth factor, SMC proliferate and express extracellular matrix (ECM) proteins such as collagen, elastin, and proteoglycans to form a fibrous cap (Figure 1.1)(Doran et al., 2008). In the advanced atherosclerotic plaque, lipid laden macrophages and SMC apoptose and release cell debris, such as tissue factor (TF) and lipids, to form a highly pro-thrombotic necrotic core (Tabas, 2010). Destabilisation of the fibrous cap is thought to result from activated inflammatory cells and EC expressing enzymes such as matrix metalloproteases (MMP), these enzymes digest ECM proteins and weaken the fibrous cap of the plaque leaving it susceptible to rupture. While the plaque itself may cause ischemia through a narrowing of the artery, acute cardiovascular events are thought to be the result of plaque rupture which leads to a release of pro-thrombotic contents of the plaque lipid core including TF. These react with blood components including platelets and form a thrombus that may either occlude the artery locally at the site of atherosclerosis, or may detach and lodge in a distal part of the vasculature causing an embolism (Libby, 1998).

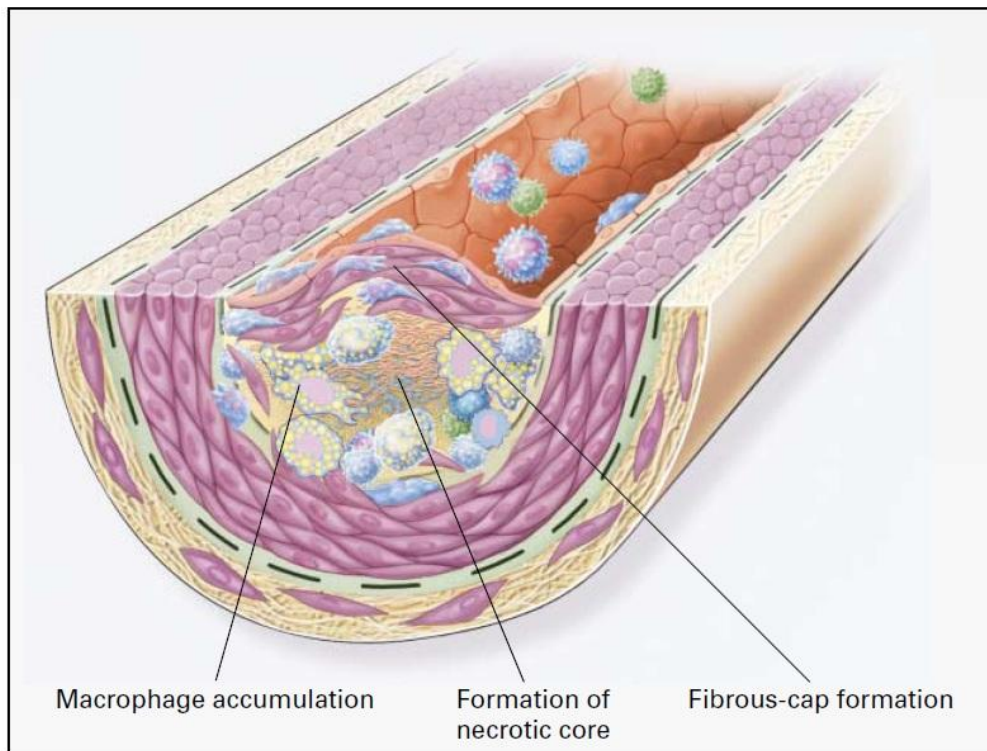


Figure 1.1 Formation of an Advanced, Complicated Lesion of Atherosclerosis. As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leukocytes, lipid, and debris, which may form a necrotic core. The principal factors associated with macrophage accumulation include macrophage colony-stimulating factor, monocyte chemotactic protein 1, and oxidized low-density lipoprotein. The necrotic core represents the results of apoptosis and necrosis, increased proteolytic activity, and lipid accumulation. (Modified from (Ross, 1999))

1.1.3. The resting endothelium

EC maintain homeostasis through constitutively expressing protective genes which inhibit leukocyte recruitment and thrombosis while at the same time repressing pro-thrombotic and pro-inflammatory genes. EC express endothelial nitric oxide synthase (NOS-3) and this generates low levels of nitric oxide (NO) which acts as an inhibitor of platelet and leukocyte recruitment, and also affects vascular SMC to modulate vascular tone (Dimmeler and Zeiher, 1999). Homeostatic EC also express genes that inhibit coagulation and thrombosis such as tissue factor pathway inhibitors (TFPI), heparin sulphate, proteoglycans and thrombomodulin (Cines et al., 1998) and also express junctional molecules to maintain junctional integrity between EC (Dejana, 2004). Additionally, EC inhibit the expression of cell surface pro-inflammatory and cell adhesion genes including E-selectin, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM)-1 and at the same time store molecules involved in leukocyte recruitment including interleukin (IL)-8, P-selectin and Von Willebrand factor (VWF), within Weibel Palade bodies (WPB) (Pober and Sessa, 2007).

1.1.4. Activation of endothelium during inflammation

Upon exposure to inflammatory stimuli, signalling pathways are activated to enable leukocyte recruitment. EC become activated by pro-inflammatory cytokines expressed in the blood and the surrounding tissue, resulting in vasodilation and increased vascular permeability (Cai and Harrison, 2000). Inflammatory signals may also induce EC injury or apoptosis, leading to the release of pro-thrombotic molecules such as TF, resulting in blood coagulation and thrombus formation (Manly et al., 2011). EC are activated by either an immediate type I inflammatory response which lasts 20-30 mins, or a delayed type II response (Pober and Sessa, 2007). Signalling towards a type I response is triggered by the binding of ligands such as angiotensin (Ang)-II to the G-protein coupled receptors (GPCR) or TLR on the surface of EC. This results in an increase in intracellular Ca^{2+} which is released from endoplasmic reticulum (ER). The increase in Ca^{2+} results in an increase in conversion of arachadonic acid to the vasodilator prostacyclin by cyclo-oxygenase (COX)1 and activation of NOS-2 to generate high levels of NO, which disrupts EC junctions and causes increased permeability. Ligation of GPCR also triggers signalling through the Rho GTPases, which in combination with increased Ca^{2+} mediates actin remodelling and disruption of adherens and tight junctions, and the exocytosis of WPB to release their contents of pro-thrombotic molecules such as VWF, as well as the release of P-selectin to the surface of EC (Goligorsky

et al., 2009). If the source of the activation continues, receptors such as GPCR and TLR become desensitised to inflammatory signalling, and the type II EC activation takes over.

Type II EC activation is induced by mediators released from leukocytes such as TNF- α , IL-1 and IFN- γ . These stimuli trigger the activation of transcription factors including NF- κ B, activator protein (AP)-1 and signal transducers and activators of transcription (STAT)1, which transactivate pro-inflammatory genes (Pober and Sessa, 2007). The type II response also activates COX II which, like COX I, induces the release of prostacyclin to mediate vasodilation (Mitchell and Warner, 2006).

1.1.5. Leukocyte recruitment

Activated EC play a central role in the recruitment of leukocytes to sites of inflammation. Leukocytes activated by pro-inflammatory stimuli are recruited through interaction with ligands on EC. The process of recruitment goes through a multi step adhesion cascade requiring pro-inflammatory cytokines, chemokines and an increased expression of adhesion molecules on EC (Ley et al., 2007; Langer and Chavakis, 2009) (Figure 1.2). EC, activated by pro-inflammatory stimuli, bind leukocytes through the interaction between selectins and their ligands. EC express E and P-selectin while leukocytes express L-selectin (Vestweber, 1992). The ligands of selectins are glycoproteins expressed on both EC and leukocytes they include P-selectin glycosylated ligand (PSGL)1, and E-selectin ligand (ESL)1 expressed on leukocytes (Stegmaler et al., 1995; Zarbock et al., 2008), whilst CD34 and mucosal vascular addressin cell adhesion molecule (MadCAM-1) are expressed on EC (Berg et al., 1993). This interaction in the presence of shear stress results in leukocyte rolling along the endothelium. Leukocytes lacking ligands for EC may also be recruited to activated endothelium by binding to other activated leukocytes (Ley et al., 2007). The binding of selectins to their ligands triggers inside-out signalling in leukocytes to induce integrin activation (Zarbock et al., 2008). Leukocyte rolling and the presence of chemokines such as IL-8 (CXCL8) also triggers signalling events in EC resulting in the increased expression of adhesion molecules of the immunoglobulin family, including VCAM-1 and ICAM-1, which bind the integrins very late antigen (VLA)-4 and lymphocyte-function associated antigen (LFA)-1 respectively. This interaction, in the presence of chemokines from EC and platelets, halts rolling and induces leukocyte arrest, leukocyte flattening and adhesion (Kinashi, 2005). Leukocytes then migrate through the endothelium in either a para-cellular or trans-cellular route through a process requiring adhesion molecules such as ICAM-1 and platelet endothelial cell adhesion

molecule (PECAM)-1 (Muller et al., 1993). Leukocyte adhesion drives EC signalling and the restructuring of junctions between adjacent EC. Junctional molecules that do not ligate leukocytes such as vascular endothelial (VE)-Cadherin are relocated away from junctions, while junctional molecules that carry ligands of leukocytes such as PECAM-1 or the junctional adhesion molecules (JAM) molecules (A, B and C) are moved to the luminal surface of EC junctions to form a gradient which promotes transmigration (Woodfin et al., 2009). Once leukocytes have passed through the EC junctions they must then migrate through the basement membrane which consists of molecules including laminins and collagen type IV. Leukocytes are thought to migrate via regions of the basement membrane that have a lower concentration of these proteins (Wang et al., 2006b). Leukocytes then migrate to the site of injury in the surrounding tissue.

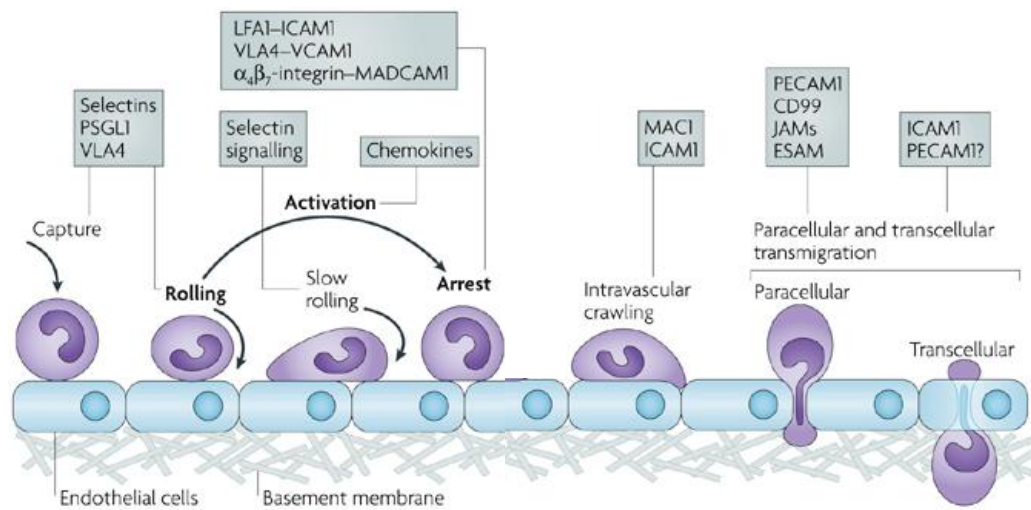


Figure 1.2 The leukocyte adhesion cascade. The four main steps are rolling, activation and arrest and transmigration. Key molecules involved in each step are indicated in boxes. ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as α L β 2-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; VCAM-1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α 4 β 1-integrin). (Modified from (Ley et al., 2007))

1.2. Inflammatory stimuli involved in leukocytes recruitment and downstream targets

Leukocyte trafficking through the endothelium requires activation of both EC and leukocytes. Many cytokines have been identified to have a role in this activation but perhaps some of the best characterised are the cytokines TNF- α , IL-1 and IFN- γ .

1.2.1. TNF- α

TNF- α plays a central role in the activation and amplification of inflammation. The TNF- α protein is the founding and most characterised member of a superfamily of 19 cytokines with diverse expression targets and functions (Aggarwal, 2003). It is a ubiquitous pleiotropic cytokine that binds two differentially expressed receptors, tumour necrosis factor receptor (TNFR) 1 and TNFR2. TNF- α induces expression of pro-inflammatory genes in EC including ICAM-1, VCAM-1 and IL-8 and triggers the activation of leukocytes. TNF- α is predominantly expressed by macrophages, T and B-lymphocytes and natural killer (NK) cells where it is expressed and recruited to the cell membrane and either remains part of the cell membrane as 26 kDa protein or is cleaved by TNF- α converting enzyme (TACE) to a 17 kDa soluble protein. Cell membrane bound TNF- α binds TNFR2 and soluble TNF- α binds TNFR1 (Grell et al., 1995). TNFR1 is expressed ubiquitously but TNFR2 expression is restricted to EC and immune cells (Aggarwal, 2003). Binding of TNF- α to TNFR induces TNFR relocation to cell membrane lipid rafts to form two alternative complexes. The first complex signals towards activation of AP-1, NF- κ B, and p38 mitogen activated protein kinase (MAPK) pathways and is anti-apoptotic, and the second complex signals towards apoptosis. Formation of the first complex leads to inhibition of the second complex (John, 2011). The signalling mechanisms that lead from TNF- α binding to its receptor to activation of NF- κ B are particularly pertinent to this thesis and so will be discussed in more detail in section 1.3.3

1.2.2. IL-1

The IL-1 family of cytokines are important regulators of inflammation, they consist of IL-1 α , IL-1 β and IL-1Ra (Dinarello, 2009). IL-1 family members are expressed in many cells including EC, epithelial cells and immune cells induced by pro-inflammatory stimuli including TNF- α , IFN- α and IFN- β ; IL-1 is also self induced. IL-1 family members are recognised by the IL1 receptors (IL1R), which are members of the TLR/IL1R receptor

family. These receptors all share a conserved signalling mechanism. Ligand bound IL-1R or TLR receptors recruit the adaptor protein MyD88, as well as phosphorylating and activating the IL-1R associated kinase (IRAK), and inhibitor of NF- κ B kinase β (IKK β) (Weber et al., 2010). This leads to the activation of the NF- κ B signalling pathway as well as activation of the MAPK pathway which triggers activation of p38 kinase and Janus kinase (JNK), which in turn activates transcription factors such as the AP-1 transcription factor family. IL-1 stimulation induces the expression of many pro-inflammatory genes. IL-1 and TNF- α both activate similar pathways and share a number of target genes, including IL-8, MCP-1 and COX-2; however, expression is often cell and stimulus specific.

1.2.3. IFN- γ

The chemokine IFN- γ is a 20 kDa protein expressed by T-cells, NK cells, and macrophages activated by inflammatory stimuli. IFN- γ is highly expressed in atherosclerotic plaques which has led to analysis of inhibitors of IFN- γ as potential therapeutic targets (Gotsman and Lichtman, 2007). IFN- γ plays an important role in the regulation of pro-inflammatory genes such as the major histocompatibility complex (MHC) class I molecules, TNF- α , VCAM-1, ICAM-1 and COX1 (Schroder et al., 2004). IFN- γ dimers bind to the IFN- γ receptors (IFR) α and IFR β and this drives oligomerisation of the receptor molecules (Marsters et al., 1995). Each receptor molecule binds a JAK tyrosine kinase which is activated upon receptor oligomerisation. This allows the transcription factor STAT-1 to bind to the receptor and become phosphorylated in a JAK/STAT dependent manner (Darnell, Jr. et al., 1994). Phosphorylated STAT-1 forms dimers which translocate to the nucleus where STAT-1 binds γ -activated sites (GAS) within the promoters of target genes containing the consensus sequence TTNCNNNAA (where N is any nucleotide). Upon ligand binding the IFR is internalised and the IFN- γ is degraded in the lysosome leaving the receptor components to be recycled to the cell membrane (Bach et al., 1997).

1.3. Role of the transcription factor NF- κ B in inflammation

The signalling pathways activated by IL-1 and TNF- α trigger the activation of one of the most important pro-inflammatory transcription factor families, the NF- κ B family.

1.3.1. NF- κ B family members and structure

The NF- κ B family of transcription factors include NF- κ B1 (p50 and its precursor p105), NF- κ B2 (p52 and its precursor p100), RelA/p65, c-Rel, and RelB (Figure 1.3). They are

characterised by the conserved amino-terminal Rel homology domain (RHD) which is responsible for DNA binding, dimerisation, inhibitor binding and nuclear localisation (Oeckinghaus and Ghosh, 2009). p65, RelB and c-Rel possess the carboxy-terminal transactivation domain which is responsible for transcriptional activation. With the exception of RelB, all NF- κ B family members can form homodimers, as well as heterodimers, with the most well studied dimer being the p65/p50 heterodimer. p65 and p50 are expressed ubiquitously, while RelB is restricted to the thymus, lymph nodes and Peyer's patches, and the expression of c-Rel is restricted to haematopoietic cells and lymphocytes (Thanos and Maniatis, 1995; Liou and Hsia, 2003).

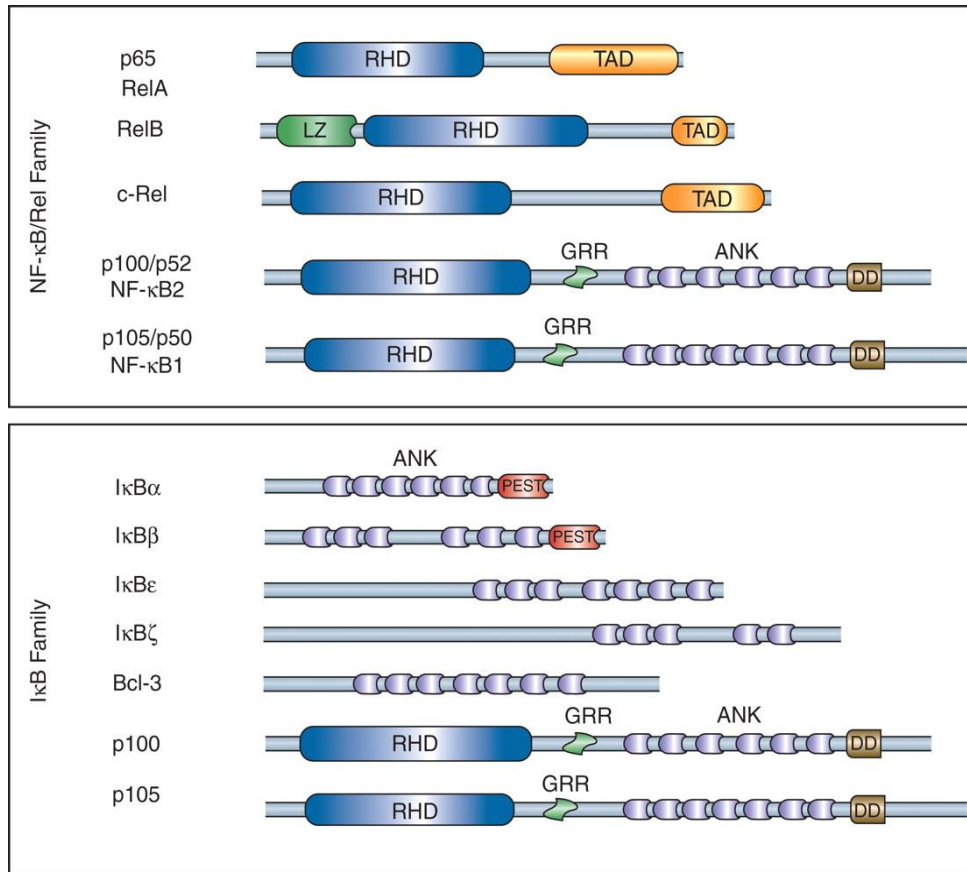


Figure 1.3 Members of the NF-κB and IκB protein families. The Rel homology domain (RHD) is characteristic for the NF-κB proteins, whereas IκB proteins contain ankyrin repeats (ANK) typical for this protein family. The domains that typify each protein are indicated schematically. DD, death domain; GRR, glycine-rich region; LZ, leucine-zipper; PEST, proline-, glutamic acid-, serine-, and threonine-rich region; TAD, transactivation domain. (Modified from (Oeckinghaus and Ghosh, 2009))

1.3.2. NF- κ B consensus sequences and specificity

The canonical NF- κ B consensus sequence is GGGRNNTYCC (where R=G or A, Y = C or T, and N is any nucleotide) also called the class I sequence; however the consensus sequence in target gene regulatory regions can vary and be bound preferentially by NF- κ B dimer combinations, as different dimer combinations have higher affinity for variations in this sequence. For example p50 and p52 homodimers prefer an 11 base pair (bp) consensus, while p50/p52 heterodimers prefer a 10 bp consensus and homodimers of RelA and cRel bind a 9 bp consensus sequence (Huang et al., 2005). Some NF- κ B target genes contain the class II consensus sequence of HGGARNYYCC (where H = A, C or T). The class II sequence lacks the 5'-guanine, and x-ray crystallography has shown this is required for p50 homodimer binding; the class II consensus is preferentially bound by cRel/RelA heterodimers (Parry and Mackman, 1994).

1.3.3. Regulation of NF- κ B

In resting cells inactive NF- κ B is predominantly localised in the cytoplasm where it is sequestered by members of the family of inhibitor of kappa B (I κ B) proteins, including, I κ B α , β , ϵ , BCL-3 and p105 and p100 which when cleaved become NF- κ B p50 and p52. I κ B binding to NF- κ B masks the nuclear localisation signal and DNA binding site of NF- κ B, therefore preventing its nuclear translocation. As I κ B α and I κ B ϵ only mask one nuclear localisation signal of the dimer, NF- κ B is constantly shuttled between the nucleus and cytoplasm (Ghosh and Karin, 2002). However, the I κ B α nuclear export signal is stronger than the nuclear localisation signal and therefore more NF- κ B is found in the cytoplasm of resting cells. I κ B β on the other hand can block both nuclear localisation signals on an NF- κ B dimer and so retains NF- κ B in the cytoplasm. Inflammatory stimuli trigger a signaling cascade which results in phosphorylation, ubiquitination and proteasome-mediated degradation of I κ B proteins allowing nuclear translocation of NF- κ B (Oeckinghaus and Ghosh, 2009). Inhibition of NF- κ B is controlled by feedback regulation as the I κ B genes are downstream targets of NF- κ B, and their induction after NF- κ B activation results in a repression of NF- κ B activity through binding and nuclear export of NF- κ B.

NF- κ B activity is induced by stimuli such as LPS and peptidoglycans, and pro-inflammatory cytokines including TNF- α and IL-1, which are recognised through TLR/IL1R or TNFR and this activation results in signalling through the canonical or non-canonical NF- κ B pathways (Vucic et al., 2011).

The classical or canonical NF- κ B pathway is activated by ligation of TLR/IL1R, TNFR and TCR which trigger complex downstream signalling pathways to converge at the point of phosphorylation of I κ B kinase (IKK) complex, which contains IKK α , β and NEMO (Figure 1.4). There are multiple possible upstream mediators of IKK phosphorylation; however, the exact mechanism is unknown. Phosphorylated IKK then phosphorylates I κ B molecules which are ubiquitinated then targeted for processing or degradation. p100 and p105 are processed to p50 and p52, and the remaining I κ B proteins are processed for degradation by the 26S proteasome, resulting in unbound NF- κ B translocating to the nucleus (Liu and Chen, 2011).

Nuclear translocation however, is not the only requirement for NF- κ B activity. NF- κ B transactivates a variety of target genes with different transcriptional activity kinetics, which are regulated in part by post translational modifications. DNA binding ability and transcriptional activity of NF- κ B family members are also regulated by post translational modifications, via I κ B independent mechanisms. Most studies of post translational modification of NF- κ B have focused on p65. Phosphorylation of p65 occurs on multiple serine and threonine residues by a variety of kinases; these affect the interaction with DNA and co-factors including co-activators such as cyclic AMP-response element binding protein (CREB)-binding protein (CBP) and co-repressors such as histone deacetylases (HDAC) (Perkins, 2006).

The transcriptional activity of NF- κ B family members is mediated through their interaction with chromatin modifying enzymes. Phosphorylated NF- κ B p65 has been shown to interact with histone acetyl transferases (HAT) including CBP and its related protein p300, which are involved in transcriptional activation (Zhong et al., 2002; Hoberg et al., 2006; Perkins et al., 1997), while p65/p50 dimers have been shown to also bind to HDAC 1, 2 and 3 which are mediators of transcriptional repression, in resting cells (Ashburner et al., 2001). Additionally, interaction with HDAC3 has been found to affect the acetylation of NF- κ B p65 and its association with I κ B α (Hoberg et al., 2006).

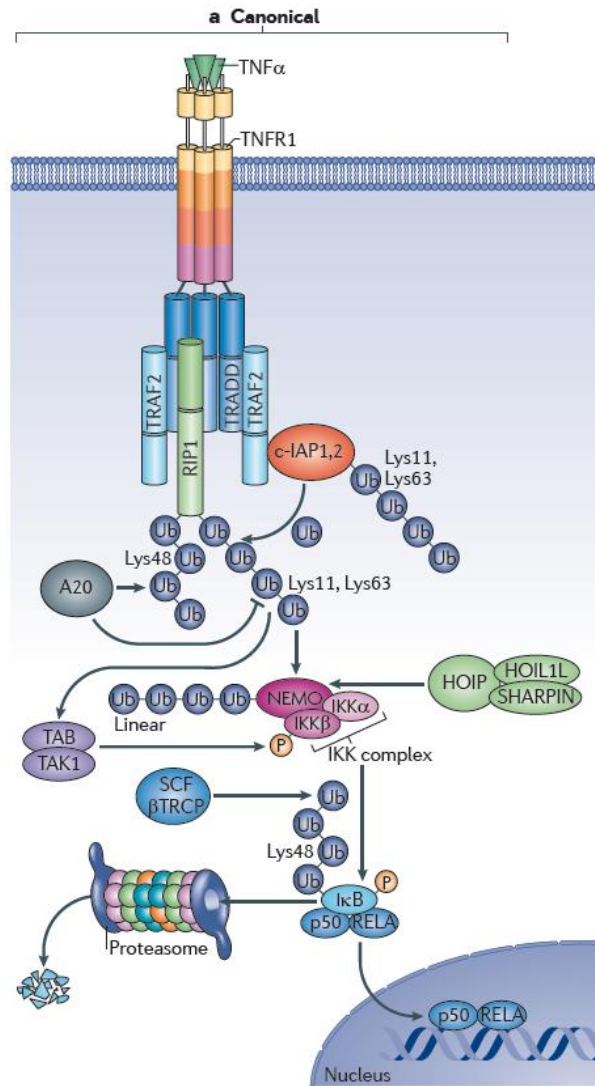


Figure 1.4 Canonical NF-κB signaling pathway. During the TNFα-stimulated canonical pathway, the E3 ligases cellular inhibitor of apoptosis 1 (c-IAP1) and c-IAP2 promote Lys11 or Lys63 polyubiquitylation of receptor-interacting protein 1 (RIP1), and also of themselves. These c-IAP-polymerized ubiquitin chains form a platform for the recruitment and activation of the inhibitor of NF-κB (IκB) kinase (IKK) complex, which phosphorylates the inhibitory NF-κB subunit IκB and the linear ubiquitin chain assembly complex (LUBAC), allowing HOIL1-interacting protein (HOIP)-mediated linear ubiquitylation of NF-κB essential modulator (NEMO), which ensures persistent activation of NF-κB signalling. Phosphorylation of IκB triggers its Lys48-linked ubiquitylation and subsequent degradation, thus liberating the NF-κB p50–p65(RelA) dimers, and allowing them to translocate to the nucleus and activate gene expression. A20-mediated deubiquitylation of Lys63 chains on RIP1 and A20-mediated Lys48 ubiquitylation of RIP1 can attenuate this signalling pathway.; HOIL1L, haeme-oxidized IRP2 ubiquitin ligase 1; SCF^{βTRCP}, Skp–cullin–F-box–βTRCP; SHARPIN, SHANK-associated RH domain-interacting protein, TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1; TNFR1, TNF receptor 1; TRADD, TNFR1-associated DEATH domain protein; Ub, ubiquitin. (Modified from (Vucic et al., 2011)).

1.4. The inflammatory molecule ICAM-1

An important pro-inflammatory target gene of NF- κ B transcriptional activity is the cell surface molecule intercellular adhesion molecule (ICAM)-1.

1.4.1. Structure and mechanisms of action in EC

ICAM-1 is an inducible cell surface glycoprotein belonging to the immunoglobulin super family with a size between 80 to 114 kDa depending on cell type and glycosylation (Van de Stolpe and Van der Saag, 1996). The extracellular portion of ICAM-1 consists of 5 repeats of an IgG-like domain forming a hinged rod which is bound to the cell by a hydrophobic transmembrane region followed by a cytoplasmic tail (Figure 1.5). ICAM-1 is expressed on EC, epithelial cells, fibroblasts, leukocytes and keratinocytes (Dustin et al., 1986; Dougherty et al., 1988). In EC, ICAM-1 binds to two integrins, LFA-1 and macrophage antigen (Mac)-1 on leukocytes, which support adhesion and transmigration of activated leukocytes through the endothelial monolayer at sites of inflammation. ICAM-1 dimerisation enhances the affinity for the LFA-1 receptor (Miller et al., 1995).

Inhibition of ICAM-1 has been found to inhibit inflammation. For instance blockade of ICAM-1 inhibits leukocyte adhesion to EC *in vitro* (Kyan-Aung et al., 1991). ICAM-1 is up-regulated on EC at sites susceptible to atherosclerosis along with other pro-inflammatory adhesion molecules. The significance of the role of ICAM-1 in atherosclerosis has been demonstrated by a number of *in vivo* studies. For example in lesion prone regions of aortas of both wild type (WT) and atherosclerosis prone apolipoprotein-E knockout (ApoE^{-/-}) mice, ICAM-1 was up-regulated (Nakashima et al., 1998). In addition, lesions in ApoE^{-/-} mice were 3 times smaller upon knockout of ICAM-1 compared to mice expressing ICAM-1 (Bourdillon et al., 2000). In humans, ICAM-1 protein expression is low in healthy aortas but is high in endothelium in both the shoulder region and fibrous cap of atherosclerotic plaques (Poston et al., 1992). This suggests that high ICAM-1 expression is a key risk factor associated with atherosclerosis and therefore a potential therapeutic target. *In vivo* data suggest that inhibition of ICAM-1 expression may inhibit plaque formation in humans.

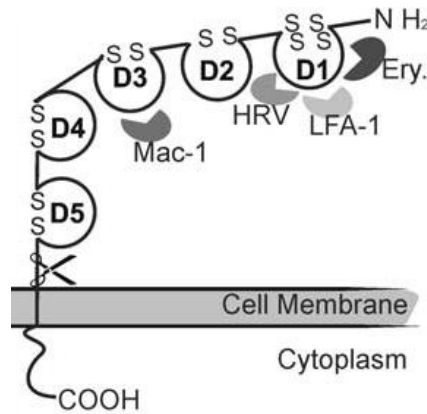


Figure 1.5 Schematic diagram of ICAM-1. With five extracellular IgG domains. Scissors represent the generation of sICAM-1 by cleavage of ICAM-1's extracellular domain from the cell surface. Binding sites for ligands, LFA-1, Mac-1, *Plasmodium falciparum* erythrocyte membrane protein-1 (Ery) and human rhinovirus (HRV) as indicated. (Modified from (Lawson and Wolf, 2009)).

1.4.2. Regulation of ICAM-1 expression

The ICAM-1 promoter has been investigated in depth and a number of regulatory elements responsive to inflammatory stimuli, such as TNF- α , retinoic acid, IFN- γ , virus infection, and oxidative stress such as H₂O₂ treatment, have been identified (Roebuck and Finnegan, 1999). Analysis of the first 5.8 kb upstream of the translation start site of ICAM-1 found a DNase I hypersensitive site 1.5 kb upstream of the translation start. The sequence upstream of the translation start site and the first intron contain a hypomethylated CpG island (Wawryk et al., 1991). These two observations are indicative of an active promoter. Two transcription start sites (TSS) at -41 and -319, relative to the start of translation, have been identified which generate the same ICAM-1 protein. The selection of TSS appears to depend on cell type or stimulus (Voraberger et al., 1991)(Figure 1.6). All ICAM-1 regulatory sites in this Thesis are expressed relative to the TSS at -41.

Deletion studies of the ICAM-1 promoter have identified an enhancer region located -2400 to -1352 bp upstream of the TSS (Jahnke et al., 1995) and a silencer region between -485 and -429 bp. Deletion of this silencer region increased promoter activity (Voraberger et al., 1991); however, no transcription factor binding site, or bound regulatory proteins have been identified within these regions.

G AATTCAGAAC TCCTCAGCCC CCCAAGAAAA AAATATCCCC GTGGAAATTC CTTGGGAATG -1302

ACCGAGGCGG GGGAAATATG CGTCTCTGGA TGGCCAGTGA CTCGCAGCCC CTTCCCGGA TAGGAAGGGC -1232

CTGCGCGTCC GGGGACCCCT CGCTTCCCT TCTGCTGCGC GACCTCCCTG GCCCCTCGGA GATCTCCATG -1162

GCGACGCCGC GCGCGCCCCA CAACAGGAAA GCCTTAGGCG GCGCGGCTTG GTGCTCGGAG ACTTAAGAGT -1092

ACCCAGCCTC GACGTGGTGG ATGTCGAGTC TTGGGGTCAC ACGCACAGGC GGTGGCCAAG CAAACACCCG -1022

CTCATATTTA GTGCATGAGC CTGGGTTCGA GTTGCCGGAG CCTCGCGCGT AGGGCAGGGG TTCGAGCGCC -952

CCTTCTCCCT GCCTCGCCTC TGCCTGGGG GCTGCTGCCT CAGTTTCGCA GCGACAGGCA GGGATTTCGA -882

GCGTCCCCCT CCCCTCCCTC GTCAAGATCC AAGCTAGCTG CCTCAGTTTC GCGCGGAGC CTGGGACGCC -812

AGCGGAGGGG CTCGCGCGCT AGGGATCACG CAGCTTCCTT CTTTTCCTG GGAGCTGTAA AGACGCCCTC -742

GCGGCCAAGG CCGAAAGGG AAGCGAGGAG GCCGCCGGG TGAGTGCCT CGGTGTAGA GAGAGGACGC -672

CGATTTCGCC GGACGTGGTG AGACCGCGCT TCGTCACTCC CACGGTTAGC GGTGCGCGG AGGTGCCTGG -602

CTCTGCTCTG GCCGCTTCTC GAGAAATGCC CGTGTCAGCT AGGTGTGGAC GTGACCTAGG GGGAGGGGCA -532

TCCCTCAGTG GAGGGAGCCC GGGGAGGATT CCTGGGCCCC CACCCAGGCA GGGGGCTCAT CCACTCGATT -462

AAAGAGGCCT GCGTAAGCTG GAGAGGGAGG ACTTGAGTTC GGACCCCTC GCAGCCTGGA GTCTCAGTTT -392

ACCGCTTTGT GAAATGGACA CAATAACAGT CTCCACTCTC CGCGGAAGTT GGCAGTATTT AAAAGTACTT -322

AATAAAGCCT TAGCGCGGTG TAGACCGTGA TTCAAGCTTA GCCTGGCCGG GAAACGGGAG GCGTGGAGGC -252

TATA TRE Distal TSS

CGGGAGCAGC CCCCGGGGTC ATCGCCCTGC CACCGCCGCC CGATTGCTTT AGCTTGAAA TTCCGGAGCT -172

GAAGCGGCCA GCGAGGAGG ATGACCCTCT CGGCCGGGC ACCCTGTCAG TCGGAATA ACTGCAGCAT -102

TTTTCGGA GGAAGGCG CGAGTTTCC GGGAAAGCAG CACCGCCCT TGGCCCCAG GTGGCTAGCG -32

CTATAAGGA TCACGCGCC CAGTCGACG TGAGCTCCTC TGCTACTCAG AGTTGCAACC TCAGCCTCGC +39

TATGGCTCCC AGCAGCCCC GGCCTCGCT GCCCGCACTC CTGGTCCTGC TCGGGGCTCT GTTCCAGGT +109

GAGTCGGGGT GGGGATTG +126

1.4.3. Transcription factors involved in the regulation of ICAM-1 expression

1.4.3.1. Regulation of ICAM-1 expression by AP-1/ETS transcription factors

There are a number of transcription factor binding sites in the ICAM-1 promoter which have been shown to be functional in basal or activated cells (Figure 1.6). H₂O₂ induced ICAM-1 expression in human umbilical vein EC (HUVEC) and EAhy926 cells, and this was found to depend on a region containing two identical repeats containing AP-1 and E-26 transformation specific (ETS) DNA binding sites located -981 and -769 bp upstream of the TSS (Roebuck et al., 1995). AP-1/ETS composite sites have been characterised in the macrophage scavenging receptor (MSR) promoter (Wu et al., 1994) where both the AP-1 and ETS sites have been shown to be required, as mutation of either the AP-1 or the ETS site or both together left a luciferase construct unresponsive. This AP-1/ETS site is responsible for the up regulation of ICAM-1 in HUVEC after cholesterol exposure, although the role of ETS factors and this interaction was not investigated in this study (Yuan et al., 2001).

1.4.3.2. Regulation of ICAM-1 expression by ETS transcription factors

The ICAM-1 promoter contains two ETS binding sites (EBS) at -118 and -98 bp relative to the TSS which have been found to regulate ICAM-1 expression. In rabbit kidney RK13 cells, ICAM-1 was up-regulated after over-expression of the ETS factors, Ets-1, Ets-2 and ERM (de Launoit et al., 1998; Maurer et al., 2003); however, the more distantly related ETS factor Pu-1 could not induce ICAM-1 promoter activity. It was found that ERM bound strongly to EBS at -118 and less strongly to the EBS at -98. Mutation of the EBS at -118 decreased basal ICAM-1 promoter activity compared to wild type and had little or no responsiveness to ERM over-expression depending on the cell type used. Mutation of the EBS at -98 did not change basal ICAM-1 promoter activity but decreased the amount of activity induced after ERM over-expression. The promoter activity of a double mutant was similar to the -118 mutant alone. The ETS factor-mediated regulation of ICAM-1 appeared to be cell type specific, because in HUVEC Ets-2 did not regulate ICAM-1 expression (Cheng et al., 2011).

The EBS at -118 was also investigated for ETS mediated repression of ICAM-1. The ETS factor fifth Ewing's variant (FEV) repressed constitutive ICAM-1 promoter activity as well as activity induced by the ETS factors, Ets-1 and ERM, in RK13 cells. FEV bound an

oligonucleotide corresponding to the ICAM-1 promoter sequence containing the -118 EBS and FEV-mediated repression was lost in the ICAM-1 promoter construct containing mutations in the -118 and -98 EBS (Maurer et al., 2003).

1.4.3.3. Regulation of ICAM-1 expression by STAT and ETS transcription factors

Mutations of the EBS at -118 and -98 also appear to be involved in IFN- γ signalling through interaction with STAT-1, which binds to the nearby GAS site at -76 bp relative to the TSS (Figure 1.6). Induction of ICAM-1 transcriptional activity by IFN- γ stimulation was decreased after mutation of these combined sites. ICAM-1 promoter constructs containing these separate mutations were still induced by IFN- γ stimulation, although to a lesser extent. FRET analysis showed that after IFN- γ treatment of COS-1 cells, Ets-1 and STAT-1 interacted, suggesting a possible interaction at the ICAM-1 promoter (Yockell-Lelievre et al., 2009). The double mutation of EBS -118 and -98 inhibited bpV(pic)-mediated up-regulation of ICAM-1 promoter activity in Jurkat cells but this was not seen on constructs with these sites mutated individually (Roy et al., 2001). bpV is a protein tyrosine phosphatase (PTP) inhibitor that stimulates the janus kinase (JAK)/STAT pathway and it has been suggested that PTP are expressed in T-cells to maintain basal expression of ICAM-1 pathway (Audette et al., 2001).

1.4.3.4. Regulation of ICAM-1 expression by NF- κ B

As well as the ETS sites and AP-1 sites, a number of other transcription factor binding sites in the ICAM-1 promoter have been investigated. The most characterised is the NF- κ B binding site at -188 bp from the proximal TSS. This site is required for ICAM-1 up-regulation after stimulation with TNF- α and IL-1 (Van de Stolpe and Van der Saag, 1996; Hou et al., 1994; Ledebur and Parks, 1995). It deviates from the canonical NF- κ B consensus of GGRRNNYYCC by having a thymine at the 5'end, a sequence shared with the NF- κ B binding site of IL-8 (Kunsch and Rosen, 1993; Ledebur and Parks, 1995). Mutational analysis of this site has highlighted the importance of nucleotides within and flanking this consensus site (Table 1.1) (Hou et al., 1994; Paxton et al., 1997; Ledebur and Parks, 1995). Binding specificities of members of the NF- κ B family to the ICAM-1 NF- κ B binding site have been studied by electrophoretic mobility shift assay (EMSA) and combinations of subunits appear to depend on cell type. In TNF- α treated HepG2 cells, p50 and p65 antisera were found to inhibit the formation of a protein complex with the ICAM-1 promoter NF- κ B binding site

(Hou et al., 1994). In HUVEC treated with TNF- α , two complexes were associated with the NF- κ B site, one containing p50/p65 heterodimers and a second containing p65 homodimers (Ledebur and Parks, 1995). Finally, in 293T cells dimer specificity depended on length of stimulation, with p65 homodimers formed after 30 mins of TNF- α stimulation and p65/cRel heterodimers formed after 4 hours of TNF- α stimulation. The activity of the NF- κ B binding site was enhanced by the presence of a C/EBP site -199 upstream of the TSS. Mutation of this site decreased TNF- α induced ICAM-1 promoter activity (Paxton et al., 1997; Hou et al., 1994).

Table 1.1. The mutations within the ICAM-1 promoter NF- κ B binding site and their effect on response to pro-inflammatory stimuli measured by luciferase assay.

NF- κ B binding site in bold (mutations underlined) AGCT GGAAATTCC GGAGCT	phenotype	Cell type	reference
AGCT <u>CC</u> AAATTCCGGAGCT	No TNF- α response	Hep G2	(Hou et al., 1994)
AGCT <u>CTAGATTAG</u> GGAGCT	No TNF- α , IL-1, LPS response	HUVEC	(Ledebur and Parks, 1995)
GGAAATTCC alone	No TNF- α response	Melanoma	(Paxton et al., 1997)
AGCT <u>AGGA</u> ATTCCGGAAGCT	No TNF- α response	Melanoma	(Paxton et al., 1997)
AGCT <u>CCGGAA</u> ATTCCGGAGCT	No TNF- α response	Melanoma	(Paxton et al., 1997)
AGCT GGAAATTG <u>C</u> AGGAGCT	No TNF- α response	Melanoma	(Paxton et al., 1997)
AGCT GGAAATTCC GGAG <u>C</u>	No TNF- α response	Melanoma	(Paxton et al., 1997)
AGCT GGAAATTCC GGA <u>ACT</u>	No TNF- α response	Melanoma	(Paxton et al., 1997)

1.4.3.5. Regulation of ICAM-1 expression by STAT-1

Another binding site previously studied in detail is the signal transducer and activator of transcription (STAT)-1 site at -76 relative to the TSS (TTTCCGGGAAA). This site was first identified by its response to IL6 and IFN- γ . EMSA was used to show that the protein complex that bound to this site in HepG2 cells contained STAT-1 (Caldenhoven et al., 1994; Hou et al., 1994). It has been suggested that ICAM-1 expression in EC is not up-regulated upon stimulation with IFN- γ , while in epithelial cells it is (Look et al., 1994).

1.4.3.6. Regulation of ICAM-1 expression by AP-1

The AP-1 binding site at -294 relative to the TSS has been studied by a number of groups (Voraberger et al., 1991; Van de Stolpe and Van der Saag, 1996; Munoz et al., 1996) and is responsive to treatment with phorbol 12-myristate 13-acetate (PMA), 12-O-tetradecanoylphorbol-13-acetate TPA, and pyrrolidine dithiocarbamate (PDTC). In HUVEC the AP-1 transcription factors cFos and cJun bound this site after treatment with PDTC, a metal chelating compound, indicating this site may be activated in conditions where a redox imbalance plays a key role, for example ischemia/reperfusion injury or atherosclerosis (Munoz et al., 1996).

1.5. Epigenetic regulation of transcription

1.5.1. Introduction

Epigenetics was originally defined as ‘the heritable or transient change in gene expression that is not due to a change in primary DNA sequence’ (Hirst and Marra, 2009). The definition has evolved as more has become understood about the mechanisms involved and epigenetics was more recently defined as ‘the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states’ (Bird, 2007). Epigenetics mechanisms are responsible for the regulation of genes in response to environmental stimuli, including nutrition, disease, hormones or drugs. Epigenetic mechanisms control gene expression through the accessibility of transcriptional machinery to DNA packaged around nucleosomes and this accessibility can be increased or decreased depending on modifications on the DNA or nucleosome. The two most characterised mechanisms of epigenetic regulation are histone modifications and DNA methylation. These modifications cause phenotypic and functional differences in genetically identical cells within an organism and are responsible for the development of different cell lineages. This Thesis will be limited to the study of

modifications of histones, as methylation of DNA is beyond the scope of this study. In the nucleus DNA is wrapped around nucleosomes which have the characteristic “beads on a string” appearance; these pack tightly together to form the highly organised chromosome structure. Nucleosomes consist of two copies of each of the four canonical histones H2A, H2B, H3 and H4 forming an octamer around which approximately 147 bp of DNA are wrapped (Figure 1.7) (Luger et al., 1997). Nucleosomes are organised with linker histone molecules of the Histone H1 family. H1 molecules are associated with the linker DNA and bind the two ends of DNA at nucleosome entry/exit sites on the surface of the nucleosome core and organise the higher order chromatin structure (Happel and Doenecke, 2009). Each core histone molecule has an N terminal tail region which can be modified by post translational modifications such as acetylation, methylation, phosphorylation and ubiquitylation (Figure 1.8). These modifications alter the binding specificity of the tail domain which changes the nucleosome structure, allowing the chromatin to become accessible to transcriptional machinery. Transcriptional activity is dynamic and can be modulated by activators or repressors that recruit histone-modifying co-factors to promoters. Certain histone modifications are thought to specify a ‘histone code’ that directs regulatory features of genes; this code causes the nucleosomes to have either an active (accessible) or repressed (closed) structure (Narlikar et al., 2002). The two most studied modifications are acetylation and methylation.

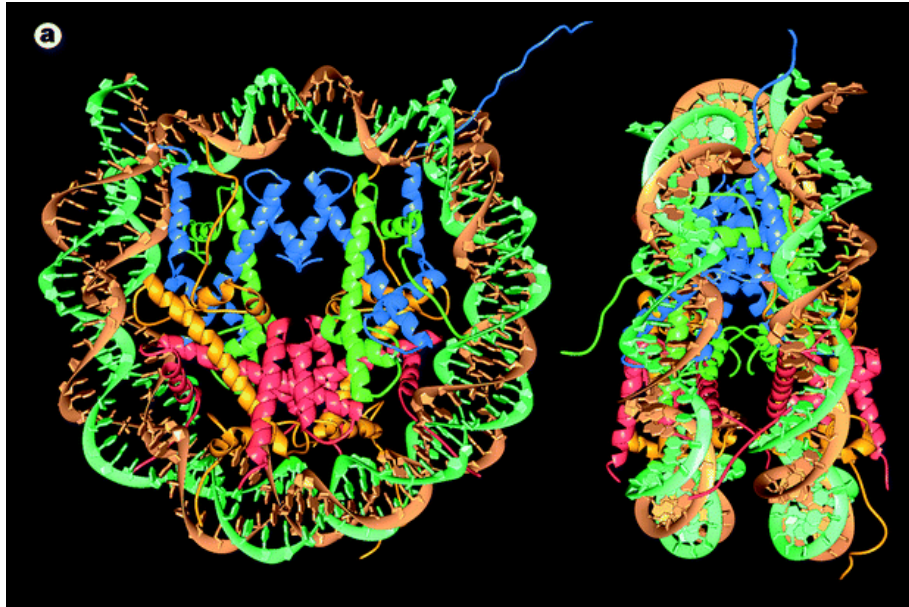


Figure 1.7. Nucleosome core particle: Ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B). The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. For both particles, the pseudo-two-fold axis is aligned vertically with the DNA centre at the top (Luger et al., 1997).

1.5.2. Histone acetylation

Histone acetyl transferases (HAT), which include CBP and its related protein p300 (CBP/p300), act as transcriptional co-activators (Kalkhoven, 2004). HAT utilize acetyl CoA as a co-factor and catalyse the transfer of an acetyl group to the ϵ -amino group of lysine side chains of histone proteins and other proteins. HAT are recruited to DNA by transcription factors and acetylate specific lysine residues on the N-terminal tails of histone molecules, for example histone (H) 3 Lysine (K) 9, H3K14 and H4K8 (Figure 1.8). Additionally, lysine residues within the histone core may also be modified. Transcriptional activation induced by acetylation is thought to result from the neutralisation of the positive charge on lysine residues which weakens the interaction between histones and DNA; alternatively, acetylation may facilitate the binding of other transcription factors which remodel nucleosomes (Lee and Workman, 2007).

The acetylation state of histones is dynamic and may be reversed by histone deacetylases (HDAC) which induce a repressive chromatin state. There are 4 classes of HDAC categorised by their homology and function. Class I consists of HDAC 1, 2, 3 and 8, which are almost exclusively found in the nucleus, with the exception of HDAC3 which can also be found in the cytoplasm. Class I HDAC are involved many biological processes including differentiation, proliferation, and some cancers (Shakespeare et al., 2011). Class II HDAC contain the similar core catalytic site to class I HDAC but differ in the structure of the entrance region surrounding the catalytic core. Class II HDAC are further subdivided into class IIa and class IIb depending on the presence or absence of homologous domains. The class IIa HDAC consist of HDAC 4, 5, 7 and 9 (reviewed in (Martin et al., 2007)). These are located both in the cytoplasm and nucleus and are important in the development of many tissues including the brain and the vascular system as well as the immune system (reviewed in (Parra and Verdin, 2010)). Class IIb HDACs are HDAC6 and HDAC10. HDAC6 is mainly located in the cytoplasm, while HDAC10 can be found either in cytoplasm or nucleus. HDAC6 acts by deacetylating non-histone proteins, most notably tubulin, which results in an increase in cell motility (Haggarty et al., 2003). Little is known about the function of HDAC10, although it has recently been shown to have a role in melanogenesis (Lai et al., 2010). Class III HDAC are the Sirtuins (SIRT) which include SIRT 1-7. SIRT are evolutionarily distinct from the other HDACs and require nicotinamide adenine dinucleotide

(NAD⁺) for their activity. They are ubiquitously expressed and have been shown to deacetylate histone molecules H1, H3 and H4, and also non-histone proteins including NF-κB and Forkhead (FOX) transcription factors (Yeung et al., 2004; Motta et al., 2004). SIRT are involved in regulating stress responses and apoptosis, metabolism, development and differentiation. The class IV HDAC family so far consists of one member, HDAC11, which has been shown to regulate the balance between immune activation and immune tolerance in CD4⁺ T-cell through inhibition of IL-10 (Villagra et al., 2009). HDAC11 is also involved in oligodendrocyte differentiation (Liu et al., 2009) and the expression of OX40 ligand in Hodgkin Lymphoma (Buglio et al., 2011).

1.5.3. Histone methylation

Histones are methylated on either lysines or arginines by histone methyltransferases (HMT) (Bannister and Kouzarides, 2005). Both lysine and arginine can be mono or di-methylated, while only lysine can be tri-methylated. Lysines are methylated by transferring a methyl group from S-adenosylmethionine (SAM) domain on the HMT to the ε-amino group on the lysine. In contrast, arginines are methylated by transfer of a methyl group from SAM to the ω-guanidino group of arginine. Most HMT have specificity to produce a certain modification on a specific histone residue. Unlike acetylation, methylation marks may be associated with either repressed or activated chromatin conformation, which is dependent on the modified residue. Histone methylation does not alter the charge or the structure of the chromatin in itself, rather it forms a substrate for specific co-factors to recognise and bind, or forms a barrier blocking binding of other histone modifying co-factors (Peterson and Laniel, 2004).

1.5.4. Other post-translational modifications of histones

Histones can be phosphorylated on serine, threonine and tyrosine residues within the histone tail. Kinases transfer a phosphate group to the hydroxyl group of the amino acid side chain, which results in a negatively charged histone tail, affecting the nucleosome structure. Phosphorylation of histone molecules affects transcription by facilitating the binding of other co-factors and can therefore have a positive or negative effect.

Other histone modifications include: ubiquitylation and sumoylation, deimination, and ADP ribosylation (Kouzarides, 2007), although less is known about the roles of these modifications in regulation of transcription.

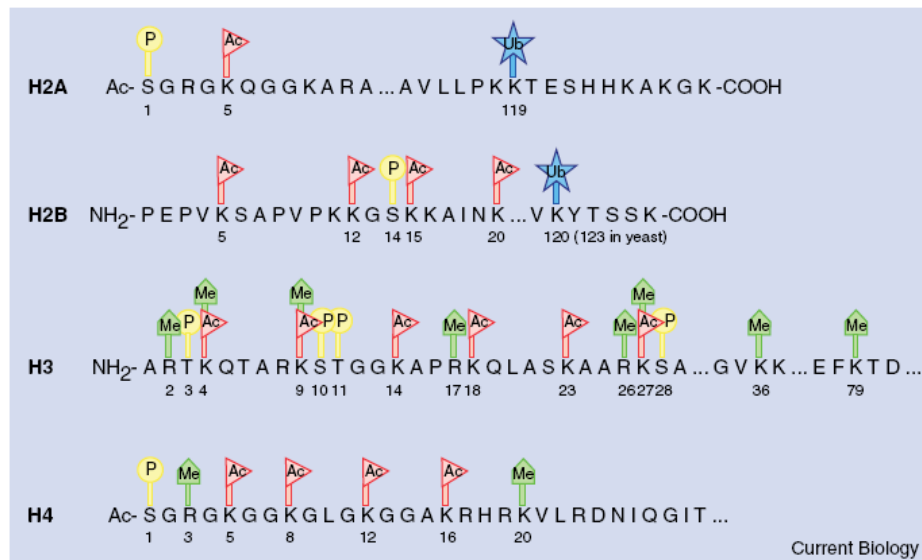


Figure 1.8. Post-translational modifications of the core histones. The coloured shapes represent known post-translational modifications of the core histones. The histone tails can be methylated at lysines and arginines (green pentagons), phosphorylated at serines or threonines (yellow circles), ubiquitylated (blue stars) and acetylated (red triangles) at lysines. (Modified from (Peterson and Laniel, 2004)).

1.5.5. The histone code

The occurrence of certain histone modifications in combination with others has led to the hypothesis of the histone code, or cross-talk (Lee et al., 2010). This suggests that a histone modification may be dependent on the modification of another histone residue, and therefore they occur in a sequential manner. For example, the GCN5 HAT recognises and acetylates H3K14 more effectively when it has been phosphorylated at H3S10 (Clements et al., 2003). As some residues, in particular lysines, can be modified by multiple post-translational modifications, there is competitive antagonism between modifications. Additionally steric hindrance may occur between co-factors at differentially modified residues. Examples of the positive or negative effects certain modifications have on other modifications are shown in Figure 1.9.

1.5.6. HDAC inhibitors

Due to the over-expression of a number of HDACs in various cancers, HDAC inhibitors (HDACi) have been developed as possible therapeutic agents for cancer treatment. Various HDAC inhibitors have been developed, which have different specificity and actions. The canonical HDAC inhibitors are trichostatin acid (TSA) and suberoylanilide hydroxamic acid (SAHA) (also known as Vorinostat), which inhibit class I, II, and IV HDACs. In addition, class and isoform specific HDACi have also been developed (Bieliauskas and Pflum, 2008). HDACi consist of a metal-binding moiety which chelates the catalytic metal atom within the HDAC active site, leaving the HDAC unable to bind Histone residues. HDAC specificity comes from the interaction of the HDACi with the residues within the entrance to the HDAC active site. HDACi have shown therapeutic potential in a number of diseases including cutaneous T-cell lymphoma (CTCL), acute myeloid leukaemia, prostate cancer, non-Hodgkin's lymphoma, and diffused large B-cell lymphoma. The HDACi Vorinostat and Romidepsin have been approved by the United States Food and Drug Administration (FDA) for the treatment of CTCL, and they act by increasing the expression of several genes that induce apoptosis in malignant cells (Lane and Chabner, 2009).

1.5.7. HDAC inhibitors and inflammation

While HDACi were originally designed to target the increased HDAC activity in cancer, they have been found to be efficacious in the treatment of inflammatory diseases at doses lower than those used for cancer treatment (Shuttleworth et al., 2010). The role of HDAC in

inflammatory pathways is complex; HDAC have a repressive effect on some pathways and an activating effect on others. In macrophages the TLR and IFN- γ signalling pathways are regulated by HDAC. LPS-induced COX2 expression in macrophages was increased by TSA treatment, but TSA treatment blocked the expression of other LPS target genes such as chemokines ligand (CCL) 2 (MCP1), CCL7 and Edn1 (Aung et al., 2006). TSA treatment of HUVEC has been found to inhibit TNF- α -induced VCAM-1 expression, but have no effect on TNF- α -induced ICAM-1 expression (Inoue et al., 2006).

1.6. The ETS family of transcription factors

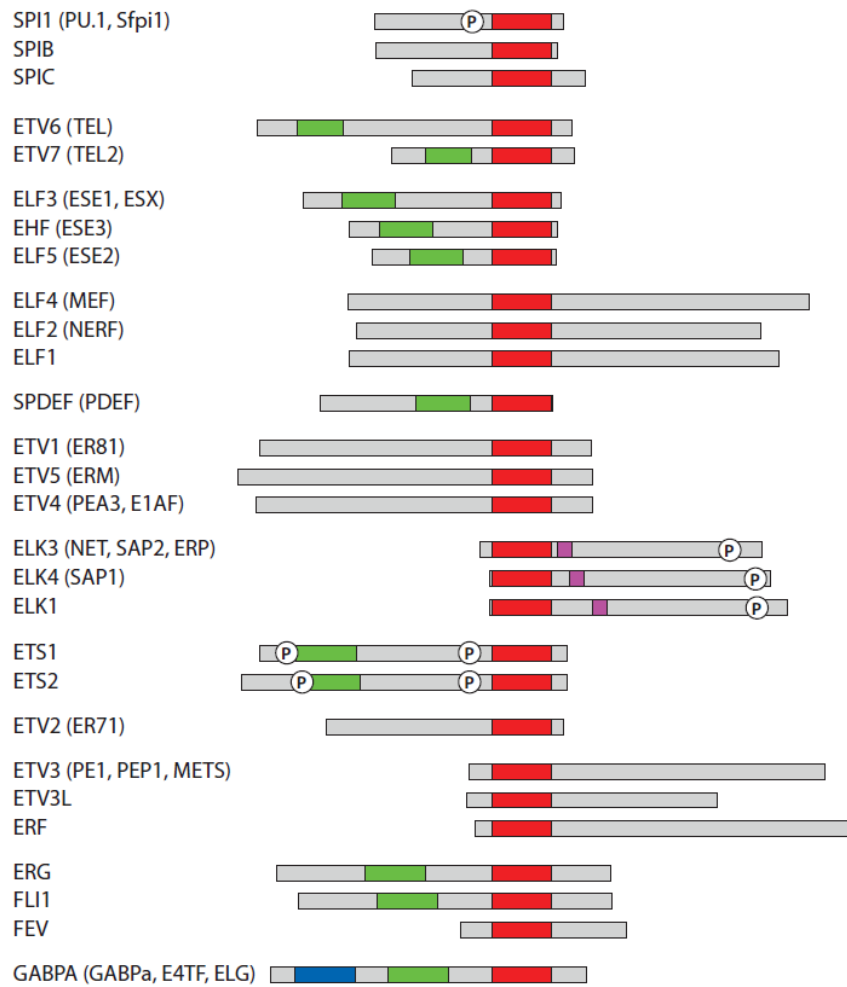
The E-26 transformation specific (ETS) family of transcription factors consist of approximately 30 mammalian members which share a conserved 85 amino acid DNA binding domain (ETS domain) that binds to the consensus DNA sequence 5'GGA(A/T)3' (Oikawa and Yamada, 2003). The name of the ETS factor family is derived from the avian erythroblastosis virus, E26, which carries the v-ets oncogene (Nunn et al., 1983). ETS factors mediate important cellular functions such as proliferation, differentiation and tumourgenesis. Several ETS factors also act as oncogenes, including Ets-1, Ets-2, Pu-1(SPI1), Fli-1, Erg and Tel (ETV6) (Seth and Watson, 2005). The ETS factors are expressed either endogenously or aberrantly after chromosomal translocations such as those affecting the ETS factors Fli-1 and Erg in Ewing's sarcoma, acute myeloid leukaemia or prostate cancer.

1.6.1. Structure and interactions of ETS factors

ETS factors have been phylogenetically classified and grouped on the basis of sequence homology within the ETS domain and the presence of other conserved domains (Figure 1.10). All ETS factors contain the highly conserved ETS DNA binding domain, which in the majority of ETS factors, is located in the C-terminal domain; however, in some ETS factors, for example ERF and Elk-1, the ETS domain is in the N-terminus. The ETS domain forms winged helix-turn-helix structure consisting of 3- α helices and a 4-stranded β sheet (Figure 1.11) which bind directly to the GGA(A/T) consensus motif (Kodandapani et al., 1996; Donaldson et al., 1996). Another homologous domain conserved in a number of ETS factors is the ~80 amino acid pointed domain (PNT) (Klamt, 1993) which is found in Ets-1, Ets-2, Erg, Fli-1, GABP α , Tel (ETV6), Tel-2 (ETV7), ESE-1 (ELF3), ESE-2 (ELF5), ESE-3 (EHF) and PDEF (SPDEF). The PNT domain consists of 4 or 5 α -helices and has been shown to function in Tel homo-oligomerisation, interaction with kinases, transcriptional co-factors, and

heterodimerisation with other ETS factors (Nelson et al., 2010; Seidel and Graves, 2002; Sharrocks et al., 2000; Lacronique et al., 1997). Another conserved domain is the B-box domain found only in the ternary complex factor (TCF) ETS factors, Elk-1, 3 and 4. This is required for the interaction with serum response factors (SRF) and subsequent binding to serum response elements (SRE) (Ling et al., 1997).

Human ETS family



■ ETS domain ■ PNT domain ■ OST domain ■ B-box

Figure 1.10 Structural and functional domains of the ETS family of transcription factors. Many ETS genes produce multiple protein products via alternative splicing/start sites, and in these cases, a single polypeptide was chosen arbitrarily. Boxes identify the DNA-binding ETS domain (red), PNT domain (green), OST domain (blue), and B-box (magenta) of the ETS factors. The circled P symbolizes a phosphorylated residue. (Modified from (Hollenhorst et al., 2011)).

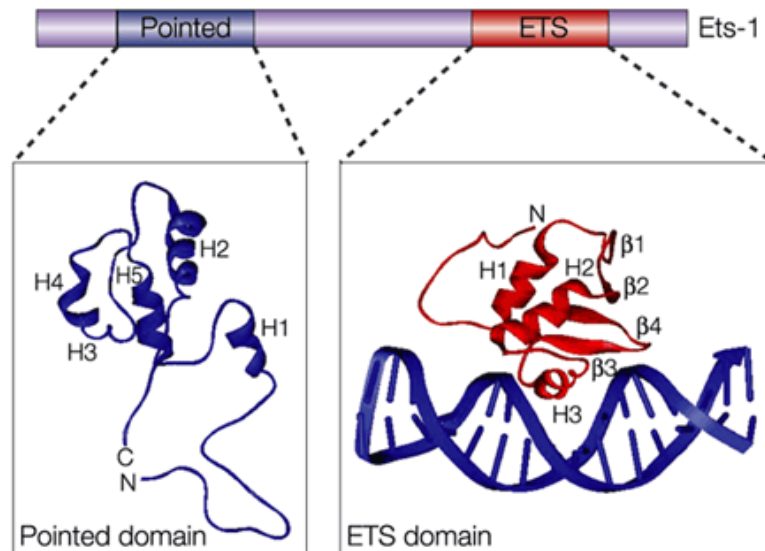


Figure 1.11 Structure of the ETS domain and pointed domain of Ets-1. The location of helices (H) and β -strands (β) within the structures of the pointed domain (blue) and ETS domain (red) of Ets-1 are shown. (Modified from (Sharrocks, 2001))

1.6.2. DNA binding and specificity of ETS factors

The ETS DNA binding domain binds to the DNA consensus GGA(A/T), with the most important DNA/protein interactions being the hydrogen bonds between two conserved arginines within helix H3 and the two guanines of the consensus sequence (Obika et al., 2003). While no other direct interactions occur with the flanking DNA, there is a preference for a surrounding variable motif of 9 bp, and ETS factors can be divided into 4 classes based on this specificity (Figure 1.12)(Wei et al., 2010; Hollenhorst et al., 2004). This lack of sequence specificity between ETS factors suggests that gene target specificity may come from surrounding sequences by facilitating the association of ETS factors with other transcription factors, or by tissue specificity and expression levels. Chromatin immunoprecipitation sequencing (ChIP-seq) studies have shown a degree of redundancy in ETS factor binding at sites not associated with any regulatory regions; however, greater enrichment of ETS factors has been shown for binding sites near transcription start sites of specific target genes (Wei et al., 2010). A number of composite binding sites for ETS factors with other transcription factors have been identified; including TCF/SRF, ETS/RUNX, FOXC/ETS, AP-1/ETS, Pu-1/IRF4 and Ets-1/PAX5 composite sites (Rabault and Ghysdael, 1994; Latinkic et al., 1996; Arman et al., 2009; De Val et al., 2008; Moulton et al., 1994; Yee et al., 1998). In some of these sites the EBS deviates from the GGA(A/T) consensus, for example the ETS binding site in the Ets-1/PAX5 composite site is GGAG (Garvie et al., 2001).

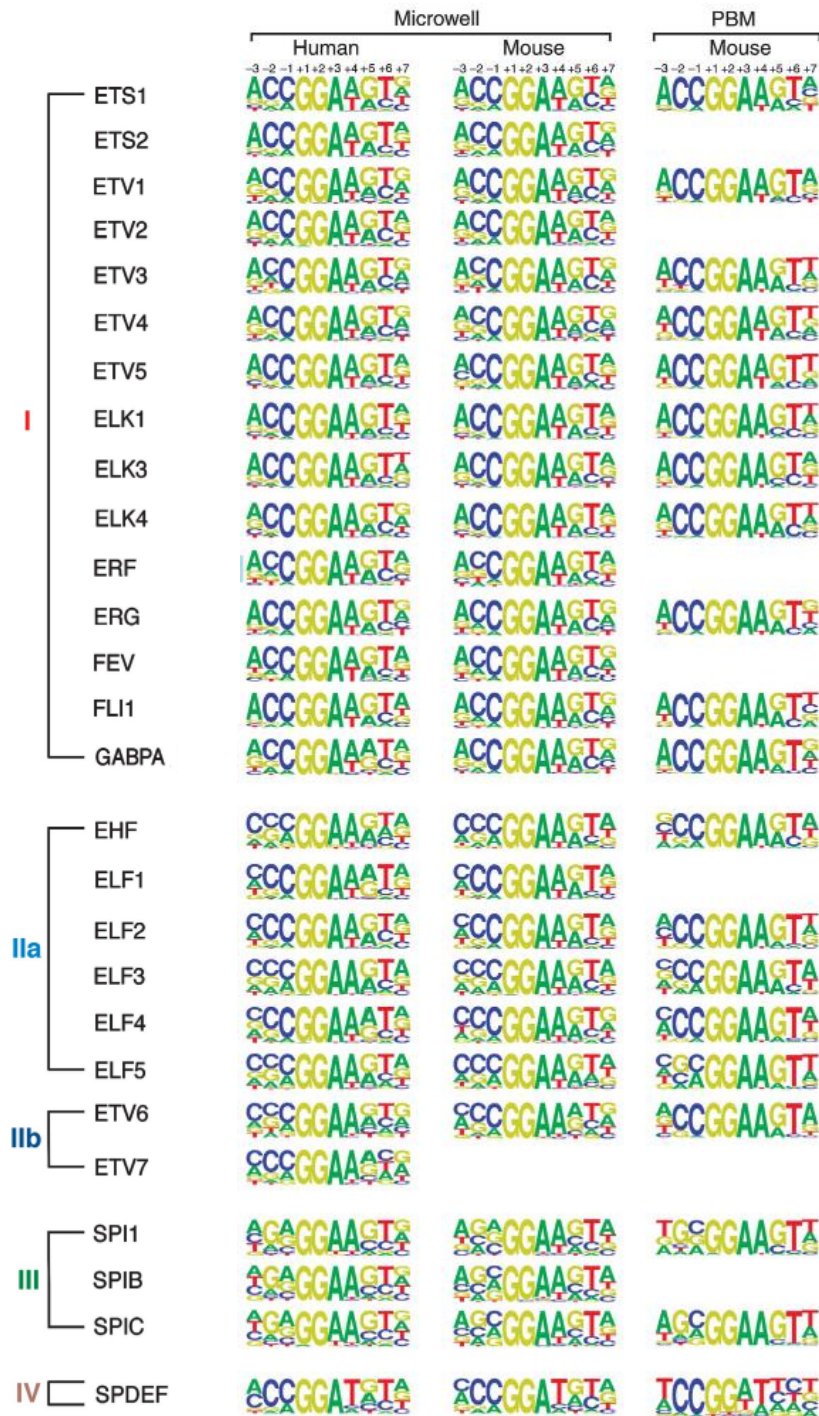


Figure 1.12 ETS factor classes and binding specificities of mammalian ETS transcription factors. ETS factors divided into classes based upon binding specificities. The first and second columns, respectively, show human and mouse ETS-binding profiles determined using microwell-based transcription factor-DNA-binding assays. The right column shows mouse ETS-binding profiles determined using protein binding microarrays. The logos are drawn using enoLOGOS, and the height of a letter at a particular position is directly proportional to the effect of that nucleotide on the binding affinity. (Modified from (Wei et al., 2010)).

1.6.3. Expression and function of ETS factors

All tissues tested contain multiple ETS factors (Hollenhorst et al., 2004), with nearly two thirds of ETS factors, including Ets-2, GABP- α and Tel expressed ubiquitously in adult tissue. However, other ETS factors have a more restricted expression, such as Pu-1 which is expressed in B-lymphocytes, macrophages, neutrophils, mast cells and erythroblasts (Lloberas et al., 1999). ETS transcription factors mediate the expression of a variety of genes and play a role in diverse cellular processes such as proliferation, apoptosis, cell/cell and cell/matrix interactions, differentiation and development (reviewed in (Oikawa and Yamada, 2003)). ETS factors can act as transcriptional activators or repressors or both, depending on the target gene or activity of the cell. The activity of many ETS factors is regulated by signal transduction cascades, which alter their sub-cellular localisation, DNA binding activity, or transcriptional activity through post-translational modification. The TCF ETS transcription factors are phosphorylated by the extracellular signal-regulated kinase (ERK)/MAPK pathway following stimulation by serum or tissue plasminogen activator (TPA); this allows association with the serum response factor (SRF) which in turn enhances DNA binding to the serum response element (SRE) in the promoters of genes such as c-fos (Gille et al., 1995).

1.6.4. ETS transcriptional activators

The majority of ETS factors have transcriptional activating properties. ETS factors that act as transactivators include: Ets-1, GABP α , Elf-1 and Nerf-1. Transactivating ETS factors bind to EBS and recruit co-activators such as the HAT CBP/p300, which lead to recruitment of transcriptional machinery to the promoters of target genes. A number of ETS factors have been found to interact with the epigenetic co-activators CBP/ p300 including Ets-1, GABP α , Elk-1, Pu-1 and ETV1 (Kang et al., 2008; O'Donnell et al., 2008; Bai et al., 2005; Goel and Janknecht, 2003). Ets-1 and Ets-2 interacted with CBP/p300 via the PNT domain and this interaction is required for the expression of Ets-1 and 2 target genes (Foulds et al., 2004; Kang et al., 2008). GABP α binds CBP through the On-SighT (OST) domain of GABP α (Kang et al., 2008), and this interaction is responsible for driving IL-6 expression in T-lymphocytes (Bannert et al., 1999).

1.6.5. ETS transcriptional repressors

While most studies have investigated the roles of ETS factors as activators of transcription, a number of ETS factors have shown repressive properties. Some ETS factors including Yan,

ERF, NET, Tel and FEV, so far appear to be exclusively repressive (Mavrothalassitis and Ghysdael, 2000; Maurer et al., 2003). However, there is little known about the mechanism behind their repressive activity: The leukaemia-associated ETS factor Tel encodes a transcription repressor which associates with silencing mediator for retinoid and thyroid-hormone receptors (SMRT) and mSin3A (Chakrabarti and Nucifora, 1999). Tel repression is abolished by sumoylation which induces nuclear export (Chakrabarti et al., 1999).

1.6.6. ETS factors with both activating and repressing properties

A growing number of ETS factors have been shown to have both repressing and activating properties, depending on activation of signalling pathways and subsequent post-translational modification of the ETS factor. They include Elk-1 (Yang et al., 2003b; Yang et al., 2001), Pu-1 (Kihara-Negishi et al., 2001; Suzuki et al., 2003), Ets-2 (Wei et al., 2003; Baker et al., 2003) Fli-1 (Czuwara-Ladykowska et al., 2001) and Erg (Yuan et al., 2009; Sperone et al., 2011). Different post-translational modifications of ETS factors can alter their activity from activation to repression, and in some instances ETS factors can both activate and repress the same gene, depending on signalling events (Wei et al., 2003; Yang et al., 2001). Fli-1 is involved in EC and megakaryocyte development and differentiation (reviewed in (Dejana et al., 2007; Oikawa and Yamada, 2003)). Fli-1 is an activator of transcription; however, Fli-1 also binds the sumo E3 ligase PIASxalpha/ARIP3 via its ETS domain and this represses the activation of multiple ETS-dependent promoters (van den Akker et al., 2005). The mechanisms responsible for the dual role of Elk-1 as a transcriptional activator and a repressor have been studied in detail. Elk-1 acts as an activator and a repressor of the immediate early gene c-fos, phosphorylation of Elk-1 results in the recruitment of co-activators to the SRE in the promoter of c-fos and phosphorylation also triggers a delayed recruitment of an mSin3A/HDAC1 complex which then shuts down transcription (Yang et al., 2001).

1.6.7. ETS factors and endothelial cells

So far, at least 19 ETS factors have been found expressed at some point during development or following activation in human EC, and the most highly expressed ETS factor in differentiated quiescent EC is Erg (ETS related gene) (Hollenhorst et al., 2004)(GM Birdsey, AM Randi, unpublished data). ETS factors are required for EC development and homeostasis. All characterised EC promoters and enhancers contain multiple essential ETS binding motifs which can be bound by more than one ETS family members (reviewed in (De

Val and Black, 2009; Randi et al., 2009)). ETS target genes in EC including genes that define the EC lineage including VE-Cadherin, VWF, endoglin, Tie-1 and 2, neuropilin (Dejana et al., 2007). Loss of function and over-expression studies have shown that many ETS factors are required for vascular development (Randi et al., 2009).

1.6.8. ETS factors and inflammation

Many ETS factors play an important role in inflammatory response, including Ets-1, Ets-2, ESE-1 and Elk-3, with the majority of these acting as transcriptional activators of genes including VCAM-1, MCP-1 and COX2 (Zhan et al., 2005; Cheng et al., 2011; Grall et al., 2005). ETS factors are regulated by pro-inflammatory cytokines, growth factors and vasoactive peptides, and ROS which may act by regulating ETS factor expression or mediate post translational modifications. A recent study investigating the role for Ets-2 in atherosclerosis found that Ets-2 expression was associated with neovessel formation of the vasa vasorum within the vulnerable plaque. Ets-2 expression in EC was found to be increased by TNF- α treatment, and Ets-2 inhibition decreased both basal and TNF- α -induced levels of VCAM-1, MCP-1 and IL-6. However, not all TNF- α target genes were affected as ICAM-1 was not regulated by Ets-2 inhibition (Cheng et al., 2011). Similarly Ets-1 expression is also induced by pro-inflammatory stimuli such as Ang II, thrombin, IL-1 β , and TNF- α , and increased expression of Ets-1 drives expression of MCP-1 and VCAM-1. The ETS transcription factor ESE is only found in epithelial cells in resting conditions, however, stimulation with IL-1 β or TNF- α induces ESE expression in EC and VSMC which in turn transactivates pro-inflammatory gene expression including NOS-2, COX-2 and angiopoietin-1 (Rudders et al., 2001; Grall et al., 2005; Brown et al., 2004). While the majority of ETS factors are pro-inflammatory, some act as repressors of inflammation. The TCF ETS factor Net is constitutively expressed in macrophages and EC; however, treatment with endotoxin decreases levels of Net mRNA which results in a loss of promoter binding by Net, and derepression of the anti-inflammatory heme oxygenase (HO)-1 and NOS2 (Chung et al., 2006; Chen et al., 2003). ETS factors also act synergistically with other pro-inflammatory transcription factors including AP-1, STAT-1 and NF- κ B (Yockell-Lelievre et al., 2009; Thomas et al., 1997; Rudders et al., 2001). In T-lymphocytes, Ets-1 acts synergistically with AP-1 to drive expression of IL-5 (Wang et al., 2006a). In an *in vivo* model of abdominal aortic aneurism (AAA), a chronic inflammatory disease of the aortic wall, treatment with a chimeric oligonucleotide containing ETS and NF- κ B binding sites, decreases the

pathophysiology of the disease, partly by decreasing expression of MMP-2 and MMP-9, VCAM-1 and MCP-1 (Nakashima et al., 2004; Miyake et al., 2006).

1.7. The ETS related gene Erg

1.7.1. Expression of Erg

The ETS transcription factor Erg is constitutively expressed in all EC tested so far including HUVEC, mammary artery, coronary artery, and venous endothelium and it is the most highly expressed ETS factor in resting EC with an mRNA copy number of 243 per cell (Hollenhorst et al., 2004). In addition to EC, Erg is endogenously expressed in myeloid (HL60) and T-cell (Jurkat) lines (Hollenhorst et al., 2004), as well as megakaryocytes (Rainis et al., 2005) and chondrocytes (Iwamoto et al., 2000). Erg is also expressed at the early stages of T-cell lineage specification and in pre-B lymphocytes but not in mature T or B-lymphocytes (Anderson et al., 1999). Erg is found in the nucleus of resting cells and there is so far no evidence of a cytoplasmic role for Erg. A recent study of the expression of Erg in the developing mouse embryo found it to be expressed in EC and in pre-cartilage and hematopoietic tissues, but not in the epithelium or lymphocytes (Mohamed et al., 2010). Additionally Erg has been found to be expressed in the myocardium of E8.5 mouse embryos (Schachterle et al., 2012). The mediators of constitutive Erg expression have not been identified; however, multiple studies have shown that Erg protein is down regulated after stimulation with the inflammatory stimuli TNF- α and LPS (McLaughlin et al., 1999; Yuan et al., 2009). Erg activity has also been found to be repressed by oestrogen receptor (ER) α , although this was not through inhibition of DNA binding, protein-protein interaction, or lack of available co-factors (Vlaeminck-Guillem et al., 2003). Little is known about the post-translational modifications of Erg. In KG1 myeloblast cells Erg is phosphorylated on a serine residue by TPA treatment, an activator of the protein kinase C pathway (Murakami et al., 1993); however, the function of this modification is unknown.

1.7.2. Erg: genomic structure and isoforms

The Erg gene is located on the reverse strand of chromosome 21 q.22.2 (Rao et al., 1987), and as such has been implicated in the haematologic disorders associated with chromosome 21 trisomy, including Down's syndrome (Ng et al., 2010), and Alzheimer's disease (Shim et al., 2003). Nine Erg isoforms which generate transcripts have so far been identified, and their expression is dependent on alternative transcription initiation sites, polyadenylation sites and

mRNA splicing (Figure 1.13). The intron/exon structure of the nine isoforms is shown in Figure 1.13. Erg-1, Erg-2, Erg-3 (p55), Erg-4 (p49), and Erg-5 (p38) have been shown to form functional proteins (Reddy and Rao, 1991; Prasad et al., 1994; Duterque-Coquillaud et al., 1993); Erg-7 and Erg-8 have open reading frames and are predicted to form functional proteins; while Erg-6 and Erg-9 isoforms are assumed to be non-functional (Owczarek et al., 2004). In EC reverse transcriptase (RT)-PCR analysis of transcripts using isoform specific primers suggests only two Erg isoforms, Erg-3 and Erg-5, are expressed (Hewett et al., 2001); however, western blot analysis from our lab suggests a third isoform, Erg-2, may also be expressed in EC (Randi et al unpublished data). There are two predicted promoter regions in the Erg gene, one 5' of exon 1, consisting of a 558 bp region conserved between mouse and human, and another putative promoter region 5' of exon 2. Additional conservation was found 2 kb and 3.2 kb 5' of exon 1, suggest putative enhancers for Erg expression (Owczarek et al., 2004). Transcription factor binding site analysis has identified 77 putative transcription factor consensus sequences in the promoter region involved in Erg regulation; however, no transcription factors have been identified which regulate Erg expression.

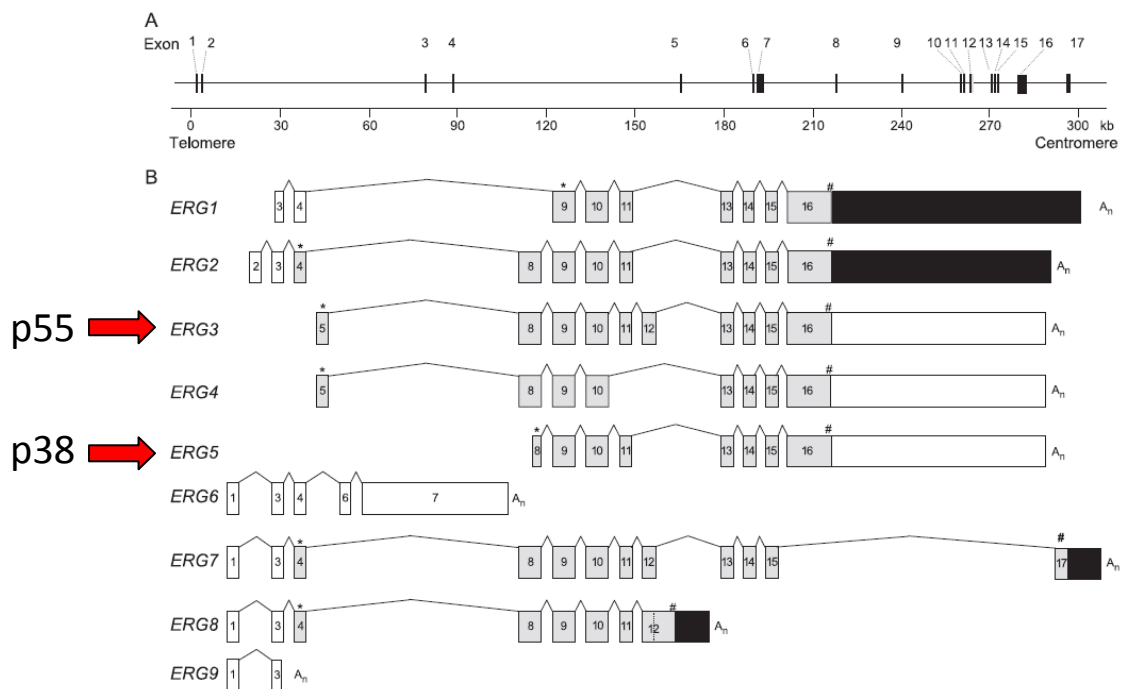


Figure 1.13. Structure of the human ERG gene. A, schematic representation of the ERG gene. Exons are indicated by black boxes, and numbered starting from the 5' exon. B, structure of alternative transcripts encoded by the ERG gene. Start codons are indicated by an asterisk (*) and stop codons are indicated by a hatch (#). Open reading frames are shown in black boxes, the 5'UTRs and 3'UTRs in white boxes, and the transcribed exons in grey boxes. Red arrows indicate the Erg isoforms expressed in ECs. (Modified from (Owczarek et al., 2004)).

1.7.3. Domains of the Erg protein

1.7.3.1. Erg ETS DNA binding domain

Deletion and homology analysis of Erg has identified domains involved in DNA binding and gene regulation, (Figure 1.14)(Siddique et al., 1993). The Erg ETS domain is located in the carboxy(C)-terminus from amino acids 290 to 372 of Erg-2 (Figure 1.14). This domain shares 80% homology with the closest related ETS factor, Fli-1. As with other ETS factors, the ETS domain of Erg is essential for DNA binding and binds a consensus sequence classified by the class I ETS binding sites (Hollenhorst et al., 2011). Site directed mutagenesis has found that Arg 367 within the ETS domain is essential for DNA binding (Verger et al., 2001). As ETS binding specificity is thought to be controlled in part by the flanking sequences, the Erg extended consensus DNA binding sequence around the core (GGAA/T) has been investigated using a number of techniques in multiple studies. Early analysis by EMSA identified the consensus of (A/C)GGAAG (Duterque-Coquillaud et al., 1993) or (C/G)(C/a)GGAA(G/a)T (Murakami et al., 1993). Further genome-wide studies analysing Erg binding site specificity using ChIP-seq, found the consensus sequence AGGA(A/t) (G/A) (Wilson et al., 2010) or (C/a/g)(A/C)GGAA(G/A/c) (Wei et al., 2010). Analysis of the binding specificity of all ETS factors indicates Erg is a class I ETS factor (Wei et al., 2010). This is the largest class, suggesting Erg recognised a similar consensus motif to many other ETS factors.

1.7.3.2. Erg Pointed domain

Erg also contains a PNT domain which is conserved in six other ETS factors and is found between amino acids 125 and 209 in Erg-2 (Figure 1.14)(Siddique et al., 1993). The Erg PNT domain forms four α -helices (H2–H5) and a short α -helix (H2') (Hollenhorst et al., 2011). A role for the Erg PNT domain in protein-protein interaction and homo/hetero-dimerisation has been suggested (Carrere et al., 1998); however, studies of soluble Erg PNT domain found no interaction with the PNT domains of Erg or other ETS factors (Mackereth et al., 2004). While no known function or ligands have been identified for the Erg PNT domain, deletion of this domain results in a 70% decrease in transcriptional activity of Erg-2 (Siddique et al., 1993)

1.7.3.3. Additional Erg functional domains

Erg also contains a carboxy terminal transcriptional activation domain between amino acids 416 and 462 which is also conserved in Fli-1, and this domain is repressed by a negative

regulatory transcriptional activation domain located between amino acids 209 and 283 (Figure 1.14) (Siddique et al., 1993).

1.7.3.4. Erg fusion proteins and the deregulation of Erg in cancer

Genomic translocations in prostate cancer and leukaemia result in dysregulated expression of full length Erg, or truncated Erg.

1.7.3.5. FUS(TLS)/Erg and EWS/Erg fusions in sarcoma and leukaemia

A number of malignant tumours are characterised by gene translocations involving the fused in sarcoma (FUS/TLS) and Ewing's sarcoma (EWS) genes. In acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), and in rare cases of Ewing's sarcoma (EWS) (Shing et al., 2003) a t(16;21)(p11.2;q22.2) translocation results in the N-terminus of Erg being replaced by the N-terminal region of FUS producing in a chimeric protein (Figure 1.15) (Ichikawa et al., 1994; Pan et al., 2008). In EWS cells, a t(21;22)(q22;q12) translocation results in the fusion of the EWS gene with the ETS DNA binding domain of Erg (Figure 1.15) (Sorensen et al., 1994; Peter et al., 1996). While the EWS/Erg fusion occurs in 5-10% of EWS, the most common (85%) fusions occur between EWS and Fli-1, the most closely related ETS factor to Erg. The FUS and EWS genes are highly related and contain conserved domains (Delattre et al., 1992). Erg fusions result in replacement of the C-terminus of EWS by the DNA-binding domain of an ETS factor and loss of the endogenous Erg promoter activity and deregulation of Erg and Erg target genes (Barr and Meyer, 2010). Little is known about the function of the EWS/Erg fusion protein, but it is assumed it functions in a similar manner to the EWS/Fli-1 fusion which can transform NIH3T3 cells (Sankar and Lessnick, 2011).

1.7.3.6. TMPRSS2/Erg in prostate cancer

ETS fusion genes are also found in prostate cancer. In about 50% of prostate cancer tissue a chromosomal translocation between TMPRSS2 and Erg, two genes normally located 3 Mbp apart on chromosome 21, is induced by exposure to androgens and DNA damage. This fusion results in the aberrant transcription of full length Erg regulated by the androgen responsive TMPRSS2 promoter (Figure 1.15) (Tomlins et al., 2005; Mani et al., 2009). While the association of the TMPRSS2/Erg fusion gene with prostate cancer has been investigated in detail, little is known about the function of this fusion. It has been suggested that expression of the TMPRSS2/Erg fusion protein in prostate cancer mediates invasion of tissue (Tomlins

et al., 2008) which may be due to the ability of TMPRSS2/Erg to induce transition from epithelial cells to mesenchymal cells (Leshem et al., 2011). Gene expression analysis has found that a number of biomarkers for prostate cancer such as genes involved in WNT and TGF- β /BMP signaling pathways are associated with the expression of the TMPRSS2/Erg fusion (Brase et al., 2011).

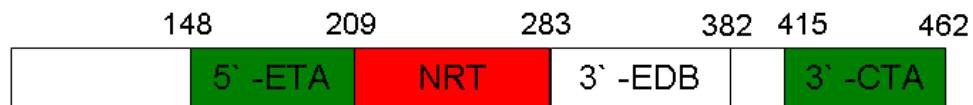


Figure 1.14. Schematic diagram of the functional domains of ERG-2. ETA, Erg/ETS transcriptional activation domain. NRT, negative regulatory transcriptional activation domain. EDB, ETS DNA binding domain. CTA, carboxyterminal transcriptional activation domain.

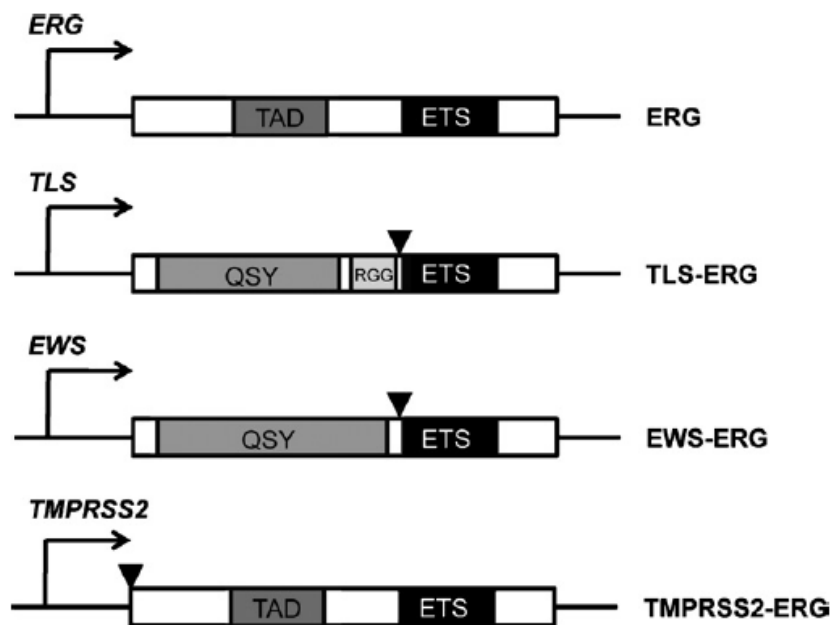


Figure 1.15 Schematic representation of ERG and ERG-fusion proteins. The arrows indicate which gene promoter drives expression of the fusion genes and the black triangles indicate the fusion breakpoints. TAD, Transcription Activation Domain; ETS, ETS DNA-binding domain; QSY, glutamine-, serine-, and tyrosine-rich domain; RGG, Arg-Gly-Gly repeat region (Modified from (Joost, 2011)).

1.8. Erg binding partners

GST-Erg-3 fusion proteins were used to analyse protein-protein interactions involving Erg (Carrere et al., 1998). Erg forms homodimers through two domains including the ETS domain and an amino-terminal domain of 200 amino acids, containing the PNT domain. Erg can also form heterodimers with some ETS factors including Ets-2, Fli-1 and Pu-1, although the domains of Erg responsible for this interaction were not identified. The ETS domain is also involved in interaction with cJun to form a ternary complex with cFos and cJun (Carrere et al., 1998; Verger et al., 2001; Camuzeaux et al., 2005).

Two separate yeast two-hybrid screen studies to identify interacting partners of Erg have been carried out. One study, using the entire *Xenopus* Erg protein to screen a stage 12 *xenopus* embryo cDNA library, found that Erg interacted with three proteins: the *xenopus* homeobox transcription factor Xvent-2, *xenopus* homeobox transcription factor Xvent-2B involved in *xenopus* development, and *xenopus* small nuclear RNP C protein, a splicing factor (Deramaudt et al., 1999). A second yeast two-hybrid screen using the amino-terminal domain of Erg as bait to screen a cDNA library from mouse hematopoietic cells, suggested that Erg interacted with the SUMO-conjugating enzyme UBC9. UBC9 has also been found to bind the PNT domain of the repressive ETS factor Tel and inhibit the repressor activity of Tel (Chakrabarti et al., 1999). In addition, Erg was shown to bind the HMT ESET (Erg Associated protein with an SET (suppressor of variegation, enhancer of zest and trithorax) domain) (Yang et al., 2002). This interaction is of particular interest for this study and will be discussed later in this chapter.

1.9. Function of Erg

1.9.1. Erg as an activator of transcription

Erg regulates the expression multiple EC genes with roles in homeostasis, junctional stability, survival, vascular development and angiogenesis. Erg plays an important role in haematopoiesis, this is partly through the Erg-mediated transactivation of RUNX1 and GATA2 which facilitates sustained haematopoiesis through the maintenance haematopoietic stem cell development (Taoudi et al., 2011). Erg binds to the promoter of, and constitutively transactivates the endothelial junctional adhesion molecule vascular endothelial (VE)-Cadherin (Birdsey et al., 2008), claudin (CLDN)5 (Yuan et al., 2012), and ICAM-2 (McLaughlin et al., 1999). Endoglin, which is expressed in EC and is essential for

development of blood vessels, is transactivated by a number of ETS factors including Erg (Pimanda et al., 2006). Erg is also implicated in the transcription of pro-angiogenic-vascular endothelial growth factor receptor (VEGFR)-1 (Wakiya et al., 1996) and VEGFR2 (Meadows et al., 2009). The coagulation factor VIII carrier protein, Von Willebrand factor (VWF), is transactivated by Erg through the binding of Erg to the VWF promoter (Schwachtgen et al., 1997; McLaughlin et al., 2001). Erg also regulates genes involved in EC cytoskeleton. Our lab has recently shown that Erg transactivates the expression of HDAC6 (Birdsey et al., 2012). Moreover, Erg drives the expression of RhoJ, a Rho GTPase which regulates the activity of Cdc42 and Rac1, and affects focal adhesion number and actinomyosin contractility (Yuan et al., 2011b)

The regulation of these genes all point to a role for Erg in angiogenesis. *In vitro* studies of Erg-depleted EC show that Erg is essential for EC tube formation within Matrigel, a model of angiogenesis (McLaughlin et al., 2001; Birdsey et al., 2008; Yuan et al., 2012). Matrigel tube formation involves the co-ordination of a number of mechanisms such as EC migration, cell-cell and cell-matrix interactions, and survival, all of which are regulated in part by Erg (Birdsey et al., 2008; Birdsey et al., 2012). Erg also regulates EC migration, as inhibition of Erg decreases EC speed and migrated distance in an *in vitro* scratch wound assay. Single cell imaging indicates that Erg inhibition results in a reduction of lamellipodia formation. Erg inhibition also results in a decrease of HDAC-6 which deacetylates tubulin, therefore decreased Erg results in increased acetylation of tubulin and increased stabilisation of microtubules (Birdsey et al., 2012). The importance of Erg for angiogenesis, survival and migration has been confirmed using the *in vivo* Matrigel plug assay. We found that Erg is required for formation of neo-vessels in mice (Birdsey et al., 2008). *In vivo* Matrigel plugs treated with Erg siRNA contained significantly more apoptotic cells than control, and EC within *in vivo* Matrigel plugs treated with Erg siRNA have lower levels of HDAC6 and higher levels of acetylated tubulin (Birdsey et al., 2012).

Developmental studies of differentiation of embryoid bodies suggest Erg is important in EC differentiation (Nikolova-Krstevski et al., 2009). Loss of function studies in zebrafish embryos treated with an Erg morpholino showed that Erg was required for normal vasculature development and maintaining vessel integrity. Loss of Erg activity resulted in haemorrhage in the head and defective intersomitic vessel formation. Moreover, combining Erg and Fli-1 morpholino constructs in the same zebrafish gave an exaggerated phenotype

indicating an additive requirement for both of these related ETS factors (Liu and Patient, 2008).

An Erg knockout mouse is currently being developed in our group and preliminary data indicates that knockout of the Erg gene in EC is embryonically lethal (Birdsey GM and Randi AM, unpublished data). This is in line with a previous study that showed that mice with a germ line mutation of Erg within the ETS DNA binding domain, resulting in a transcriptionally dead mutant, are embryonic lethal at day E13.5 and studies in heterozygotes highlighted the role for Erg in haematopoiesis (Loughran et al., 2008). As found in zebrafish, loss of Erg and Fli-1 function in mouse has a more dramatic effect than loss of either gene alone suggesting a co-regulation of Erg and Fli-1 target genes (Kruse et al., 2009).

1.9.2. Erg as a repressor of transcription

Microarray analysis has recently been carried out in our lab to investigate the genome-wide effects of Erg inhibition using antisense oligonucleotides (Genebloc) (Birdsey et al., 2012). As is consistent with the role for Erg as a transcriptional activator, 1511 genes were significantly down regulated by Erg inhibition; but surprisingly, 1138 genes were significantly up-regulated by Erg inhibition (Figure 1.16), suggesting that Erg represses the transcription of these genes in quiescent EC. Erg may repress these genes through direct binding and repression of their promoters, or indirectly through repressing or activating a mediator of their expression. Amongst the Erg-repressed genes are genes involved in inflammation including ICAM-1, VCAM-1 and IL-8 which suggests a role for Erg in repression of inflammation (Sperone et al., 2011; Birdsey et al., 2012). The microarray data suggests Erg represses a large number of genes in EC, and there have been two studies confirming this data which have both focused on the repression of pro-inflammatory genes by Erg.

1.9.3. Erg as a repressor of inflammation

Erg has recently been shown to repress expression of the pro-inflammatory chemokine IL-8 in HUVEC. Erg inhibition resulted in an increase in IL-8 expression in EC, and Erg was shown to repress an IL-8 reporter construct. Erg bound to the promoter of IL-8 in quiescent EC and mutation of the EBS at this binding site abolished repression (Yuan et al., 2009). In addition, a study carried out by our group has shown Erg represses the expression of ICAM-1, VCAM-1 and IL-8 (Sperone et al., 2011). Recent *in vitro*, *in vivo*, and pathology data have

confirmed a role for Erg as a repressor of inflammation. In an *in vitro* leukocyte adhesion assay, inhibition of Erg resulted in a 2-fold increased recruitment of leukocytes compared to control (Yuan et al., 2009). Our group has shown Erg over-expression inhibits TNF- α -induced paw swelling in an *in vivo* model of inflammation. Moreover, in eccentric atherosclerotic plaques of coronary arteries from clinical specimens, the endothelium overlying the shoulder region of the plaque, a site of leukocyte recruitment, does not express Erg, while Erg is expressed in the non-leisonal endothelium (Sperone et al., 2011). This data suggest an important role for Erg in inhibition of inflammation and the requirement for Erg to maintain EC homeostasis through repressing basal activity of pro-inflammatory genes.

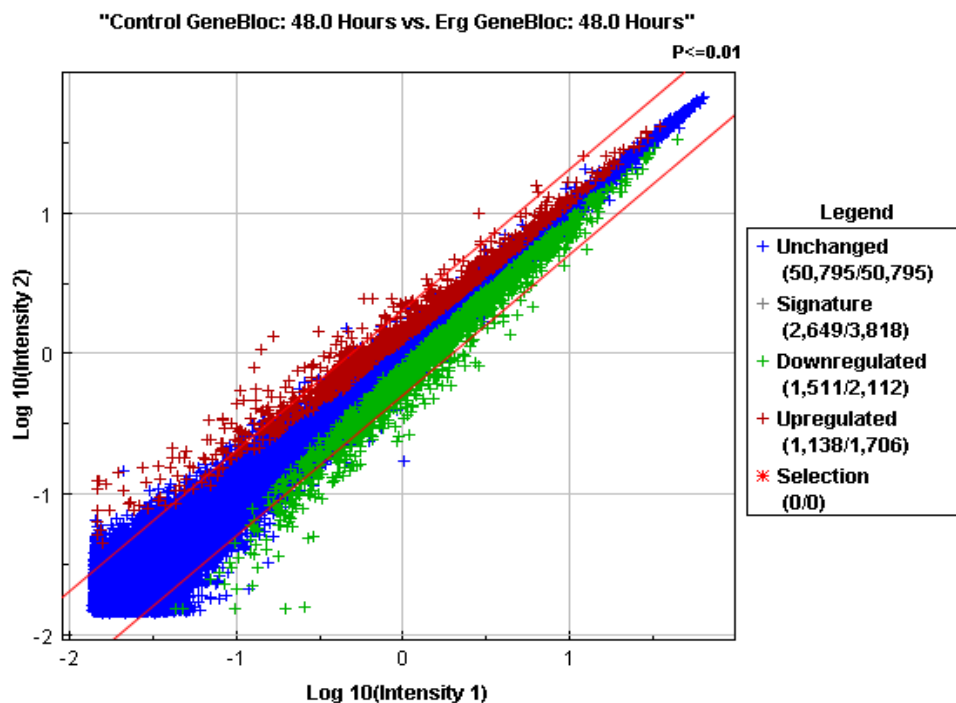


Figure 1.16 Microarray data from HUVEC treated with Erg GB. Intensity plot showing the expression values of genes that are significantly different between control GB and Erg GB at 48 hours. Red crosses represent genes significantly up-regulated, green crosses represent genes significantly down regulated and blue crosses represent genes that are unchanged. One-way ANOVA, $p < 0.01$.

1.10. Epigenetic co-regulators and Erg

While the epigenetic mechanisms involved in the regulation of genes by some ETS factors are known, the mechanisms behind Erg-mediated transactivation or repression are unknown. As described in section 1.7.4, a yeast-two hybrid screen of a mouse hematopoietic cDNA library investigating binding partners for the N-terminus of Erg identified an interaction with the HMT ESET (human homologue SETDB1). ESET specifically tri-methylates histone 3 lysine 9 (H3K9me3) which is a histone modification associated with transcriptional repression (Yang et al., 2002). ESET was shown to bind to HDAC1 and 2 and the co-repressors mSin3A and B to form a repressive complex (Yang et al., 2003a). This complex was found to have repressive properties, and the repression was associated with the interaction between ESET and mSin3A and was reversed by the HDACi TSA. Surprisingly this repressive activity was not dependent on the HMT activity of ESET. The role for Erg in the ESET repressive complex is yet to be investigated, but the interaction between Erg and the ESET repressive complex is a potential mechanism responsible for Erg repression of pro-inflammatory genes.

1.11. Purpose of investigation

The importance of Erg as a regulator of endothelial homeostasis through the transcriptional activation of protective genes is well characterised; however, the role for Erg as a repressor is still a new field, moreover, little is known about the mechanisms of Erg-mediated repression. Genome-wide expression analysis suggests that in addition to transactivating genes Erg may have a dual role as a repressor of large numbers of genes. The aim of this study is to investigate the mechanisms behind the role of Erg as a transcriptional repressor of pro-inflammatory genes to ultimately identify novel therapeutic targets of diseases caused by chronic inflammation including atherosclerosis.

Chapter Two

Erg represses ICAM-1 expression by binding specific ETS binding sites within the ICAM-1 promoter.

2.1. Introduction

2.1.1. Genome-wide gene expression profiling analysis suggests Erg is a repressor of inflammation

Results of previous studies from our lab and others have highlighted the role for the ETS transcription factor Erg in maintaining EC homeostasis through regulating expression of endothelial protective genes. In order to investigate the genome-wide targets of Erg, microarray analysis of endothelial gene expression after Erg inhibition was carried out (Birdsey et al., 2012). HUVEC were treated with either control or Erg Genebloc (GB) for 24 and 48 hours and a microarray was carried out to identify changes in gene expression. This identified that a number of pro-inflammatory genes including ICAM-1, VCAM-1, and IL-8 were up-regulated following Erg inhibition (Birdsey et al., 2012; Sperone et al., 2011). We and others have previously investigated Erg as a transcriptional activator of genes involved in EC homeostasis, and as expected, the microarray analysis identified the decreased expression of many genes after Erg inhibition. However, surprisingly Erg inhibition resulted in increased expression of a number of other genes, which suggests they are repressed by Erg (Figure 1.16). Gene ontology analysis identified a group of genes repressed by Erg that have a role in pro-inflammatory pathways (Birdsey et al., 2012; Sperone et al., 2011). Erg may repress these pro-inflammatory genes either directly, by binding to their promoters, or indirectly, by affecting a gene upstream of these regulated genes. Moreover, Erg may use a single mechanism or multiple mechanisms to repress this set of pro-inflammatory genes. The identification of these mechanisms could lead to the discovery of novel therapeutic targets for diseases where inflammation and EC dysfunction occur, including atherosclerosis.

2.1.2. Regulation of ICAM-1

From the list of pro-inflammatory genes repressed by Erg, we chose to study ICAM-1. ICAM-1 is an important mediator of the leukocyte adhesion cascade; additionally, the regulatory elements in the promoter of ICAM-1 have been studied in detail (reviewed in (Roebuck and Finnegan, 1999)). As described in Chapter One, ICAM-1 is expressed at low levels in resting EC and is up-regulated after exposure to pro-inflammatory stimuli including TNF- α , IL-1 β , retinoic acid, IFN- γ and H₂O₂ (Voraberger et al., 1991; Wawryk et al., 1991; Roebuck et al., 1995; Aoudjit et al., 1994). Activation of the ICAM-1 promoter after inflammatory stimuli depends on the NF- κ B, AP-1, ETS and STAT families of transcription

factors. The binding sites for these transcription factors on the ICAM-1 promoter have been investigated (Van de Stolpe and Van der Saag, 1996; Hou et al., 1994; Ledebur and Parks, 1995; Caldenhoven et al., 1994; Roy et al., 2001; Yuan et al., 2001; Yockell-Lelievre et al., 2009) and are shown in Figure 1.6. Little is known about the regulation of basal ICAM-1 expression in EC. Despite low ICAM-1 expression levels in resting EC, nuclear extracts contain proteins capable of binding to the ICAM-1 promoter sequence *in vitro*, and these proteins may be involved in basal regulation of ICAM-1 (Hou et al., 1994; Paxton et al., 1997; Ledebur and Parks, 1995).

2.1.3. ETS factors and ICAM-1 regulation

A role for ETS factors as activators and repressor of ICAM-1 in non-EC has been shown. In rabbit kidney RK13 cells the ETS factors ERM, Ets-1, and Ets-2 were found to drive ICAM-1 activity via the EBS located 118 bp and 96 bp upstream of the transcription start site (de Launoit et al., 1998; Maurer et al., 2003). All three ETS factors are expressed constitutively in EC, although Ets-1 and ERM are expressed at low levels (Hollenhorst et al., 2004). In the same study in RK13 cells, over-expression of the ETS factor Pu-1 did not regulate ICAM-1 expression, suggesting specificity for certain ETS factors in regulating and binding EBS in the ICAM-1 promoter. ETS factors are also involved the regulation of ICAM-1 by inflammatory stimuli. Recently functional cooperation between Ets-1 and STAT-1 has been demonstrated (Yockell-Lelievre et al., 2009). Double mutation of the EBS at -118 and -96 inhibited ICAM-1 up-regulation by IFN- γ in COS-1 cells; however this effect was not seen in HEK293 cells. This suggests that both ETS factor specificity and cell specificity are important in the regulation of ICAM-1. ETS factors have also been found to repress ICAM-1. The ETS factor FEV repressed ICAM-1 expression induced by Ets-2 in RK-13 cells (Maurer et al., 2003). FEV is closely related to Erg and Fli-1, sharing 90.21%, and 91.99% amino acid-similarity respectively within the ETS DNA binding domain (Peter et al., 1997). However FEV is not expressed ubiquitously; it is not expressed in EC and has only been found in Dami Megakaryocytic cells and serotonergic neurons (Maurer et al., 2003; Maurer et al., 2004). Therefore, ETS factors activate or repress ICAM-1 in a cell and stimulus specific manner.

2.1.4. Potential mechanisms of repression

Transcription factors can repress target genes in a variety of ways. They may inhibit or sequester specific transcriptional activators away from the promoters of target genes. They

may bind to a specific sequence in the promoters of repressed genes and directly block binding of transcriptional activators. Or they may repress indirectly by binding to the promoters of and altering the activity of transcriptional activators. Finally, transcriptional repressors may repress by binding to target gene and recruiting co-factors that modify the chromatin structure, leaving the gene inaccessible to transcriptional machinery (reviewed in (Payankaulam et al., 2010)). Erg appears to act as a transcriptional activator and repressor for different target genes within the same cell and Erg may act as a repressor through any of the mechanisms described above.

We hypothesised that Erg maintains EC homeostasis by repressing basal pro-inflammatory gene expression. This chapter investigates the mechanisms involved in the repression of ICAM-1 by Erg.

2.1.5. Aims

- Confirm whether Erg represses ICAM-1 at a transcriptional level in primary EC.
- Investigate the interaction of Erg with the ICAM-1 promoter and confirm that Erg repressed ICAM-1 directly.
- Investigate whether Erg binds directly to the ICAM-1 promoter by carrying out a study of the EBS in the ICAM-1 promoter to identify which EBS are involved in the Erg-mediated repression of ICAM-1 promoter binding sites.

2.2. Materials and Methods

2.2.1. Antibodies

Antibodies used in this chapter include: Polyclonal antibodies anti-Erg-1/2/3 sc-353 (Santa Cruz Biotechnology), anti-NF- κ B p65 ab7970 (AbCam), anti-Fli-1 sc-356 (Santa Cruz Biotechnology) anti-GAPDH cat MAB374 (Millipore), anti-ICAM-1 (clone 15.2, kind gift of Prof. Nancy Hogg), rabbit IgG isotype control PP64 (Millipore). Anti-V5 46-0705 (Invitrogen), NA931 anti-mouse/HRP (1:10000 in PBS-T, Amersham Biosciences), RPN4301 anti-Rabbit/HRP (1:5000 PBS-T Amersham Biosciences).

2.2.2. HUVEC isolation

The veins of human umbilical cords were washed twice with 20 ml HBSS and incubated with 0.5 mg/ml collagenase in 20 ml HBSS at 37°C and 5% CO₂ for 8 mins. The cell suspension

was collected in a 50 ml tube and centrifuged for 9 mins at 306 *g* at room temperature. The cell pellet was re-suspended in 5 ml complete M199 medium, and transferred to a T25 flask pre-coated with 1% gelatin (Sigma). The following day the medium was changed and cells were passage after reaching confluence. Cells were used between passage 2 and 4.

2.2.3. Cell culture

Human umbilical vein ECs (HUVEC) were maintained in M199 medium with 20% foetal bovine serum (FBS), 1 U/ml penicillin/ 0.1 mg/ml streptomycin, 2 mM L-glutamine, 300 µg/ml EC growth factor (ECGF, BD Bioscience) in heparin (100 U/ml, CP Pharmaceuticals) at 37°C in 5% CO₂, in plates pre-coated with 1% gelatin. Cells were passaged every 3-4 days by trypsinization: cells were washed twice with PBS without Ca²⁺ and Mg³⁺, then incubated with 0.5 mg/ml trypsin in EDTA (MP Biomedicals) for 3 mins at 37°C and 5% CO₂ and re-suspended in growth medium. HeLa cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1 U/ml penicillin/ 0.1mg/ml streptomycin and cultured at 37°C in 5% CO₂ and passaged 1:5 every 3 days.

2.2.4. Erg inhibition by Genebloc

HUVEC were seeded in a 6-well gelatin coated plate at a density of 1x10⁵ cells per 35 mm diameter well in EGM2 medium (Lonza) and incubated overnight. The following day cells were treated with control or Erg antisense genebloc (GB). GB (final concentration 100 nM) and transfection lipid AtuFect 01 (final concentration, 1 µg/ml) (Silence Therapeutics) were mixed at 5 times concentration in OptiMEM medium (Invitrogen) at 37°C for 30 mins in polystyrene tubes. After incubation, the lipid/GB mix was added to HUVEC to give a 1x concentration and incubated for either 24 or 48 hours.

2.2.5. Erg inhibition by siRNA

HUVEC were seeded in a 6-well gelatin coated plate at a density of 1x10⁵ cells per 35 mm diameter well, or in a gelatin coated 100 mm diameter dish at a density of 1x10⁶ cells in EGM2 medium (Lonza) and incubated overnight. The following day cells were treated with Allstars negative control siRNA (Qiagen), Hs_Erg_7, Hs_Fli-1_7, Hs_Ets-2_7 or Hs_GAPBα_10 siRNA (Qiagen). siRNA (final concentration 10 nM) and transfection lipid AtuFect 01 (final concentration, 1 µg/ml (Silence Therapeutics) were mixed at 5 times concentration in OptiMEM medium (Invitrogen) at 37°C for 30 mins in polystyrene tubes.

After incubation, the lipid siRNA complex was added to HUVEC to give a 1x concentration and incubated for either 24 or 48 hours.

2.2.6. Adenovirus amplification and titration

The Adenovirus expressing p55-Erg was generated (as described in (Sperone et al., 2011)) and plaque forming units (PFU) were titrated using pAd/CMV/V5-DEST (Invitrogen) which p55 Erg had been cloned into. The volume of virus used to infect the cells was calculated using the formula; number of infected cells x desired multiplicity of infection (MOI) = Total plaque forming units (PFU) needed / PFU/ ml = ml virus.

Adenovirus was amplified in human embryonic kidney (HEK) 293A cells and purified using the AdenoX™ virus purification kit (Clontech) following manufacturer's instructions. Briefly HEK 209A cells were grown in 175 cm² tissue culture flask (BD falcon) until confluent. Cells were infected with 200 µl adenovirus and incubated for 5 days or until cytopathic effects are seen. The adenovirus was then purified from the cells using the kit components and eluted in a volume of 3 ml.

Adenoviral titre was then determined using the Adeno-X Rapid titre kit following manufacturer's instructions.

2.2.7. Erg over-expression with adenovirus

HUVEC were seeded in 16 mm diameter gelatin coated well of a 24 well plates at a density of 3x10⁴ cells per well. The following day cells were infected with 50 MOI AdErg or AdLacZ in 250 µl of EGM2. After 3 hours the media was changed and cells were incubated for 24 hours before addition of luciferase reporter vector (See 2.2.18.2).

2.2.8. RNA extraction and cDNA generation

Cells were harvested with 350 µl RLT buffer (Qiagen) containing 3.5 µl 2-mercaptoethanol (Sigma). The lysate was passed 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. 350 µl of 70% ethanol was added to the homogenized lysate. The sample was transferred to an RNeasy spin column (Qiagen) placed in a 2 ml collection tube, and centrifuged for 15 secs at 8000 *g*. The flow-through was discarded. 350 µl buffer RW1 (Qiagen) were added to the RNeasy spin column. The column was centrifuged for 15 secs at 8000 *g*, and the flow-through was discarded. 30 Kunitz units of DNase I (Qiagen) diluted in RNase-free water were added to 70 µl buffer RDD (Qiagen), and added to the RNeasy spin column membrane.

After 15 mins 350 µl buffer RW1 (Qiagen) were added to the RNeasy spin column. The column was centrifuged for 15 secs at 8000 *g*, and the flow-through was discarded. 500 µl buffer RPE (Qiagen) were added to the RNeasy spin column. The column was centrifuged for 2 mins at 8000 *g*, and placed in a new 2 ml collection tube. 50 µl RNase-free water (Qiagen) were added to the spin column membrane. The column was centrifuged for 1 min at 8000 *g* to elute the RNA. The purity and the concentration of the RNA were analyzed using the Nanodrop spectrophotometer (Thermo Scientific).

2.2.9. cDNA synthesis

cDNA was synthesised using the SuperScript® III RT kit (Invitrogen) following manufacturer's instructions. Briefly, 1 µg of total RNA was added to 1 µl oligo(dT)₂₀ (50 µM) (Promega) and 1 µl 10 mM dNTP Mix (Promega), and adjusted to a final volume of 13 µl with water. The mixture was heated to 65°C for 5 mins and incubated on ice for 5 mins. 4 µl of 5x first-strand buffer, 1 µl of 0.1M dithiothreitol (DTT), and 1 µl of SuperScript III reverse transcriptase were added to the mixture. The reaction was incubated at 50°C for 60 mins and inactivated by heating it to 70°C for 15 mins.

2.2.10. Quantitative real-time PCR

5 µl of template cDNA (pre-diluted 1:50) or chromatin was amplified using the following reagents; 12.5 µl Sybr ® Green JumpStart™ Taq ReadyMix™ (Sigma); 5.5 µl H₂O, 0.4 µM forward primer, 0.4 µM reverse primer. The cDNA or DNA was denatured by heating the mixture at 95°C for 3 mins, and amplified according to the following conditions, repeated for 50 cycles: 95°C for 15 secs, 60°C for 30 secs. Real-time PCR were performed using the iCycler thermocycler (Biorad). The results were analysed using iCycler IQ optical system software (Biorad).

Table 2.1 Oligonucleotides used for RT PCR

Erg	Fwd 5'-GGAGTGGGCGGTGAAAGA-3' Rev 5'-AAGGATGTCGGCCTTGTAGC-3'
Fli-1	Fwd 5'-ACGGGACTATTAAGGAGGCTCTG-3' Rev 5'-GTTGACCCTCACTGGCCTGATTG-3'
Ets-2	Fwd 5'-GAGTTTCAGATGTTCCCAAGTC-3' Rev 5'-CTCCGCACCGTTCTCAGG-3'
GABP α	Fwd 5'-AAGTGACAAGATGGGCTGCT-3' Rev 5'-CTCCCCGAAATGTTGAGTGT-3'
GAPDH	Fwd 5'-CAAGGTCATCCATGACAACCTTG-3' Rev 5'-GGGCCATCCACAGTCTTCTG-3'
ICAM-1	Fwd 5'-CTGAAACTTGCTGCCTATTGGG-3' Rev 5'-ACACATGTCTATGGAGGGCCA-3'
cIAP2	Fwd 5'-TGCCAAGTGGTTTCCAAGGTGTGAG-3' Rev 5'-GGGCTGTCTGATGTGGATAGCAGC-3'
TNFAIP3	Fwd 5'-GCGTTCAGGACACAGACTTC-3' Rev 5'-TTCATCATTCAGTTCCGAGTATC-3'

2.2.11. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot

Cells were washed twice with ice-cold PBS, harvested with CelLytic™ M Cell Lysis Reagent (Sigma), containing 1:100 dilution of protease inhibitor cocktail (Sigma) and incubated at 4°C for 15 mins. Lysates were centrifuged for 15 mins at 2000 *g* at 4°C and supernatants containing proteins were collected.

Immunoblotting was carried out using 15 µg of lysate, determined with Bio-Rad protein assay (Bio Rad Laboratories GmbH): the absorbance of the samples was obtained with Synergy HT spectrophotometer (BIO-TEK). Samples were added to laemmli sample buffer (BioRad) containing 0.7 M 2-mercaptoethanol and boiled for five mins at 100°C. When samples were loaded in non-reducing conditions, 2-mercaptoethanol was omitted from laemmli sample buffer.

Proteins were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore), blocked with PBS-T milk (PBS with 0.1% Tween-20 (PBS-T); 5% low-fat milk

powder) for 1 hour at room temperature, and probed with the following antibodies; anti-ESET (1:5000 in PBS-T); anti-ICAM-1 (1:1000 in PBS-T); anti Erg (1:400 in PBS-T); anti-Tubulin (1:10000 in PBS-T); anti-GAPDH (1:10000 in PBS-T milk). These antibodies were incubated on the membrane for 1 hour at room temperature, except anti-ESET which was incubated overnight at 4°C. The membrane was rinsed in PBS-T, then incubated for 1 hour at room temperature with the following secondary antibodies conjugated to horseradish peroxidase (HRP): NA931 anti-mouse/HRP (1:10000 in PBS-T), RPN4301 anti-Rabbit/HRP (1:10000 in PBS-T). The membrane was rinsed in PBS-T, and then washed three times for 5 mins with PBS-T. Detection was visualized by enhanced chemiluminescence (ECL™) detection system (Amersham™ GE Healthcare) and Kodak®BioMax® Light Film (Sigma).

2.2.12. Sequence alignment

Human (accession number X59286.1) and Mouse (accession number M90551.1) ICAM-1 promoter sequences were aligned using ClustalW2 analysis software, <http://www.ebi.ac.uk/Tools/msa/clustalw2/>, using default settings. (Larkin 2007 *Bioinformatics*, 23, 2947-2948)

2.2.13. Preparation of sheared chromatin from HUVEC

Chromatin immunoprecipitation (ChIP) was carried out using the ChIP-IT™ express kit (Active Motif), following manufacturer's instructions. Approximately 7.5×10^6 passage-3 HUVEC were seeded onto a 15 cm diameter dish pre-coated with 1% gelatin and incubated for 48 hours. HUVEC were fixed by incubating with 1% paraformaldehyde (Sigma) in basal M199 media (Invitrogen) for 10 mins at room temperature on an orbital shaker (Bibby scientific) at 60 rpm. Plates were rinsed with 10 ml PBS followed by a glycine solution for 5 mins. After one further rinse with ice-cold PBS, cells were collected into a 15 ml tube using 2 ml PBS containing 10 µl PMSF, then centrifuged for 10 mins at 1000 *g* and 4°C.

The pellet was then re-suspended in 1 ml ice-cold lysis buffer (Active Motif) containing 5 µl protease inhibitor cocktail (Active Motif) and 5 µl PMSF (Active Motif) and incubated on ice for 30 mins. The cell membranes were disrupted by passing cells through in an ice-cold dounce homogeniser and absence of cell membrane integrity was assessed by trypan blue staining. The homogenised cell mix was centrifugation 2400 *g* at 4°C to pellet the nuclei. The pellet was re-suspended in 350 µl shearing buffer (Active Motif) supplemented with 1.75 µl PMSF. Nuclei were sheared using a Bioruptor (Diagenode, Belgium) water bath sonicator,

using 5 cycles of 30 secs on 30 secs off. The sheared chromatin was then centrifuged at 18,000 *g* for 10 mins at 4°C and the supernatant containing the sheared chromatin was stored at -80 °C.

2.2.14. Assessment of chromatin fragment sizes.

150 µl dH₂O and 10 µl 5M NaCl was added to 50 µl of sheared chromatin. This was heated at 95°C for 15 mins. When the sample returned to room temperature 1 µl RNase A was added and incubating at 37°C for 15 mins, 1 µl of Proteinase K (Active Motif) was then added to the sample which was incubated at 67°C overnight. The sample was then purified and concentrated using a Minelute PCR purification kit (Qiagen), and the Chromatin was eluted in a final volume of 15 µl. This 15 µl was then run on a 2% agarose gel with at 100 bp DNA marker (New England Biolabs).

2.2.15. Chromatin Immunoprecipitation

Chromatin was immunoprecipitated using the ChIP-IT™ express kit (Active Motif), following manufacturer's instructions. 100 µl of sheared chromatin was added to a siliconised microcentrifuge tube containing 25 µl protein G magnetic beads (Active Motif), 20 µl ChIP buffer 1 (Active Motif), 1 µl protease inhibitor cocktail, 3 µg of antibody (anti-Erg, Rabbit IgG control) and dH₂O to give a final volume of 200 µl. This was then incubated overnight at 4°C with constant mixing. The antibody and chromatin complexes bound to magnetic beads were separated from the chromatin solution using a magnetic bar. Beads were then washed twice with 800 µl ChIP buffer 1 followed by 2 washes with 800 µl ChIP buffer 2 (Active Motif). Chromatin was then eluted from the washed beads by adding 50 µl of elution buffer AM2 (Active Motif), and incubating on an end-to-end rotator for 15 mins at room temperature. 50 µl of reverse cross-linking buffer (Active Motif) was then added to eluted chromatin and beads. This solution containing the chromatin was removed from the beads using a magnetic stand and collected in a PCR tube. An "input DNA" sample was made by taking 10 µl of sheared chromatin and adding 88 µl of ChIP buffer 2 and 2 µl 5 M NaCl. This and the immunoprecipitated chromatin samples were incubated at 95°C for 15 mins to reverse the cross links. 2 µl of proteinase K was then added and samples were incubated at 37°C for 1 hour to digest proteins.

Table 2.2 Oligonucleotides used in ChIP

ICAM-1 -25 to -204	Fwd 5'-GCCCCGATTGCTTTAGCTTGG-3' Rev 5'-TTTATAGCGCTAGCCACCTG-3'
ICAM-1 region 1	Fwd 5'-GGGAAATATGCGTCTCTGGA-3' Rev 5'-CAGAAGGGGAAGCGAAGG-3'
ICAM-1 region 2	Fwd 5'-CTCAGTTTCCCAGGGACAG-3' Rev 5'-AGGAAGGAAGCTGCGTGAT-3'
ICAM-1 region 3	Fwd 5'-GACACAATAACAGTCTCCACTCTCC-3' Rev 5'-GCTGCTCCCGGCCTCCAG-3'
ICAM-1 region 4	Fwd 5'-TTGGAAATTCCGGAGCTGAA-3' Rev 5'-TGCTGCAGTTATTTCCGGACT-3'
ICAM-1 region 5	Fwd 5'-GGCGCGTGATCCTTTATAGC-3' Rev 5'-ATTTGTTCCGGAGGG-3'
cIAP2	Fwd 5'-GCCACGGTTAAGAGTCATGC-3' Rev 5'-AAATCCCCACCCCTATCTGT-3'
TNFAIP3	Fwd 5'-GCCTACAACCCGCATACAAC-3' Rev 5'-CTGGGGGTGTGATCTCTCTT-3'
GAPDH	Fwd 5'-ATGGTTGCCACTGGGGATCT- 3' Rev 5'-TGCCAAAGCCTAGGGGAAGA-3;

2.2.16. Promoter analysis by MatInspector

Putative transcription factor binding sites were identified using MatInspector (Genomatix) (Cartharius et al., 2005).

2.2.17. Development of ICAM-1 promoter driven luciferase construct**2.2.17.1. Plasmids**

pGL4.10[luc2] (Promega Madison USA) Firefly Luciferase empty vector, lacking a promoter sequence, was used as a control.

pGL4.73[hRluc/TK] (Promega Madison USA) Renilla luciferase vector was used as an internal control in the luciferase assay.

pGL4.13 [luc2/sv40] (Promega Madison USA) was used as a parallel control to measure transfection efficiency.

2.2.17.2. Plasmid development

Luciferase reporter plasmid, pBH ICAM-1 1.3 containing the ICAM-1 promoter sequence 1.3 kb upstream of the transcription start sight (kindly provided by Dr.C. Stratowa, Boehringer, Vienna), was digested with restriction enzymes KpnI and SalI to extract the 1.3 kb insert of the ICAM-1 promoter (Voraberger et al., 1991). Firefly luciferase reporter vector, pGL4.10 [luc2] (Promega Madison USA) was digested with restriction enzymes KpnI and XhoI and the 1.3 kb fragment of the ICAM-1 promoter was ligated into the pGL4 backbone. Correct insertion was confirmed by digesting the ligated plasmid pGL4 ICAM-1 1.3 with KpnI and EcoRV and sequencing of the plasmid using forward and reverse primers from the 5' and 3' region of the ICAM-1 insert. The ligated plasmid pGL4 ICAM-1 1.3 was then transformed into JM109 bacterial cells and grown up in LB broth containing 100 µg/ml ampicillin for selection. Plasmids were purified by using the Qiagen plasmid mini prep kit, if plasmids were used for sequence analysis, or by Maxiprep, using the Qiagen Endotoxin free maxiprep kit, if plasmids were to be used in assays.

2.2.18. ICAM-1 reporter construct transfection

2.2.18.1. Microporation

Either 500 ng of pGL4 control or pGL4 ICAM-1 1.3 Firefly luciferase reporter plasmid and 500 ng of pGL4 Renilla luciferase control plasmid DNA were delivered into 5×10^4 HUVEC suspended in buffer R (Invitrogen) by microporation using the MP100 (Invitrogen) using settings of 1 pulse, for 30 ms, at 1400 V. Microporated cells were seeded in a 24 well dish pre-coated with 1% gelatin, containing 0.5 ml EGM-2 (Lonza) without gentamycin.

2.2.18.2. Transfection

A 1% gelatin coated 24 well plate was seeded with 3×10^4 HUVEC per well. The following day cells were transfected with 250 ng pGL4 ICAM-1 1.3 or pGL4.10 empty vector and 250 ng pGL4.73 Rluc/TK control renilla plasmid using transfection lipid Genejuice (Novagen) transfection reagent: 1.5 µl lipid was added to 50 µl of OptiMEM mixed by vortexing and

incubated at room temperature for 5 mins. Plasmid DNA was then added to lipid/OptiMEM and mixed gently with pipette. Following 15 mins incubation lipid/OptiMEM/plasmid mix was added to HUVEC and incubated for 24 or 48 hours. Transfected cells were then treated with media containing 10 ng/ml TNF- α or media alone for the final 6 hours of each time-course

2.2.19. Luciferase assay

Luciferase expression levels were measured using the Dual-luciferase® reporter assay system (Promega). HUVEC transfected with pGL4 ICAM-1 1.3 or pGL4 control and pGL4.73[hRluc/TK] were lysed with 100 μ l passive lysis buffer (Promega) per well of a 24 well plate, for 15 mins at room temperature. Lysates were collected and 20 μ l of each lysate was assessed in triplicate. Dual-luciferase assay was carried out using the Synergy HT spectrophotometer (BIO-TEK) plate reader with injectors. 100 μ l of luciferase assay reagent II (LARII) was injected into each well containing 20 μ l of lysate and luminescence from firefly luciferase was measured for 10 secs. Then 100 μ l of Stop and Glo® reagent was added and luminescence from renilla luciferase was measured for 10 secs. Dual luciferase ratio (DLR) was calculated by dividing the firefly luciferase signal by the renilla Luciferase signal.

2.2.20. Site directed mutagenesis of ICAM-1 promoter construct

Mutagenesis of ETS binding sites in the ICAM-1 promoter of pGL4 ICAM-1 1.3 was carried out using the Quickchange Lightning Multi site directed mutagenesis Kit (Agilent technologies). Mutant strand synthesis was carried out by adding 100 ng of a single mutagenic primer sequence to a mix containing 100 ng of pGL4 ICAM-1 1.3, 2.5 μ l 10x quickchange lightning multi buffer, 1 μ l dNTPmix, 1 μ l quickchange lightning multi enzyme blend and H₂O up to a volume of 25 μ l. This mixture was then processed on a Veriti 96-well thermal cycler (Applied Biosystems) with cycling parameters of:

95 °C for 2 mins,

95 °C 20 secs,

55 °C 30 secs,

65 °C 3 mins,

65 °C for 5 mins.

} x30 cycles

The parental strand of plasmid was then digested by adding 1 µl of DpnI enzyme (Agilent) and incubated for 5 mins at 37°C. 2 µl DpnI treated sample was added into 45 µl of XL-10 gold ultra competent *E.coli* which had previously been thawed on ice and mixed with 2 µl of 2-mercaptoethanol (Agilent). This mix was incubated on ice for 30 mins. XL-10 cells were then heat-shocked at 42°C for 30 secs then placed on ice for 2 mins before 500 µl of warm S.O.C. medium (Invitrogen) was added to each tube. This mix was incubated at 37°C for 1 hour before 100 µl was plated out on LB agar plate containing 50 µg/ml ampicillin and colonies were grown at 37°C over-night. Single colonies were picked and amplified in 5 ml LB broth containing 50 µg/ml ampicillin. Plasmid DNA was purified from the 5 ml culture using a Qiagen miniprep kit, following manufacturer's instruction. 10 µg of plasmid DNA was then sequenced using primers up and downstream of mutation site (Imperial College London MRC core sequencing facility). And sequences were checked for correct mutagenesis. Clones containing the correct mutation were amplified and purified using the Endo-free plasmid maxi kit (Qiagen). Plasmid concentration was analysed using the Nanodrop spectrophotometer (Thermo Scientific).

Table 2.3 Oligonucleotides for generating ICAM-1 promoter mutant constructs. EBS are highlighted in bold in WT sequence. Mutation sites are underlined in MUT sequence.

ICAM-1 promoter mutation	Oligonucleotide sequences WT/Mut
EBS-96	GGAAATAACTGCAGCATTTG TTCC GGAGGGGAAGGC WT GGAAATAACTGCAGCATTTGTT <u>GGG</u> GAGGGGAAGGC MUT
EBS-118	GGGGCACCTGTCAGTCC GGAA ATAACTGCAGCATTT WT GGGGCACCTGTCAGTCC <u>CC</u> AAATAACTGCAGCATTT MUT
EBS-156	GGCCAGCGAGGG GGAT GACCCCTCTCGG WT GGCCAGCGAGGG <u>ACC</u> ATGACCCCTCTCGG MUT
EBS-181	CCGATTGCTTTAGCTTGAAAA TTCC GGAGCTGAAGCG WT CCGATTGCTTTAGCTTGAAAT <u>TGGG</u> GAGCTGAAGCG MUT
NF-κB	CCACCGCCGCCGATTGCTTTAGCT TGGAAATTCC GGAGCTGAAGCGGC WT CCACCGCCGCCGATTGCTTTAGCTT <u>CTAGATTAGG</u> GAGCTGAAGCGGC MUT
EBS-358	AACAGTGTCCTCTCCGG GGAA GTTGGCAGTATTTAAAA WT AACAGTGTCCTCTCCGG <u>CCA</u> AGTTGGCAGTATTTAAAA MUT
EBS-834	GCTAGCTGCCTCAGT TTCC CCGCGGAGC WT GCTAGCTGCCTCAGTTT <u>GGCC</u> GCGGAGC MUT
EBS-907	GCTGCTGCCTCAGT TTCC CAGCGACAGGCA WT GCTGCTGCCTCAGTTT <u>GGC</u> AGCGACAGGCA MUT

2.2.21. Deletion constructs

ICAM-1 deletion constructs were generated by amplifying fragments of pGL4 ICAM-1. Forward primers consisted of the ICAM-1 promoter sequence with containing KpnI restriction enzyme sites at the 5' end. Reverse primer sequence was within pGL4 to amplify the pGL4 multiple cloning site. 28 ng pGL4 ICAM-1 1.3 was amplified using 2.5 units PFU-turbo taq polymerase (Stratagene), 10 mM dNTP, 100 ng forward and reverse primers, 5 µl PFU reaction buffer, then the volume was made up to 50 µl with water. The PCR reaction mix was amplified on the thermocycler (Veriti thermocycler, Applied Biosystems) with

95°C 1 for min,

95°C for 30 secs,

Primer Tm-5°C for 30 secs

72°C for 1 min.

} 30 cycles

Final incubation of 72°C for 10 mins.

PCR product and pGL4.10[luc2] (Promega Madison USA) were then digested using KpnI and EcoRV restriction enzymes. Digested products were run on a 0.8% gel. Bands from digested fragments were then excised and purified using the Qiaquick gel-extraction kit (Qiagen), then ligated overnight using T4 DNA ligase (Promega). After ligation, plasmids were transformed into JM109 *E.coli*, and plated onto LB agar containing 50 µg/ml ampicillin and incubated overnight at 37 °C. Colonies were then picked, amplified and sequenced as in 2.2.20.

Table 2.4 Oligonucleotides for generating ICAM-1 deletion constructs

ICAM-1 deletion construct primer	Oligonucleotide sequences
IC1 305 fwd	ATGCGGTACCCGGTGTAGACGTGATTCAA
IC1 163 fwd	ATGCGGTACCCAGCGAGGGAGGATGAC
IC1 103 fwd	ATGCGGTACCATTTGTTCCGGAGGGGAAG
pGL4 reverse	AACAGTACCGGATTGCCAAG

2.2.22. EMSA

2.2.22.1. Preparation of nuclear lysate

Nuclear extracts were taken from a confluent monolayer of HUVEC using the Active Motif Nuclear extract kit following manufactures instructions. Briefly cells were washed with ice cold PBS containing phosphatase inhibitors (PBS-PI), then scraped into 3 ml of PBS-PI and collected into a 15 ml falcon tube which was centrifuged at 500 rpm for 5 mins. The cell pellet was then resuspended in 500 µl hypotonic solution and incubated in a 1.5 ml microcentrifuge tube on ice for 15 mins. 25 µl of detergent was then added and the sample was vortex at the highest setting for 10 secs to break open the cell membranes. Nuclei were collected by centrifugation at 14,000 g for 30 secs, and the pellet was resuspended in 50 µl complete lysis buffer. To ensure complete nuclear lysis, samples were shaken on ice at 150 rpm on an orbital shaker for 30 mins before vortexing for 30 secs at the highest setting. Protein amounts in the nuclear lysate were then quantified using the BioRad DC protein assay.

2.2.22.2. Electrophoretic mobility shift assay

EMSA was carried out using the LightShift® Chemiluminescent EMSA kit (Thermo Scientific) following manufacturer's instructions. Briefly 2 µg of nuclear extract was incubated with 20 fmol biotin end-labelled target double stranded oligonucleotide in a buffer containing 2 µl 10x binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5) 0.05 µg/µl Poly(dI•dC), 1 µl 2.5% glycerol and water to a final volume of 20 µl. Protein/oligo complexes were detected by the addition of 2 µg antibody, or saturating amounts (4 pmol) of unlabelled probe. Samples were incubated at room temperature for 20 mins before addition of 5 µl loading buffer. A 6% tris borate EDTA (TBE) gel was pre-electrophoresed for 30 mins at 100 V in an XCell SureLock® Mini-Cell system (Invitrogen) containing 0.5X TBE buffer prior to loading samples. 15 µl of each sample was loaded and electrophoresed at 100 V for approximately 2 hours until bromophenol blue dye had migrated $\frac{3}{4}$ down the length of the gel. Oligonucleotides were then transferred to a Hybond N⁺ Nylon membrane (GE healthcare) using the XCell II™ Blot Module (Invitrogen) containing 0.5x TBE cooled to 4°C and run at 380 mA for 45 mins. The membrane was then removed and briefly dried bromophenol blue side-up before being place face down on a transilluminator containing 312 nm bulbs for 15 mins to cross-linking transferred DNA to membrane. Biotin labelled oligonucleotides were detected using the chemiluminescent nucleic acid detection module

(Thermo Scientific). Briefly, the membrane was blocked with pre-warmed (37°C) blocking buffer for 15 mins gently shaking. The blocking buffer was removed and a conjugate/blocking buffer containing streptavidin-horseradish peroxidase was added to the membrane and incubated at RT for 15 mins. The membrane was then washed four times for 5 mins in 1x wash solution before being incubated with substrate equilibration buffer for 5 mins. Finally the membrane was incubated with substrate working solution and incubated for 5 mins before exposing membrane to autoradiographic film (Biomax light film. Kodak). Films were then developed by using a compact X4 automatic X-Ray film developer (X-ograph).

Table 2.5 Oligonucleotides used in EMSA

118 WT	5'-biotin- ATGACCCTCTCGGCCCGGGCACCCTGTCAGTCCGGAATAACTGCAGCAT- 3'
118 MUT	5'- ATGACCCTCTCGGCCCGGGCACCCTGTCAGTCCCCAAATAACTGCAGCAT- 3'
181 WT	5'-biotin-GATTGCTTTAGCTTGGAATTCCGGAGCTGAAGCGGCC-3'
181 MUT	5'GATTGCTTTAGCTTGGAATTGGGGAGCTGAAGCGGCC-3'

2.2.23. Statistical analysis

Results were expressed as mean $\pm$ SEM and analysed using GraphPad Prism 4.0 (Graph Pad Software, CA, USA). Data were analysed using Student T-Test. P values less than 0.05 were considered to be statistically significant.

2.3. Results

2.3.1. Erg represses ICAM-1 protein and mRNA

In order to validate the microarray data that indicates Erg represses ICAM-1, ICAM-1 protein and mRNA levels were measured in Erg depleted HUVEC. Inhibition of Erg expression in HUVEC with Genebloc (GB) antisense, resulted in a significant decrease in Erg mRNA expression to 11% of control GB treated cells. This resulted in a significant up-regulation of ICAM-1 mRNA expression to 6.15 fold compared to control GB treated cells (Figure 2.1A). Erg protein expression was significantly decreased to 37% in HUVEC treated with Erg antisense compared to control antisense. Moreover this Erg inhibition resulted in a significant 3.15 fold increase in ICAM-1 protein expression (Figure 2.1B). We then studied the effect of over-expressing Erg using an adenovirus expressing Erg3 (AdErg). Over-expression of Erg resulted in a significant increase in Erg mRNA levels compared to HUVEC treated with control adenovirus, expressing β -galactosidase (AdLacZ). Erg over-expression also induced a significant decrease in ICAM-1 mRNA levels (Figure 2.2A). As both AdErg and AdLacZ expressed proteins with a V5 tag, expression of AdErg and AdLacZ protein was detected using an anti-V5 antibody; this showed similar expression levels of both proteins (Figure 2.2B). Over-expression of Erg resulted in a significant decrease in basal ICAM-1 protein expression to 39% of AdLacZ treated (figure 2.2C) (Sperone et al., 2011). These results demonstrate that Erg represses ICAM-1 expression in resting HUVEC.

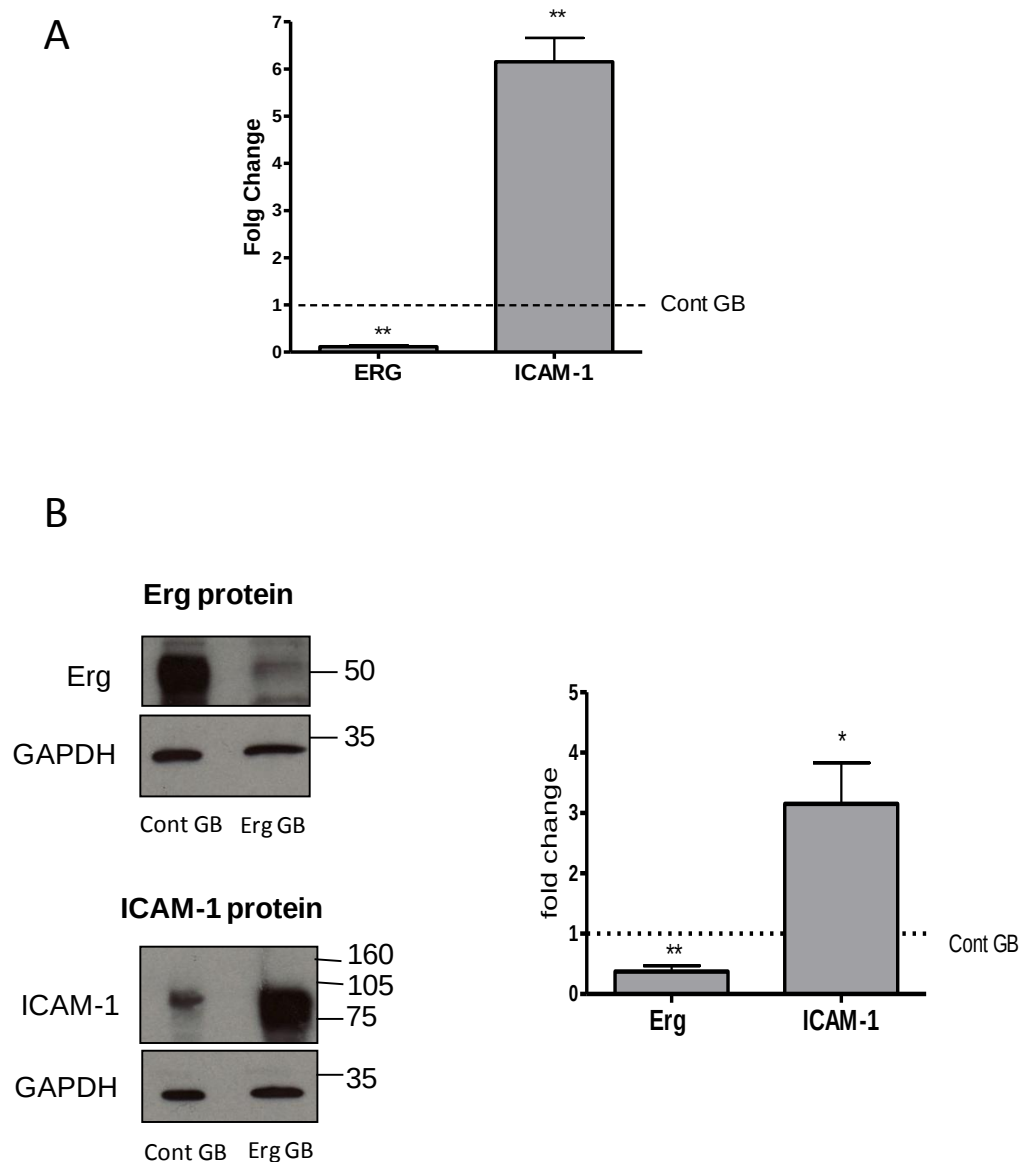


Figure 2.1. Repression of Erg up-regulates the expression of ICAM-1 mRNA and protein. A) mRNA levels of Erg and ICAM-1 in HUVEC treated with Erg or control GB for 48h results are normalised to GAPDH and expressed fold change relative to control GB. N=3 ** P <0.01 Experiment carried out by Dr Graeme Birdsey B) Western blot analysis of total protein from HUVEC lysates treated with control or Erg GB, protein levels were normalised to GAPDH and expressed fold change compared to control GB. N=5. * P= <0.05, ** P= <0.01 Experiment carried out by Dr Andrea Sperone

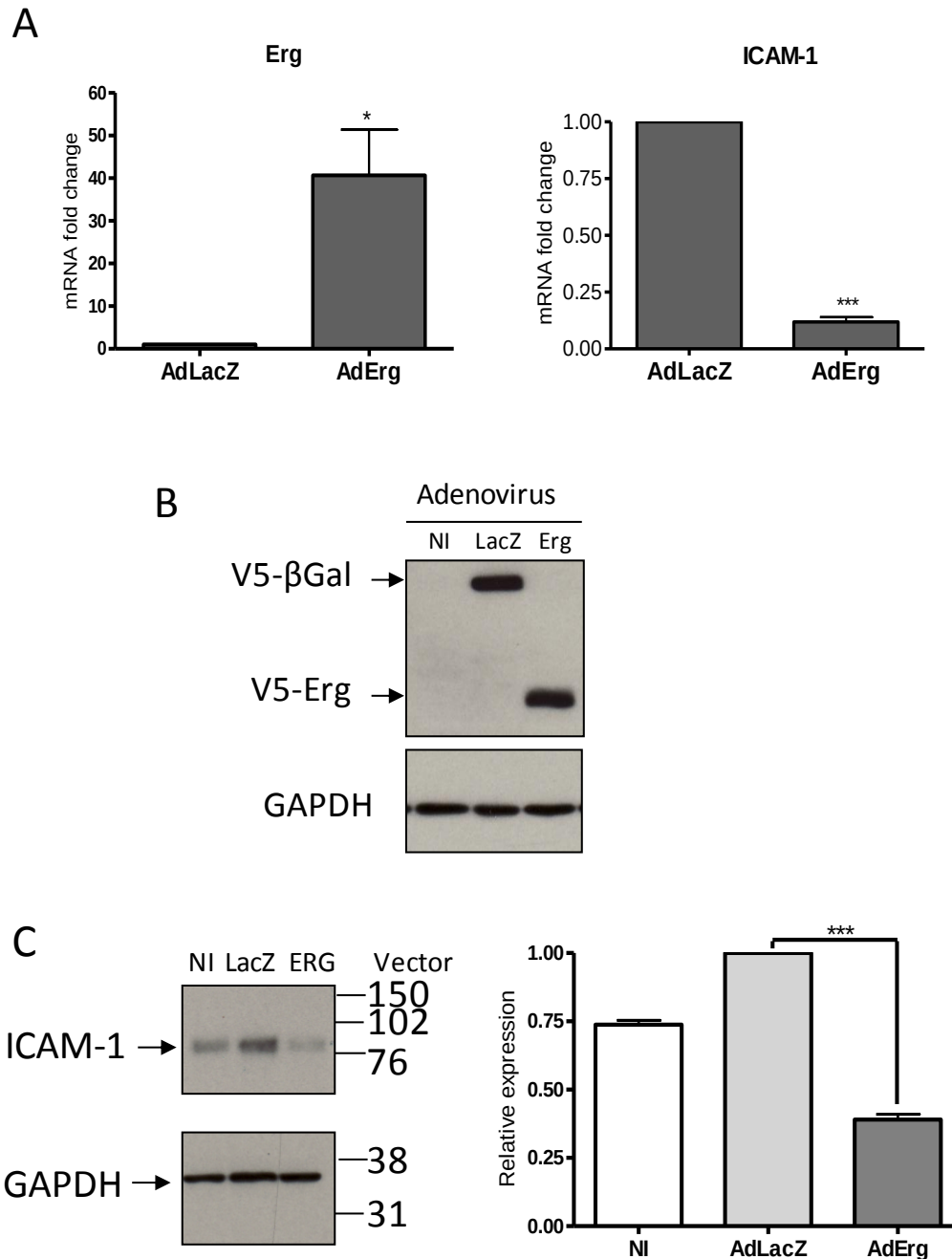


Figure 2.2 Over-expression of Erg represses basal ICAM-1 expression. HUVEC were treated with 50 MOI of AdErg or AdLacZ for 48hours. A) mRNA levels of Erg and ICAM-1. Results are normalised to GAPDH mRNA levels and expressed fold change relative to AdlacZ. N=3 * P= <0.05 *** P=<0.001. B) And C) Total protein lysates were analysed by SDS PAGE and western blot. B) Not infected (NI), AdErg and AdLacZ expression was detected with an anti-V5 antibody. C) ICAM-1 levels were detected with an anti ICAM-1 antibody. Protein levels were normalised to GAPDH and expressed relative to AdLacZ control. N=3 p=<0.001. Experiments carried out by Dr Andrea Sperone

2.3.2. ETS factors Fli-1, Ets-2 and GABP α do not repress ICAM-1 expression

As a number of ETS factors are constitutively expressed in resting EC (Hollenhorst et al., 2004) we investigated whether other ETS factors repressed ICAM-1 in EC. Three ETS factors constitutively expressed in EC are Fli-1, Ets-2 and GABP α . Fli-1 is the ETS factor with the closest homology to Erg, Ets-2 over-expression has been shown to induce ICAM-1 promoter activity in RK-13 cells (de Launoit et al., 1998), Whilst GABP α can act as an activator or repressor of transcription. Using siRNA we inhibited the individual expression of Erg, Fli-1, Ets-2 or GABP α in HUVEC and measured Erg, Fli-1, Ets-2, GABP α and ICAM-1 mRNA expression levels. Erg, Fli-1, Ets-2, and GABP α gene expression was significantly inhibited after 24 hours treatment with their respective siRNA compared to control siRNA (17%, 9%, 23%, 27% respectively) (Fig 2.3A). Inhibition of expression of each ETS factor did not affect the expression of any of the other 3 ETS factors analysed (Figure 2.3A). As seen with antisense treatment, inhibition of Erg by siRNA significantly induced ICAM-1 mRNA levels compared to control siRNA by 1.5 fold. Inhibition of Fli-1, Ets-2 or GABP α did not significantly affect ICAM-1 mRNA levels (Figure 2.3B), suggesting that the repression of ICAM-1 is specific for Erg and not a general function of all constitutively expressed ETS factors in EC.

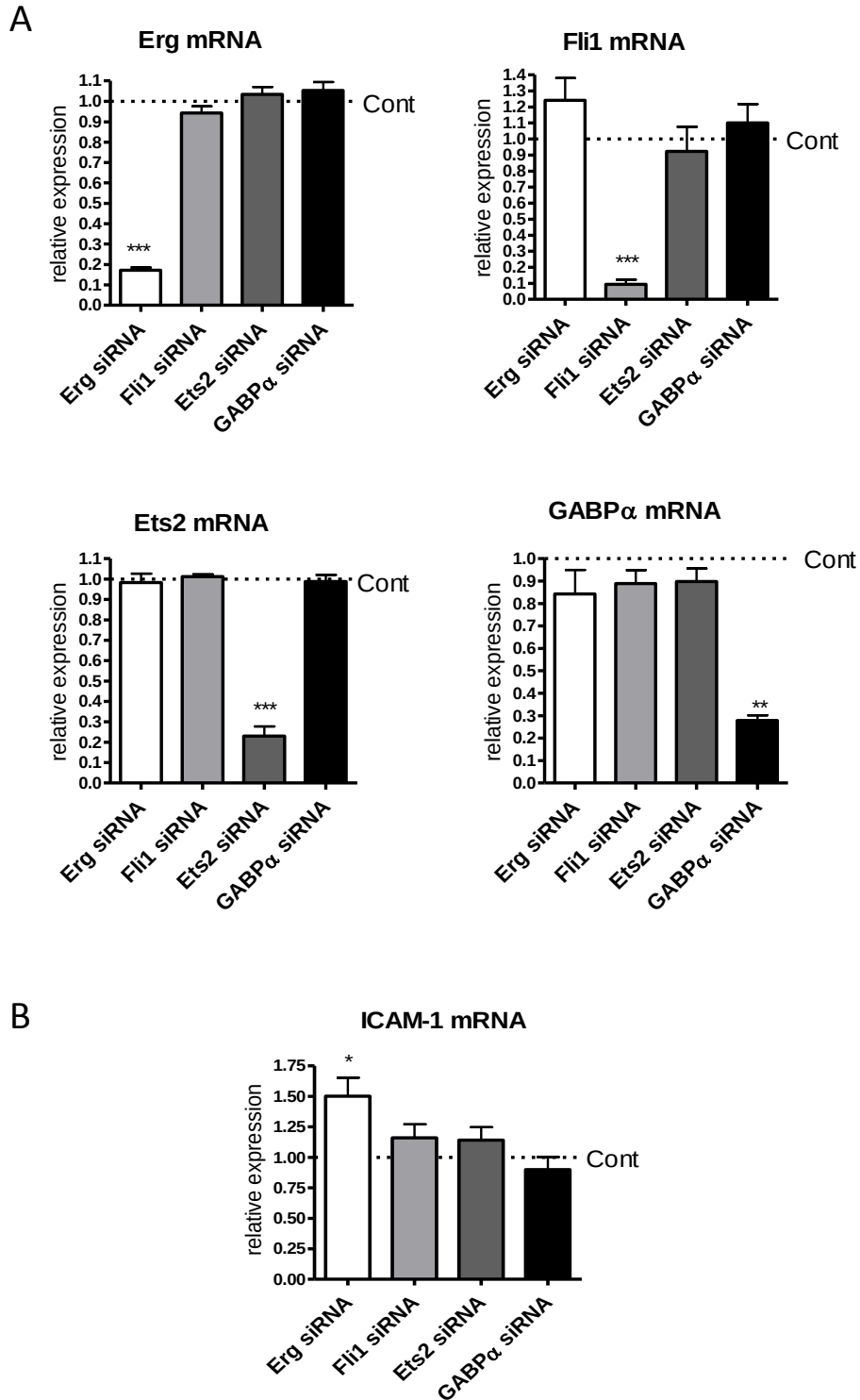


Figure 2.3 Erg, but not Fli-1 or Ets-2 represses ICAM-1. (A and B) HUVEC treated with siRNA for Erg, Fli-1, Ets-2 or GABPα for 24 hours; relative expression of Erg, Fli-1, Ets-2 and GABPα mRNA (A) or ICAM-1 mRNA (B) was quantified by RTPCR normalised to GAPDH expression and expressed as relative to control siRNA treatment (A) N=4 (B)N=7.

2.3.3. Alignment of murine and human ICAM-1 promoter sequences

The evolutionary conservation of nucleotides in gene promoters signifies their importance in the regulation of genes. The presence of conserved sites suggests a nucleotide sequence is beneficial for survival of the organism and leads to positive selection, as mutation of base pairs may lead to loss of function of the gene and death of the organism. As stated in the introduction, many regulatory regions have been characterised in the human ICAM-1 promoter. To identify conserved regulatory elements in the ICAM-1 promoter we aligned the mouse and human ICAM-1 promoter sequences using ClustalW2 (EMBL-EBI) (Larkin et al., 2007; Goujon et al., 2010) to identify common putative transcription factor binding sites, and in particular, conserved EBS. Figure 2.4 shows the alignment of the human and mouse ICAM-1 promoter sequences. The ICAM-1 promoter is highly conserved between mouse and human, in the proximal promoter region. The NF- κ B site at -188 bp relative to the transcription start site in the human sequence is identical to the sequence at -211 bp in the mouse genome (Figure 3.4 red box). Similarly both sequences contain the interferon regulatory element (GAS) site at -76 in human and -99 in mouse (Figure 3.4 green box). This conservation suggests that the response to NF- κ B and IFN- γ are important in the function of ICAM-1. There are 7 putative EBS conserved between mouse and human at positions -70, -75, -89, -96, -118, -181 and -186 bp in the human promoter (Figure 3.4 purple boxes). An SP-1 binding site suggested to be involved in basal ICAM-1 promoter activity (Jahnke et al., 1995), is also conserved between human and mouse (Figure 3.4 blue box). The previously described silencing motif located at approximately -321 bp is not conserved (Jahnke et al., 1995)(Figure 3.2 orange line). The AP-1/ETS sites (shown in figure 1.6) were not conserved in the murine promoter from this alignment, and a similar sequence could not be found elsewhere in the murine ICAM-1 promoter, this suggests that although these sites may be responsive to H₂O₂, they are not essential for ICAM-1 promoter function in the mouse. Additionally, there are a number of areas of conservation upstream of the NF- κ B binding site; however no specific binding motifs have been identified within these regions. In summary, the analysis of conserved regions, suggests the most important regions in the ICAM-1 promoter to maintain function of the gene are within the first 187 bp upstream of the transcription start site.

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GATTAAAGAGGCTGCGTAAGCTGGAGAGGG-AGGACTTGAGTTCGGACC -407 H
GGTTAAAGAGGCTTGCAGTAGT TGGGGAATCAGGACTTGATTTCGGATC -403 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *
CCCTCGCAGCCTGG--AGTCTCAGTTTACCGCTTTGTGAAATGGACACAA -359 H
CTCGAGGATCCTGCGAAA TCCGAGCCTCAGTTTATCCCTTTGAGGGGA -353 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Silencer element
TAACAGTCTCCACTCTCCG GGAAGTTGGCAGTATTTAAAAGTACTTAAT -309 H
TGGCGGTCTGATGTCGCAGGGGACTAGGCAGTAGTCAA-----TCAGT -309 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

AAAGCCTTAGCGCGGTGTAGACCGTGATTCAGCTTAGCC TGGCCGGGAA -259 H
TAACC---AGGAGCGCTGACTCCTGG---AGGCCGG---GGCTTCTCT -269 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ACGGGAGGCGTGGAGGCCGGAGCAGCCCGGGGTCATCGCCCTGCCAC -209 H
CCG---GACTCACCTGCTGG-----TCTCTGACACCACCTCCC-----C -233 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

NF-κB
CGCCGCCCGATTGCTTTAGCTTGGAAATTCCGGAGCTGAAGCGGCCAGCG -159 H
CCACATGTCACTTACTTCAGTTTGGAAATTCCCTAGATCGCAGGGGCCAGCG -183 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

EBS EBS EBS
AGGGAGGATGACCC-TCTCGGCCGGGCACCTGTCTAGTCCGGAAATAAC -110 H
AGGCAGGACCACCCCTCTCTGCAGGGCACAGTCTCCACC CGGAATA-C -134 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GAS
TGCAGCATTTCTTCGGAGCGGAAGGCGCGAGGTTTCGGGGAAG----- -65 H
CGAAGCCCTCGTTTCGGAGCGGAAGGCGCGAGGTTTCGGGGAAGSTGGCC -84 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

EBS EBS EBS EBS
----CAGCACCGCCCCCTGGCCCCCAGGTGGCTAGCG-CTATAAAGG-AT -21 H
CCGACAGCACCGCCCCCTCGCCCCCGTGAGCCAGAGACTATAAAGCGC -34 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

SP-1
CACGCGCCCCAGTCGAC-----GCTGAGCTCCTCTGCTACTC 17 H
CGCCCGCCTCAGTCTGCACCAAGTCTAGTGCTGAGCTCCGCTGCTACCT 17 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

AGAGTTGCAACCTCAGCCTCGCTATGGCTCCAGCAGCCCCCGGCCCGCG 67 H
GCACCT--TGCCCTGGCCCTGCAATGGCTTCAACCCGTGCCAAGCCACG 65 M
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

Figure 2.4. Alignments of the murine and human ICAM-1 promoters. Alignment was carried out using ClustalW2 (EMBL-EBI). The human (H) promoter is shown on the top line and the murine (M) promoter on the bottom line. Conserved sequences are marked with an * below nucleotide. Conserved transcription factor binding sites are highlighted as EBS-purple, GAS-green, SP-1-blue, NF-κB-red. Unconserved silencing element marked with orange line. ChIP PCR amplicon marked with black line. Transcription start site is marked with an arrow

2.3.4. Erg binds to the ICAM-1 promoter

2.3.4.1. Chromatin Immunoprecipitation: ethidium bromide gel analysis

Erg-mediated repression of ICAM-1 could occur through either Erg binding directly to the ICAM-1 promoter, or alternatively through Erg regulating another transcriptional activator or repressor of ICAM-1. To investigate whether Erg represses ICAM-1 by binding to the ICAM-1 promoter we carried out chromatin immunoprecipitation (ChIP). Initially the binding of Erg to the proximal promoter region was studied using primers spanning base-pairs -25 to -204 from the transcription start site. This region as shown in Figure 2.4 was chosen because it contains seven putative EBS including a functional EBS-118 which had previously been shown to be bound and transactivated by the ETS factor ERM (de Launoit et al., 1998). This domain also contains the functional NF- κ B binding site, which overlaps with two ETS consensus sequences, and one of these sites, EBS -181, was identified to be a putative EBS by MatInspector. ChIP for the ICAM-1 promoter was carried out using anti-Erg or IgG control antibody and PCR products were detected by ethidium bromide gel electrophoresis. Immunoprecipitation with an anti-Erg antibody resulted in greater enrichment of chromatin containing the ICAM-1 proximal promoter region compared to immunoprecipitation using an isotype control antibody (Figure 2.5). There was no difference in enrichment for a control region, the promoter of GAPDH, using either anti-Erg or IgG control antibodies, indicating no non-specific enrichment. The enrichment at the proximal promoter region is therefore specific for Erg binding to the ICAM-1 promoter. These results indicate that Erg binds to the ICAM-1 promoter in resting EC, and suggest that Erg represses ICAM-1 directly.

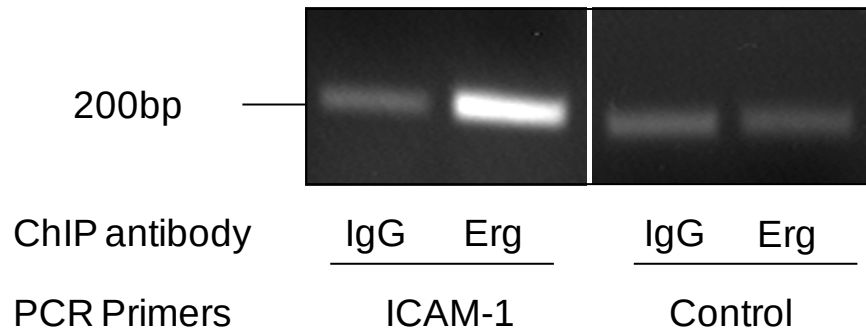


Figure 2.5 Erg binds to the ICAM-1 promoter. Sheared chromatin was immunoprecipitated with anti-Erg or an isotype control antibody. Enriched chromatin was then evaluated by PCR for the ICAM-1 proximal promoter region or a negative control region of the GAPDH gene. Representative image of 3 separate experiments.

2.3.4.2. Chromatin Immunoprecipitation: quantitative-PCR analysis

Ethidium bromide detection of PCR products and quantification by densitometry does not allow accurate quantification of the level of ChIP enrichment. Additionally, accurate direct comparison can be problematic; it is difficult to ensure the PCR has been stopped in the logarithmic phase of amplification and that all samples have been amplified equally. Therefore, quantitative PCR was used to analyse the Erg ChIP samples. There are 16 putative EBS, as identified by MatInspector, spread across the first 1.3 kb of the ICAM-1 promoter (Figure 1.6). As chromatin for ChIP is sheared in fragments with an average size of 500 bp, there are many possible sites within detected fragments that Erg may bind to; we wanted to ensure we were identifying the correct region of the ICAM-1 promoter bound by Erg. Primer pairs were designed to cover regions (R) 1 through to 5 as indicated (Figure 2.6A). Sheared chromatin from resting HUVEC was immunoprecipitated using an anti-Erg or IgG control antibody, and enriched chromatin was detected by quantitative PCR. Results are expressed as fold change compared to IgG control antibody, normalised for both total input levels and the control GAPDH region. Erg enrichment on the ICAM-1 promoter is at its greatest on R4 (Figure 2.6B). This region contains three ETS binding sites, one named EBS-118 previously shown to bind ERM, and two within the NF- κ B site named EBS-181 and EBS-188. In order to confirm that the enrichment was due to Erg binding to the ICAM-1 promoter, and not due to the Erg antibody binding non-specifically at this site, ChIP was repeated on chromatin which had been treated with control siRNA or Erg siRNA for 24 hours to inhibit Erg levels. Erg enrichment at the ICAM-1 promoter R4 was significantly decreased in HUVEC treated with Erg siRNA compared to control siRNA (Figure 2.6C). This confirms that Erg specifically binds to the ICAM-1 promoter in R4 and suggests that Erg acts directly to repress ICAM-1 by binding to the ICAM-1 promoter.

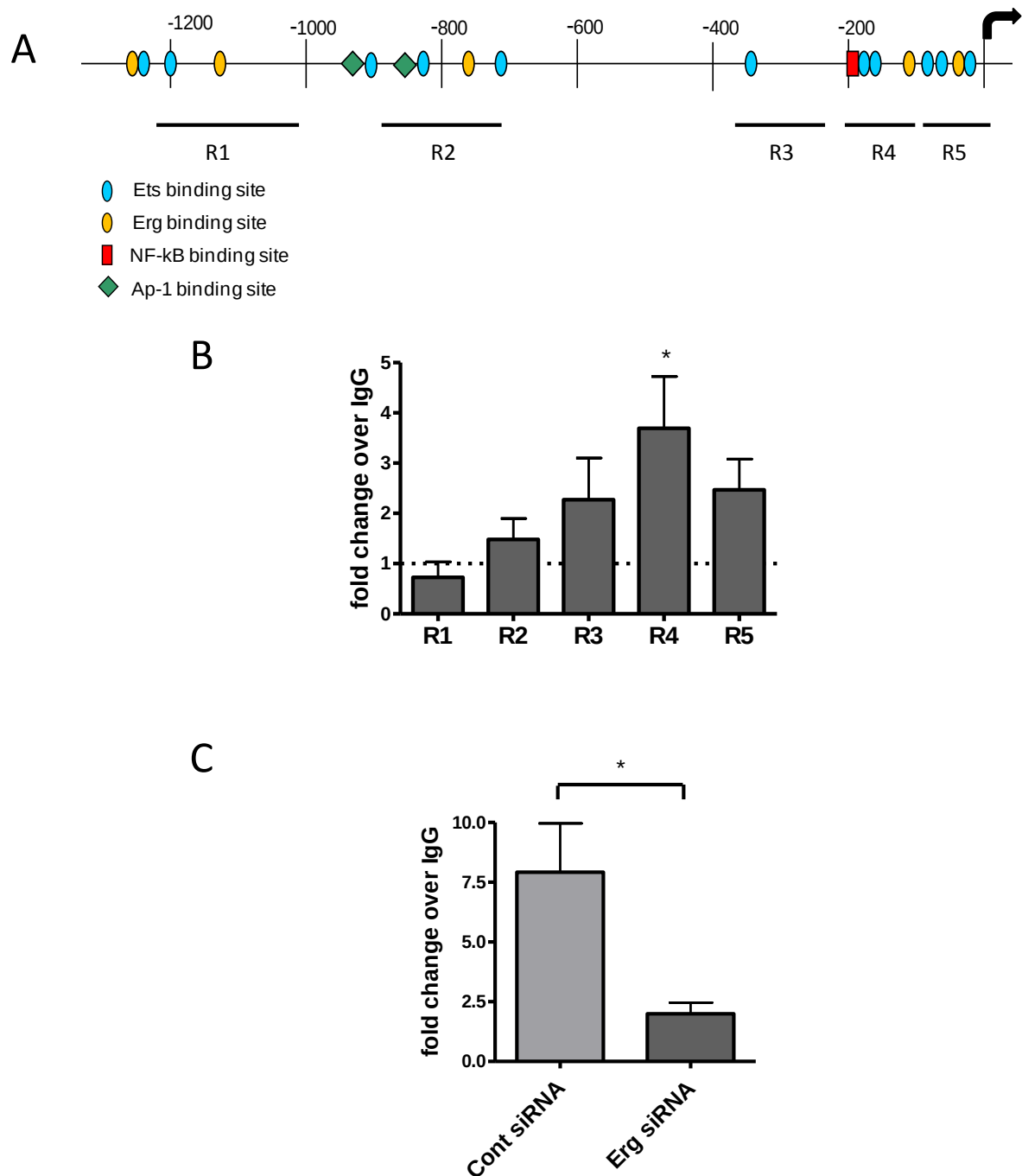


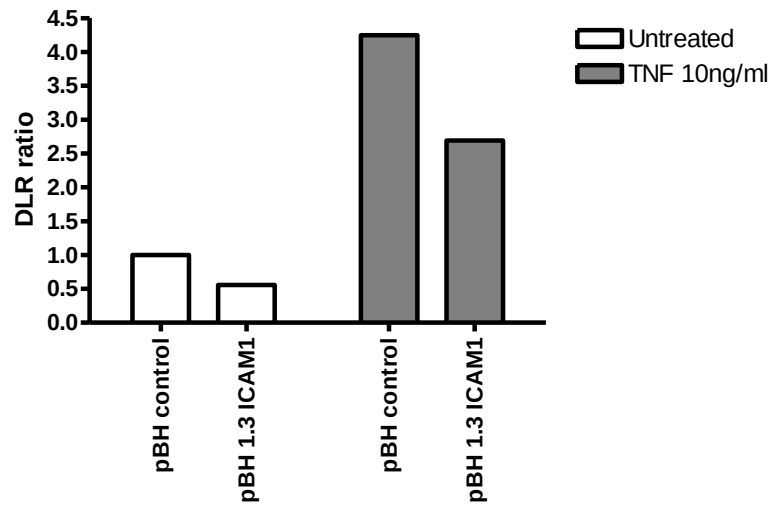
Figure 2.6 Erg binds to the ICAM-1 promoter region 4 in resting EC. A) Analysis of 1.3 kb of the ICAM-1 promoter identified putative EBS (blue ovals) including putative Erg binding sites (yellow ovals), AP1/ETS sites (green diamonds) and an NF-κB site (red rectangle) location of quantitative PCR amplicons covering R1, R2, R3, R4, and R5 are indicated by black lines below. (B) ChIP was carried out on sheared chromatin from confluent resting HUVEC (B) or HUVEC treated with Erg or control siRNA (C) using an anti Erg or control IgG antibody. Immunoprecipitated DNA was analysed by qPCR with primers covering ICAM-1 promoter regions 1-5 (B) or region 4 (C) and negative control region. Results are expressed as fold change compared to IgG normalised to input and negative control region. N=6 (B), N=5 (C), *P<0.05.

2.3.5. Investigation of the role of Erg in ICAM-1 promoter activity

2.3.5.1. Optimisation of ICAM-1 luciferase reporter gene constructs

To investigate whether Erg repressed ICAM-1 by repressing the activity of the ICAM-1 promoter, we utilised a luciferase reporter plasmid, pBH ICAM-1 1.3 which contains the 1.3kb ICAM-1 promoter sequence shown in Figure 1.6 inserted upstream of the firefly luciferase gene as (kindly provided by Dr.C. Stratowa (Boehringer, Vienna)). Initially we tested whether the pBH ICAM-1 1.3 construct was functional and responded to inflammatory stimuli as shown previously by treating with TNF- α (Voraberger et al., 1991). HeLa cells were co-transfected with either 250 ng of pBH empty vector or pBH ICAM-1 1.3, and pGL2 renilla to control for transfection efficiency. Following 16 hours of transfection HeLa were stimulated with 10 ng/ml TNF- α for 24 hours before luciferase levels were measured. We found that the luciferase activity of pBH ICAM-1 1.3 was 55% of the luciferase activity of pBH control, indicating a low basal activity of this promoter (Figure 2.7A). After 24 hours of TNF- α stimulation, the pBH ICAM-1 1.3 promoter activity was approximately 5 fold greater than untreated; however the pBH control vector activity was 4.2 fold greater than untreated. This suggests that the control vector, pBH, contains a TNF- α -responsive element. As we were unable to distinguish between the activities of the ICAM-1 promoter sequence or the pBH vector sequence, this vector backbone was unsuitable to study ICAM-1 promoter activity. Therefore, we subcloned the ICAM-1 promoter from pBH ICAM-1 1.3 into the pGL4 firefly luciferase backbone (Promega), which is designed to contain very few responsive elements, and investigated the response of pGL4 ICAM-1 1.3 to TNF- α . HeLa cells were transfected with either pGL4 ICAM-1 1.3 or pGL4 control vector, and co-transfected with pGL4 Renilla, followed by stimulation with TNF- α , for 4, 6, or 8 hours before luciferase activity was measured. The basal activity of pGL4 ICAM-1 1.3 was approximately 3-fold higher than pGL4 control, suggesting some basal activity of the ICAM-1 promoter in HeLa cells (Figure 3.7B). TNF- α stimulation increased promoter activity by approximately 6 fold compared to pGL4 control at 4 hours and this was maintained at 6 hours and 8 hours, suggesting that the ICAM-1 promoter sequence is responsive to TNF- α stimulation in HeLa cells. In addition pGL4 control was unaffected by TNF- α treatment, indicating this construct is suitable for the investigation of promoters responsive to inflammatory stimuli. This experiment was performed only once, as further experiments were carried out in more relevant primary EC.

A



B

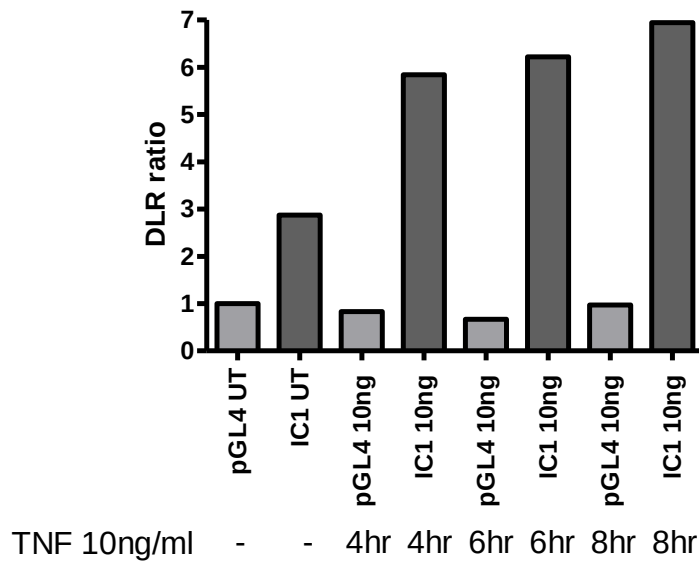


Figure 2.7 Response of ICAM-1 promoter constructs to TNF- α treatment. A) HeLa cells co-transfected with either pBH1.3 ICAM-1 1.3 or control, and pGL3 renilla followed by treatment with TNF- α for 12 hours. B) HeLa cells co-transfected with either pGL4 IC1 1.3 or control, and pGL3Renilla, then treated with TNF- α for the times indicated. (A) And (B) Luciferase levels were measured and expressed as dual luciferase ratio (DLR) of firefly luciferase over renilla luciferase normalised to empty vector control. N=1

2.3.5.2. Optimization of transfection of HUVEC with luciferase reporter gene constructs: Microporation and lipofection

Primary cells, such as HUVEC, are difficult to transfect to a high efficiency. We wished to transfect HUVEC with the ICAM-1 promoter to a detectable level, while maintaining a resting phenotype to measure the basal level of promoter activity. In order to identify the most suitable transfection protocol we investigated the effect of transfection by microporation and by lipofection. Microporation is a form of electroporation which differs in the physical components compared to traditional electroporation systems to generate a more uniform electric field (Kim et al., 2008). Microporation forms temporary pores in the phospholipid bilayer of plasma membrane, which allow plasmids and siRNA to pass into the cell. Lipofection, in contrast, involves the packaging of plasmids and other nucleic acids into liposomes using a transfection lipid which then fuses to the plasma membrane and delivers the contents into the cell (Felgner et al., 1987).

2.3.5.3. Microporation

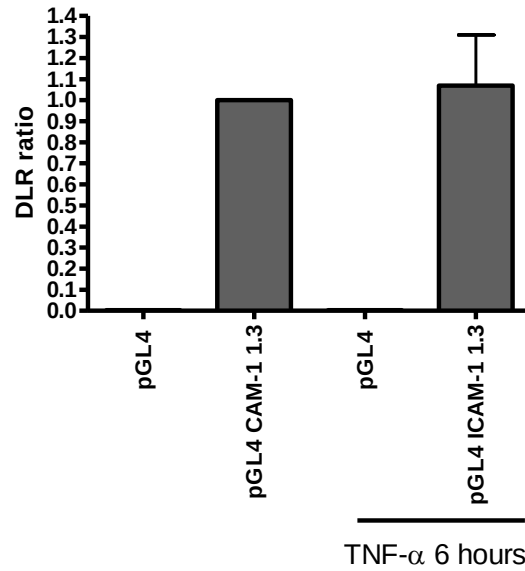
In our lab transfection by microporation has previously resulted in high transfection efficiency (Birdsey GM. and Randi AM. unpublished data). To investigate whether microporation is a suitable method for transfection of the pGL4 ICAM-1 plasmid, HUVEC were co-transfected with 0.5 µg pGL4 renilla and either 0.5 µg pGL4 ICAM-1 1.3 or pGL4 control by microporation using the settings of 1 pulse at 1400 V for 30 ms. After 42 hours of culture HUVEC were left untreated or were treated with 10 ng/ml TNF- α for 6 hours and luciferase activity was measured. In resting conditions the basal activity of pGL4 ICAM-1 1.3 was approximately 400-fold higher than pGL4 control (Figure 2.8A). This was much higher than the 3-fold difference between pGL4 and pGL4 ICAM-1 1.3 seen in HeLa cells (Figure 2.7.3). Treatment of HUVEC with TNF- α did not increase the activity of pGL4 ICAM-1 1.3 compared to untreated. The high basal activity and lack of response to TNF- α suggests microporation may activate the cells, inducing pro-inflammatory transcriptional pathways and ICAM-1 expression. This suggests that under our conditions, transfection by microporation is not suitable to study regulation of inflammatory gene expression.

2.3.5.4. Lipofection

We investigated whether lipofection was a more suitable method to transfect HUVEC with pGL4 ICAM-1 1.3. HUVEC were transfected with 0.5 µg pGL4 Renilla and either 0.5 µg pGL4 ICAM-1 1.3 or pGL4 control using the lipid Genejuice® transfection reagent

(Novagen). The promoter activity of pGL4 ICAM-1 1.3 was significantly higher than pGL4 alone. 6 hours of TNF- α treatment resulted in a significant increase in ICAM-1 promoter activity by 4 fold compared to no TNF- α treatment (Figure 2.8B). As expected, pGL4 empty vector was not affected by TNF- α treatment, indicating there are no TNF- α responsive elements in the pGL4 backbone, and suggesting the response seen in pGL4 ICAM-1 1.3 is due to activity of the ICAM-1 promoter. From these results we decided to use lipofection to study the effect of Erg on ICAM-1 promoter activity.

A



B

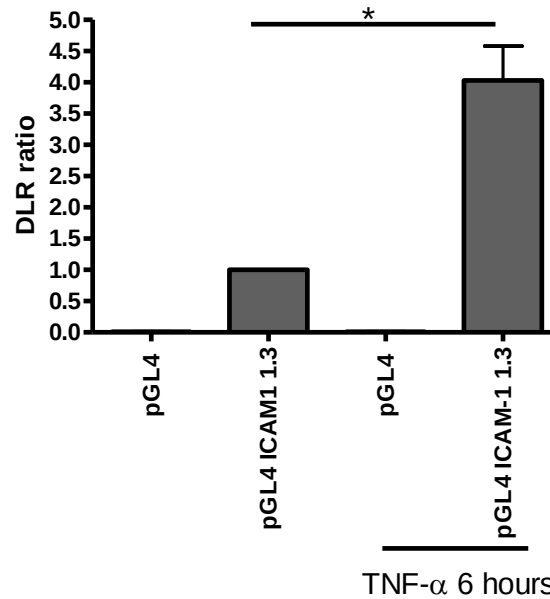


Figure 2.8 Optimisation of transfection protocol. HUVEC transfected using microporation (A) or lipofection (B) HUVEC were transfected with 0.5 μ g pGL4 ICAM-1 or control for 42 hours followed by treatment with TNF 10 ng/ml for 6 hours before luciferase activity was measured. Activity is expressed as the ratio of pGL4 ICAM-1 firefly luciferase to pGL4 renilla luciferase (A) N=3. (B) N=3 paired t-test * P= 0.0316

2.3.5.5. Erg represses ICAM-1 promoter activity

We have shown that inhibition of Erg expression in HUVEC using Erg GB or siRNA results in an up-regulation of ICAM-1 protein and mRNA levels. We have also shown that Erg binds directly to the ICAM-1 promoter. To determine whether this repression occurred at the transcriptional level, the effect of Erg GB on ICAM-1 promoter-driven luciferase levels was studied. Erg expression was first inhibited in HUVEC using Erg GB; 24 hours later cells were co-transfected, by lipofection, with pGL4 ICAM-1 1.3 or pGL4 empty vector control and pGL4 renilla for a further 24 hours before luciferase levels were measured. Inhibition of Erg by GB significantly increased the activity of the ICAM-1 luciferase reporter by 1.3 fold compared to cells treated with control GB (Figure 2.9A). This is in agreement with the increase in ICAM-1 mRNA and protein levels seen in HUVEC treated with Erg GB, and suggests Erg represses ICAM-1 expression by inhibiting promoter activity.

As Erg over-expression repressed basal ICAM-1 mRNA levels, we investigated whether over-expression of Erg inhibited basal levels of ICAM-1 promoter activity. Transduction of HUVEC with AdErg increases Erg mRNA expression approximately 40 fold (see Figure 2.2A). HUVEC were transduced with 50 multiplicities of infection (MOI) AdErg or AdLacZ control. After 24 hours, HUVEC were transfected with either pGL4 ICAM-1 1.3 or pGL4 control for 24 hours and luciferase levels were measured. AdErg over-expression resulted in significant repression of ICAM-1 luciferase levels to approximately 25% of levels after AdLacZ over-expression (Figure 2.9B). There was no significant difference between ICAM-1 promoter activities of untreated or AdLacZ transduced HUVEC. Moreover, the activity of pGL4 empty vector control did not change in response to transduction with AdErg compared to untreated or AdLacZ, suggesting the decrease in luciferase activity by AdErg is due to repression of ICAM-1 promoter activity, and not due to Erg repressing any elements within the pGL4 vector. These results indicate that Erg represses ICAM-1 expression in EC by repressing the activity of the ICAM-1 promoter.

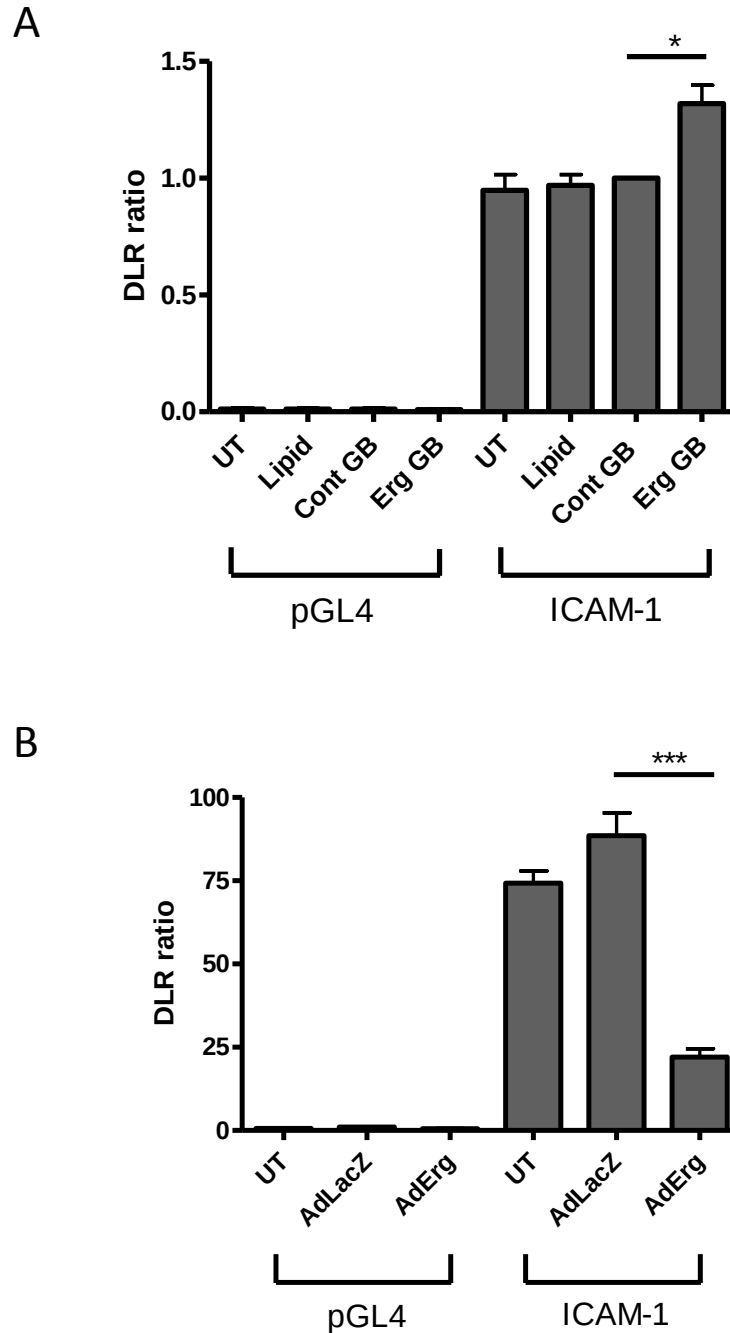


Figure 2.9. ICAM-1 promoter driven luciferase levels are increased upon inhibition of Erg expression. HUVEC were transfected with either Erg GB or control GB (A) or AdErg or AdLacZ (B) for 24 hours. (A and B) HUVEC were co-transfected with either pGL4 ICAM-1 1.3 or pGL4 control, and with the pGL4 renilla control vector. Results are presented as dual luciferase ratio (DLR) of firefly luciferase normalised to pGL4 Renilla luciferase. (A) N=4, P=0.0262 (B) N=3, P=<0.0018

2.3.5.6. ICAM-1 promoter mutants

ChIP analysis suggests Erg represses the ICAM-1 promoter by binding directly to the promoter and inhibiting basal activity; however due to the resolution limitations of the technique we could not distinguish the specific EBS bound by Erg using ChIP. In order to investigate the EBS utilised by Erg to repress ICAM-1, we generated ICAM-1 promoter constructs containing mutations within EBS using site directed mutagenesis. The ICAM-1 promoter was mutated in single or double EBS where two EBS had been previously shown to function together (Figure 2.10) (de Launoit et al., 1998; Roebuck et al., 1995). In addition to mutating the EBS, we mutated the NF- κ B site as described previously (Ledebur and Parks, 1995) in order to investigate whether this would result in a different response compared to mutating the EBS within NF- κ B binding site. We mutated the TGGAAATTCC NF- κ B sequence to TctAgATTag. This mutation in the NF- κ B site has previously been shown to result in loss of response to TNF- α (Ledebur and Parks, 1995). Oligonucleotide sequences used for the mutations are listed in Table 2.3.

A

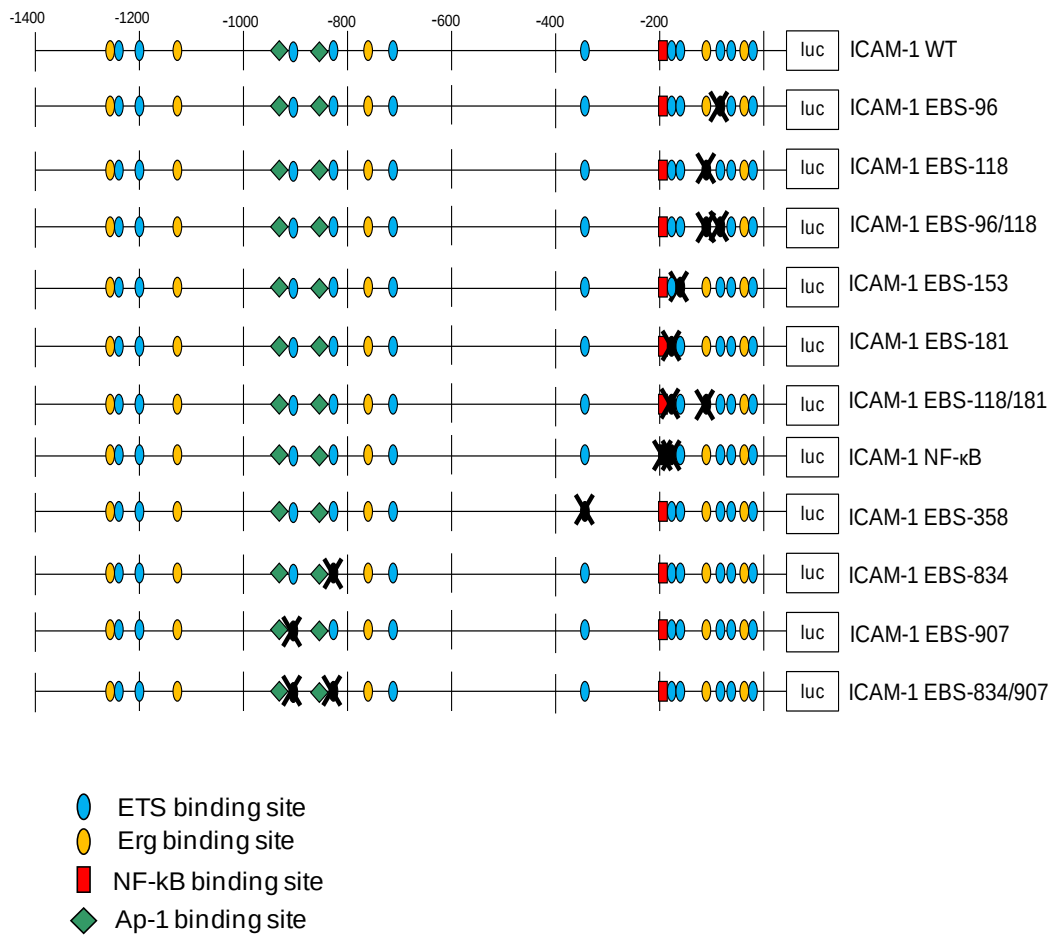


Figure 2.10 ICAM 1 promoter mutants. The pGL4 ICAM-1 1.3 promoter luciferase construct was mutated in single or double EBS, or within the NF-κB binding site as marked by a cross. Transcription factor binding sites are marked as indicated.

2.3.5.7. ICAM-1 promoter EBS mutants: basal activity

As discussed in the introduction, previous studies have shown that ETS factors both transactivate and repress ICAM-1 expression in non-EC. Over-expression of ETS factors including Ets-1, Ets-2 and ERM increased ICAM-1 promoter activity (de Launoit et al., 1998), while over-expression of FEV inhibited ICAM-1 promoter activity (Maurer et al., 2003). This regulation was shown to depend on the EBS located 118 bp and 96 bp up-stream of the transcription start site. The involvement of these ETS factors in basal ICAM-1 expression was not investigated in these previous studies; however we have shown that inhibition of Ets-2, Fli-1, or GABP α does not change the levels of endogenous ICAM-1 mRNA. As the WT ICAM-1 promoter is repressed by constitutively expressed Erg, we hypothesised that if we mutate the EBS required by Erg for repression, Erg will no longer bind to the promoter resulting in a higher basal promoter activity. Initially we investigated whether the mutation of ICAM-1 promoter EBS had an effect on the basal ICAM-1 promoter activity. HUVEC were co-transfected with 250 ng of either pGL4 ICAM-1 1.3 or one of the pGL4 ICAM-1 promoter mutants, and 250 ng pGL4 renilla. Cells were incubated for 24 hours before protein lysates were assayed for luciferase activity. The basal activity of each of the ICAM-1 promoter EBS mutants is shown in Figure 2.11A. Basal promoter activity increases above WT in only one of the mutants, EBS-96, suggesting that this EBS is bound by a repressive factor that can no longer repress the ICAM-1 promoter when this site is mutated. Basal activity was lower in a number of the EBS mutants compared to WT ICAM-1. The activity of the EBS -118 and EBS -96/118 mutants was lower than WT ICAM-1, indicating that these EBS are required for ICAM-1 basal activity. Interestingly while the activity of EBS -96 was higher compared to WT ICAM-1, combining this mutation with the EBS -118 mutation reverses the higher basal activity. This suggests that the phenotype induced by the EBS -118 mutation is dominant, and the factor responsible for increasing ICAM-1 promoter activity after the EBS -96 mutation requires a functional EBS at -118. Mutations of EBS -181, EBS -118/181 and NF- κ B all cause the ICAM-1 promoter basal activity to decrease. The EBS -181 sequence is at the 3' end of the NF- κ B binding site. The lower basal activity of these ICAM-1 promoter mutants suggests a role for the NF- κ B binding site in the basal expression of ICAM-1 in HUVEC. In summary, mutation of a number of EBS in the ICAM-1 promoter resulted in altered basal activity. One site, EBS -96, appears to be involved in basal repression, and two EBS, -118 and -181, and the NF- κ B site is involved in basal activity.

2.3.5.8. ICAM-1 promoter EBS mutants: response to Erg inhibition

As Erg inhibition increased ICAM-1 promoter activity, and there was a decreased enrichment of Erg at the ICAM-1 promoter after Erg siRNA by ChIP, we hypothesised that the inhibition of Erg allows the access and binding of activating transcription factors to the ICAM-1 promoter. By mutating the EBS required by Erg for repression, Erg binding will be inhibited, therefore treatment with Erg GB will result in no difference in ICAM-1 promoter activity. To test this hypothesis, HUVEC were treated with Erg or control GB for 24 hours before transfection with the ICAM-1 promoter EBS mutants for 24 hours. Cell lysates were then measured for luciferase activity. As shown before, inhibition of Erg significantly increased ICAM-1 promoter activity by 1.6 fold compared to control GB treated (Figure 2.11B). The promoter activity of ICAM-1 promoter mutants EBS -96, EBS -153, EBS -358, EBS -834, EBS -907, and EBS -834/907 was also significantly increased after Erg GB treatment, indicating that as with WT ICAM-1 promoter activity, constitutive Erg is still able to repress these promoter mutants; therefore Erg does not repress ICAM-1 via these EBS. Interestingly, the ICAM-1 promoter activity of the EBS -96 mutant was increased after Erg GB treatment compared to control GB, suggesting the increase in basal activity of this mutant is not due to the loss of the repressive effects of Erg, and suggests some other factor is repressing via this site. The ICAM-1 promoter mutants EBS -118, EBS -96/118, EBS -181, EBS -118/181 and NF- κ B did not respond to Erg inhibition, suggesting Erg may repress ICAM-1 via these sites. As these sites also had lower basal activity, the absence of an increase in activity after Erg inhibition may be because these promoters are defunct. To ensure the lack of response to Erg inhibition was not because these mutants were transcriptionally defunct, we stimulated transfected cells with IFN- γ . IFN- γ was chosen instead of TNF- α because previous studies had shown that mutation of the NF- κ B site inhibited ICAM-1 promoter activation by TNF- α (Hou et al., 1994; Ledebur and Parks, 1995). IFN- γ stimulation increased WT ICAM-1 promoter activity by approximately 19.4 fold, and each of the ICAM-1 promoter mutants studied showed increased promoter activity after treatment with IFN- γ (Figure 2.11D). This suggests that while the EBS -118, EBS -181 and NF- κ B mutants no longer respond to Erg inhibition, they are not defunct and still respond to inflammatory stimuli. This indicates that Erg repression of the ICAM-1 promoter involves these sites.

2.3.5.9. ICAM-1 promoter EBS mutants: response to Erg over-expression.

Erg over-expression with AdErg represses the basal activity of the ICAM-1 promoter (Figure 2.2). To identify which of the EBS are involved in this repression, we transduced HUVEC with either AdErg or AdLacZ and the following day transfected with the mutant ICAM-1 promoter constructs for 24 hours. We compared the ICAM-1 promoter luciferase activity of the EBS mutants after transduction with AdErg compared AdLacZ. Nearly all of the ICAM-1 promoter mutants were repressed by AdErg over-expression to similar levels to wild type (Figure 2.11C), which suggest the EBS involved in Erg-mediated repression of ICAM-1 is still present and functioning in these mutants. The ICAM-1 promoter mutant EBS -118/181 was not repressed by Erg over-expression, suggesting these two sites are required for the Erg-mediated repression of ICAM-1. Erg was still able to repress each of these sites mutated separately. Suggesting the presence of either site is sufficient for Erg mediated repression. The loss of function after mutation of EBS -118/181 suggests that both of these sites are involved in Erg-mediated repression of the ICAM-1 promoter.

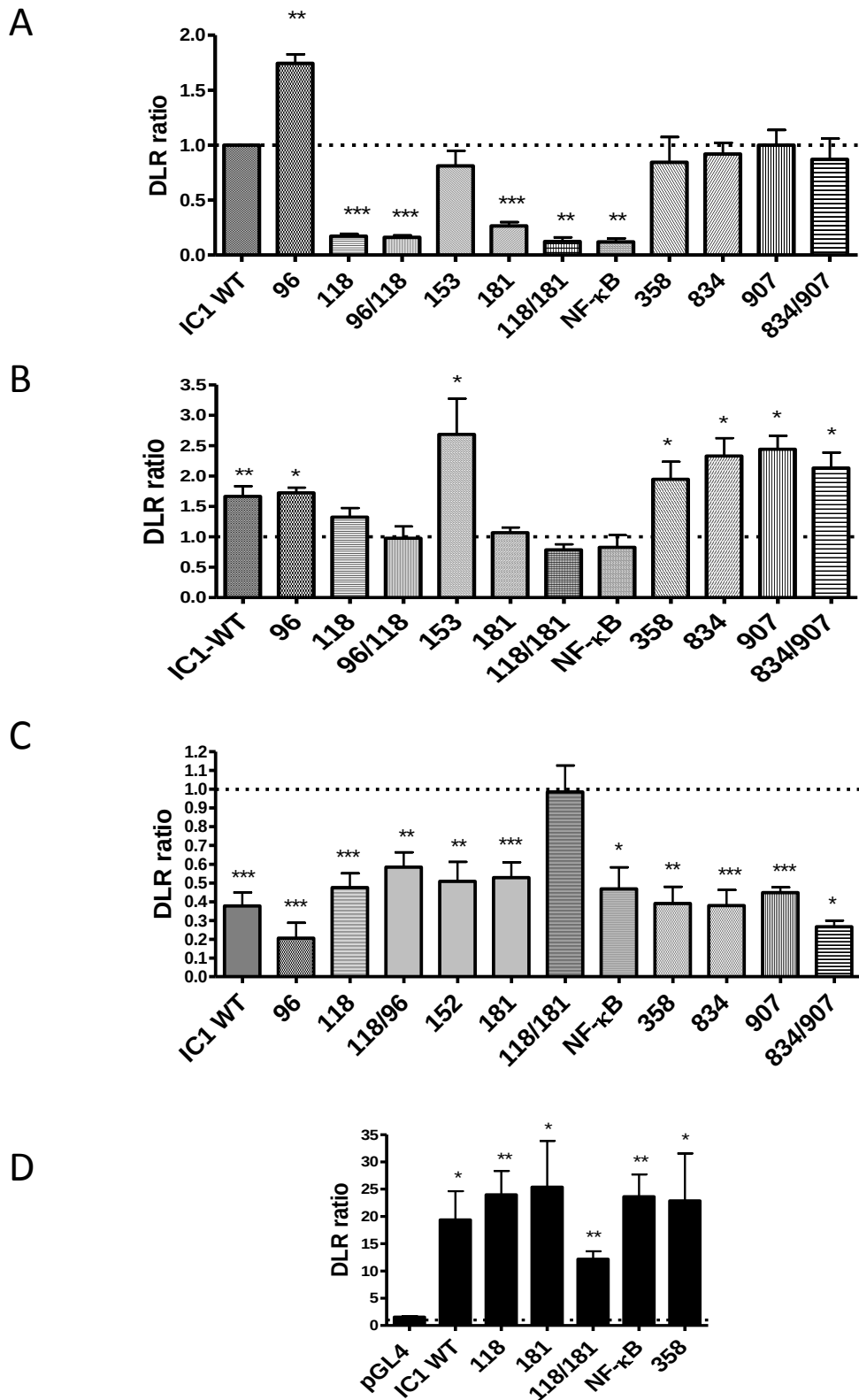
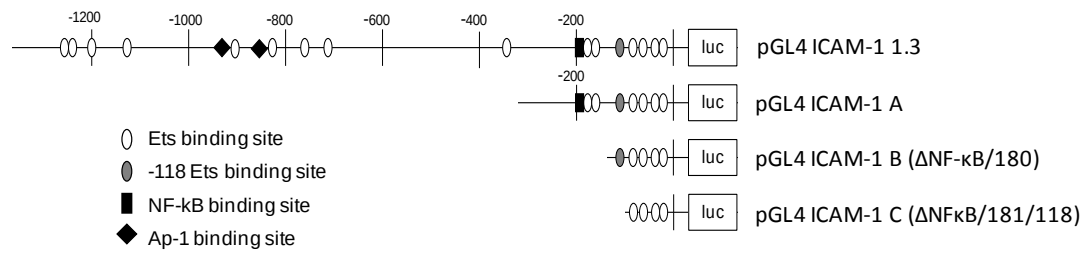


Figure 2.11 Identification of DNA binding site involved in Erg-mediated repression of ICAM-1. A) Basal ICAM-1 promoter activity of EBS mutants. Results are expressed as luciferase activity relative to ICAM-1 WT promoter. N=4-14. B) ICAM-1 promoter activity of EBS mutants after Erg Genebloc treatment. Results are expressed as luciferase activity relative to control Genebloc treated cells. C) ICAM-1 promoter activity of EBS mutants after AdErg treatment, expressed as luciferase activity relative to AdLacZ. N=3-7. D) Promoter activity of EBS mutants after treatment with IFN-γ. N=3. *P<0.05; **P<0.01, ***P<0.001

2.3.5.10. Erg repression of ICAM-1 involves promoter sequences between -164 and -103.

To confirm Erg represses ICAM-1 through the EBS at sites -118 and -181, we generated deletion constructs of the ICAM-1 promoter. We hypothesised that by deleting the region of the ICAM-1 promoter responsible for Erg-mediated repression we would no longer be able to repress ICAM-1 promoter activity by AdErg over-expression. Three deletion constructs were generated (Figure 2.12A); pGL4 ICAM-1 A consists of the ICAM-1 promoter sequence up to -305 bp upstream of the transcription start site, and contains both the EBS-181 and EBS-118; pGL4 ICAM-1 B consists of the sequence up to -164 bp upstream of the transcription start site, which lacks the EBS-181 and the NF- κ B site, but still contains EBS-118; and pGL4 ICAM-1 C containing sequence up to -103 bp upstream of the transcription start site, which lacks both the EBS-181 and EBS-118. The basal activity and response to AdErg over-expression were measured (Figure 2.12B). The basal activity of pGL4 ICAM-1-A was similar to pGL4 ICAM-1 1.3, and was repressed by AdErg over-expression, indicating that the sites required for Erg repression of ICAM-1 are located within this construct. The basal activity of pGL4 ICAM-1 B was lower than pGL4 ICAM-1 1.3, but was still repressed by AdErg over-expression, suggesting that the region present in A but lacking in B is involved in basal ICAM-1 promoter activity. pGL4 ICAM-B was still repressed by Erg over-expression, indicating the sites required for Erg-mediated repression are still present in the construct B. The basal activity of pGL4 ICAM-1 C was even lower than pGL4 ICAM-1 B and approximately 10 fold higher than the pGL4 control, indicating that this small sequence is capable of some basal activity. This region contains the TATA box which was previously reported to have 20% basal activity of the full 1.3 kb (Voraberger et al., 1991). Erg over-expression did not significantly inhibit this vector which lacks EBS-118, confirming EBS-118 is required for Erg-mediated repression of ICAM-1 promoter activity.

A



B

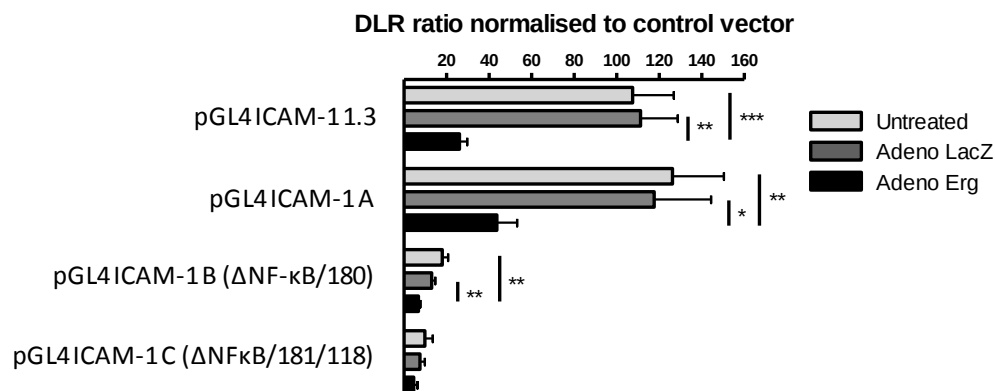


Figure 2.12 Identification of promoter regions responsible for Erg mediated repression.

A) ICAM-1 deletion constructs: pGL4 ICAM-1 A containing both the NF-κB sites and the ETS binding site -118, pGL4 ICAM-1 B which was cut downstream of the NF-κB site but still contained the EBS -118. And pGL4 ICAM-1-C which lacked both the NF-κB site and the EBS -118. B) ICAM-1 promoter deletion construct luciferase activity. Expressed as DLR ratio of firefly luciferase to renilla luciferase normalized to pGL4 empty vector. N=6 *P<0.05; **P<0.01, ***P<0.001

2.3.6. Investigation of Erg binding to ICAM-1 promoter EBS by electrophoretic mobility shift assay (EMSA)

The above experiments suggest a role for the EBS -118 and -181 in the repression of ICAM-1 by Erg. While the ChIP experiments suggest Erg binds the ICAM-1 promoter within a region encompassing these EBS, we cannot deduce which specific EBS are bound by Erg using this technique. We therefore investigated whether Erg bound to the EBS -118 and EBS -181 by EMSA. Biotinylated double stranded oligonucleotides were designed from sequences of the ICAM-1 promoter containing EBS -118 or EBS -181. Nuclear lysate from a confluent HUVEC monolayer was incubated with EBS -118 or EBS -181 biotinylated oligonucleotides and resulting oligonucleotide/protein complexes were detected by SDS-PAGE. Figure 2.13 is a representative image of an EMSA using the oligonucleotide containing the EBS -118. Lane 1 shows at least 4 shifted bands of complexes formed by proteins interacting from the pool of unbound probe, indicating protein/oligo interactions. Two of these bands, indicated by arrows, are specific for the labelled probes as they are competed-off with excess unlabelled probe (Figure 2.13 lane 2) but not by a probe containing mutation within EBS -118 (Figure 2.13 lane 3). The addition of an anti-Erg antibody results in a larger oligonucleotide/protein complex which does not migrate as far down the gel (arrow-head lane 4) causing a super-shift. Super-shifts appear when an antibody binds to a protein complex, this results in a decrease in intensity of one of the other bands. The presence of this band indicates Erg is bound to the oligonucleotide containing the EBS -118. It appears that the band indicated by a solid arrow is less intense in lane 4 compared to lane 1, and this may be the origin of the super-shift. This super-shifted band is competed-off with excess unlabelled probe which shows this is a specific interaction of protein with oligonucleotide (lane 5); however an excess of unlabelled probe with a mutation in the EBS -118 cannot compete-off the super-shifted band (lane 6). This suggests that the EBS-118 is required for Erg to bind the oligonucleotide and therefore Erg is binding to the -118 oligonucleotide probe via EBS-118. Addition of an IgG control antibody did not result in a super-shift of the oligonucleotide/protein complex (lanes 7), indicating that these antibodies did not recognise proteins in the oligonucleotide/protein complex and that the super-shift induced by addition of the Erg antibody was specific. These results suggest Erg specifically binds to EBS -118 on the ICAM-1 promoter.

A

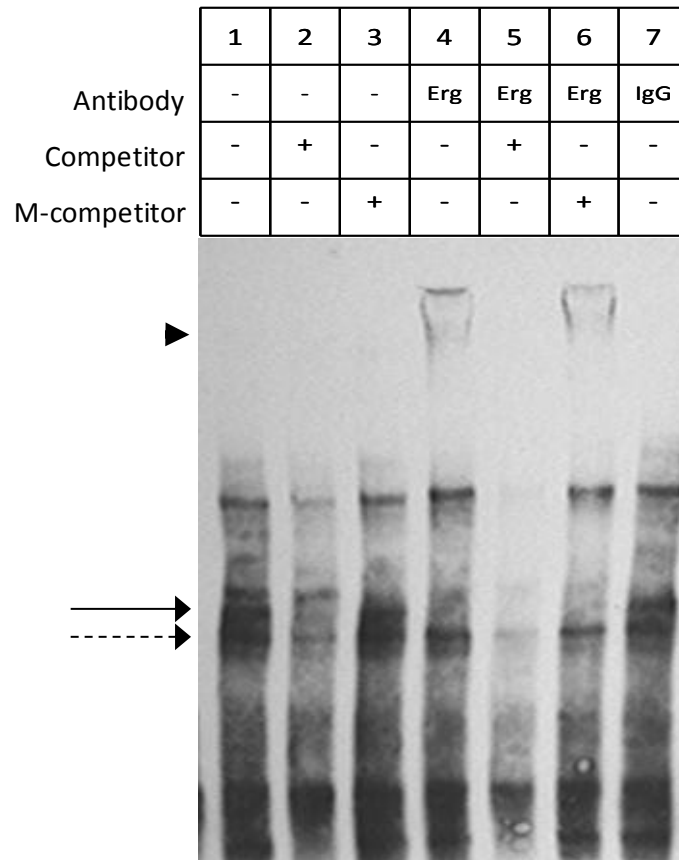


Figure 2.13 Erg binds to the ICAM-1 promoter EBS -118. Biotinylated oligonucleotides containing sequences from the ICAM-1 promoter EBS -118 were incubated with nuclear lysate from resting HUVEC. Anti-Erg antibody or IgG control were incubated with nuclear extract/oligonucleotide complexes as indicated. Specificity was measured by addition of saturating amounts of competing oligonucleotide (competitor) or competing oligonucleotide with a mutation in the EBS -118 (competitor M). Specific shifted protein/oligonucleotide complexes indicated by arrow and super-shifted complexes indicated by arrow head. This is a single representation of 3 separate experiments. This experiment was carried out with the assistance of Dr Silvia Martin Almedina.

We then investigated whether Erg bound to the EBS -181. Figure 2.14 shows a representative image of an EMSA using the oligonucleotide containing the EBS -181, this oligonucleotide sequence has been previously studied in HUVEC treated with TNF- α (Ledebur and Parks, 1995) which showed that NF- κ B p65 binds to the NF- κ B binding site within this oligonucleotide. We found that nuclear lysate from a confluent HUVEC monolayer resulted in the formation of three specific bands in lane 1 (Figure 2.14), indicated by arrows, these bands are specific for the interaction of the oligonucleotide with proteins as they are competed-off with excessive amounts of unlabeled probe (lane 2), however they are not competed off with excess amounts of a probe containing mutations in EBS -181(lane 3). Addition of an anti-Erg antibody decreases the intensity of the upper two bands suggesting Erg is part of this complex (lane 7 to 10) and the addition of IgG control does not decrease the intensity of these bands suggesting this decrease is specific for Erg. We were unable to detect the formation of a super-shifted band caused by the addition of the anti-Erg antibody and this may be because the complex was too large to be resolved on the gel. A similar pattern was observed with a different anti-Erg antibody (data not shown), suggesting that the absence of a super-shift is not due to a technical issue related to a single antibody. As NF- κ B p65 present in nuclear lysate from TNF- α -treated HUVEC was previously shown to interact with this oligonucleotide (Ledebur and Parks, 1995), we investigated whether the band produced from resting HUVEC nuclear lysate also contained NF- κ B p65. Addition of an anti-NF- κ B p65 antibody resulted in a dramatic decrease in the intensity of all three bands (lanes 4-6); however again, no super-shifted band was observed. This result suggests that Erg and NF- κ B p65 are both capable of binding to the oligonucleotide containing this EBS. This result also suggest that in resting HUVEC, with low levels of ICAM-1 expression, there is some NF- κ B p65 present in the nucleus that is capable of binding to DNA.

B

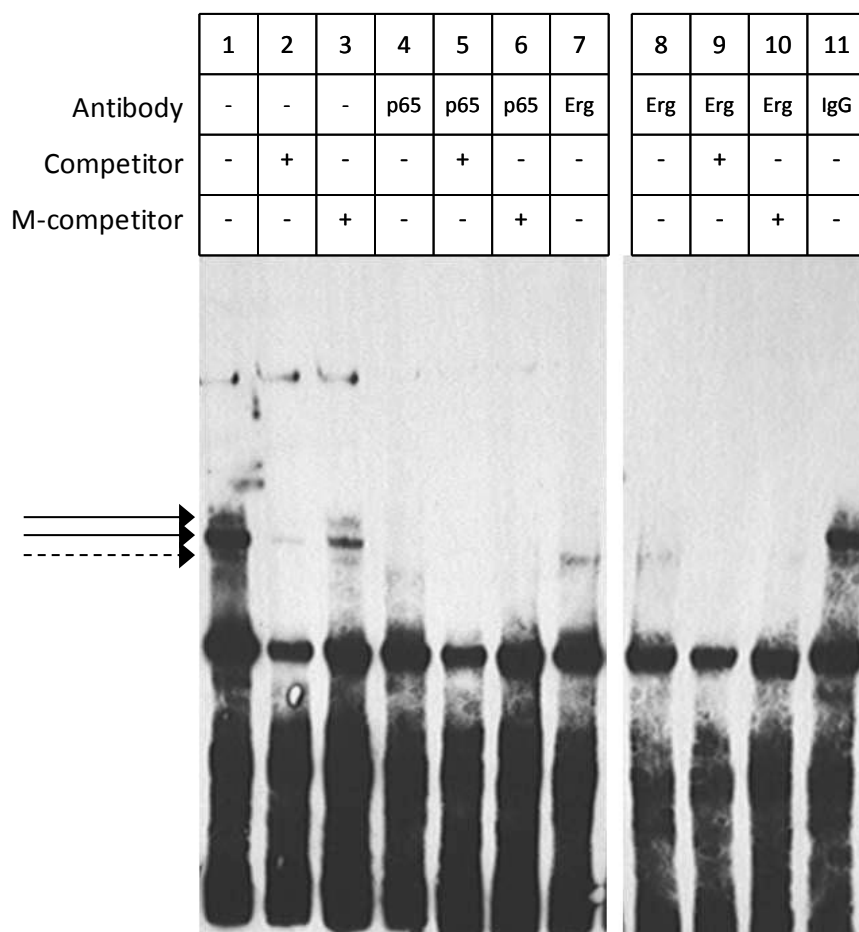


Figure 2.14 Erg and NF- κ B bind to the ICAM-1 promoter EBS-181. Biotinylated oligonucleotides containing sequences from the ICAM-1 promoter EBS-181 were incubated with nuclear lysate from resting HUVEC. Antibodies to Erg, NF- κ B p65 and IgG were incubated with nuclear extract/oligonucleotide complexes as indicated. Specificity was measured by addition of saturating amounts of competing oligonucleotide (Competitor) or competing oligonucleotide with a mutation in the EBS-181 (Competitor M). Shifted protein/oligonucleotide complexes indicated by arrows. Images are a single representation of 3 separate experiments. This experiment was carried out with the assistance of Dr Silvia Martin Almedina.

2.4. Discussion

A microarray study and gene ontology analysis carried out in our lab identified that a number of pro-inflammatory genes are up-regulated after inhibition of Erg. One of these genes is the leukocyte adhesion molecule ICAM-1. We found that Erg regulated ICAM-1 expression levels through direct binding to the ICAM-1 promoter in regions conserved between human and mouse. We found that Erg represses the ICAM-1 promoter at a transcriptional level, and repression by Erg was dependent on two EBS, one at -118 and a second at -181 relative to the transcription start site. The latter site is part of the functional NF- κ B binding site responsible for TNF- α -mediated ICAM-1 expression.

In this chapter we have shown that Erg binds to the ICAM-1 promoter by various techniques. We used ChIP to show that in the native chromatin conformation of resting HUVEC, Erg binds to the repressed ICAM-1 promoter. We found that inhibition of Erg results in a decrease in Erg binding to the ICAM-1 promoter and a corresponding increase in ICAM-1 expression levels. While ChIP is a powerful technique to study Erg-DNA binding in the context of native chromatin, the resolution of binding sites is limited by the size of sheared chromatin fragments which is an average of 500 bp. As the region of the ICAM-1 promoter bound by Erg contained more than one putative EBS, we use *in vitro* techniques to identify specific binding site involved. To investigate the mechanism of ICAM-1 repression by Erg further, we studied the promoter using a reporter assay and also through investigating Erg binding to oligonucleotides encoding ICAM-1 promoter sequences. This gave us the ability to investigate important binding sites by mutagenesis. This technique also has its own caveats; although plasmid DNA forms nucleosomes (Reeves et al., 1985), the plasmid may not be subject to the same structural regulation as native chromatin; therefore may not be subject to possible regulatory mechanisms that interact with the chromatin structure of eukaryotic DNA. We carried out our plasmid based ICAM-1 promoter studies in resting HUVEC instead of cell lines to avoid any non-specific effects due to the loss of endothelial specific factors, and to ensure the presence of endogenous co-factors that may be required for Erg-mediated repression. Indeed, preliminary experiments suggest Erg is not able to repress the ICAM-1 promoter construct in HeLa (Dryden NH. and Randi AM. data not shown). We discuss the importance of cell specificity in gene regulation in Chapters Three and Four.

Our study showed that mutation of EBS at -118 and mutation within the NF- κ B binding site at EBS -181 not only affected their response to Erg but also resulted in lower basal activity,

suggesting these sites are involved in basal activity. Previous mutational and deletion studies of the ICAM-1 promoter in EC have identified regulatory elements involved in basal activation including base pairs -206 to -177 relative to the TSS, which contain the NF- κ B site at -188 and EBS at -181 (Hou et al., 1994; Van de Stolpe and Van der Saag, 1996; Ledebur and Parks, 1995), base pairs -126 to -97 which contain the EBS-118, base pairs -66 to -57 containing a putative SP-1 binding site at -59 and a putative AP-2 site at -48 (Van de Stolpe and Van der Saag, 1996; Jahnke et al., 1995), and base pairs -46 to -37 (Hou et al., 1994). While these sites have been shown to be involved in basal transactivation, there is no evidence of their binding or regulating the ICAM-1 promoter in resting EC. SP-1 is constitutively expressed in EC, (Khachigian et al., 1995) so it is possible this does play a role in basal ICAM-1 activity. Nuclear fractions from resting hepatoma cells contain proteins capable of binding each of these promoter fragments by EMSA, suggesting that as yet unidentified transcription factors are involved in basal regulation (Hou et al., 1994). The importance of these sites is highlighted with the alignment of the human and mouse ICAM-1 promoter sequences which suggest an important role for the NF- κ B site, 7 putative EBS, a GAS site, and a putative SP-1 site in the function of ICAM-1.

Previous studies have identified various regions that may be involved in basal repression of ICAM-1. Deletion of the ICAM-1 promoter from -769 to -496 results in an increase in promoter activity, indicating loss of a repressive region; however, no transcription factor binding sites have been identified in this region (Voraberger et al., 1991). The nucleotide base pairs from -166 to -157 and -96 to -87 have been shown to contain elements involved in basal repression (Hou et al., 1994). Whilst no EBS or other transcription factor consensus sequence has been identified within base pairs -166 to -157, the ICAM-1 promoter sequence -97 to -87 contains the EBS-96. Mutation of EBS -96 in the current study also induced an increase in ICAM-1 promoter activity; however we were unable to show that this was due to loss of repression by Erg, as Erg inhibition resulted in an increase in promoter activity of this mutant. We have shown that mutation of EBS -118, -181, and the NF- κ B site results in lower basal ICAM-1 promoter activity. However the role of EBS in basal activity may be complex and involve both transactivators and transrepressors through the same EBS, therefore studying basal activity after EBS mutation may be misleading. We have shown that Erg binds and represses the ICAM-1 promoter through both EBS -118 and EBS -181, therefore we would predict that mutation of these sites should result in higher basal activity; however the

reverse is true, suggesting EBS -118 and -181 may be under the control of a balance between transcriptional activators and repressors.

Our investigation found that Erg binds to the EBS -181 which is part of the NF- κ B binding site; and Erg repression of ICAM-1 involves this site, as the ICAM-1 up-regulation induced by Erg depletion was lost in the EBS -181. Over-expression of Erg repressed the ICAM-1 promoter containing a mutation in the EBS -181, Suggesting Erg represses by binding both EBS-181 and -118. The function of the NF- κ B binding site is very sensitive to mutation, and modification of nucleotides flanking the consensus sequence can inhibit TNF- α -responsiveness (Paxton et al., 1997), therefore mutation of the EBS within this site could inhibit both NF- κ B binding and ETS factor binding. ICAM-1 is transactivated by TNF- α through the activation and nuclear translocation of NF- κ B p65. While nuclear localised NF- κ B p65 has been shown in resting EC previously (Ledebur and Parks, 1995; Sperone et al., 2011; Collard et al., 1999), the function of p65 has not been investigated. We showed that nuclear lysate from resting HUVEC contain NF- κ B p65 that binds to the EBS-181 oligonucleotide containing the NF- κ B site. The pro-inflammatory cytokine IL-8 is also repressed by Erg in EC, and this has been shown to require an EBS within the NF- κ B binding site of IL-8 (Yuan et al., 2009). Mutation of this site inhibited Erg-mediated repression, and Erg was found, using ChIP, to bind to the IL-8 promoter in a region including the NF- κ B binding site.

The DNA sequence including and surrounding EBS-118 is TCCGGAAATA, and EBS-181 is TCCGGAATTT with the EBS underlined. Preferential Erg binding sites have been investigated previously through oligonucleotide screens and ChIP sequencing (ChIP-seq) studies. A screen of random sequences to identify EBS which are preferentially bound by Erg, identified the consensus (C/G)(C/a)GGAA(G/a)T (Murakami et al., 1993). Whilst two separate studies investigating Erg binding sites by ChIP-seq have identified Erg consensus sequences of (C/a/g)(A/C)GGAA(G/A/c)(t/c/g/a) in VCaP prostate cancer cells (Wei et al., 2010) and (C/G/G)AGGA(A/t)(G/A)(T/C) in hematopoietic progenitor cell line HPC-7 (Wilson et al., 2010). Whilst EBS -118 matches the first two predicted Erg motifs, it does not exactly match the last, and EBS -181 differs from these consensus sequences in that it contains a T 3' of the GGAA binding site. This suggests some flexibility in Erg binding, and might indicate a cell specific preference for binding sites; however no analysis of binding patterns has yet been carried out in EC. ETS factors can be divided into 4 classes depending on their DNA binding specificity (Figure 1.12). Erg is a member of class I along with 14

other ETS factors including Ets-1, Ets-2, Fli-1, Elk-1 and ERM (Wei et al., 2010). The overlap in binding specificities for ETS factors with different target genes implies that the specificity of each ETS factor for a binding site may be controlled by other factors such as expression levels, post-translational modification, or interaction with other co-factors. In HEK293 and RK13 cells, the ETS factors ERM, Ets-2 and Ets-1 transactivated the ICAM-1 promoter via EBS -118 and EBS-96, while the class III ETS factor Pu-1 did not (de Launoit et al., 1998). Erg is not constitutively expressed in HEK293 or RK13 cells so any repressive effect of Erg would not have been seen in these studies, but in HUVEC where Ets-2 and Erg are both constitutively expressed (Hollenhorst et al., 2004), we found that inhibition of Ets-2 did not have an effect on ICAM-1 expression, which highlights the importance of cell type in the functional activity of ETS factors.

We have shown that Erg represses ICAM-1 through interaction with the ICAM-1 promoter, and we used a promoter fragment 1.3 kb upstream of the transcription start site to characterise this, however we have not investigated the possibility that Erg may also repress ICAM-1 through interaction with enhancers or other regulatory elements. ChIP-seq analysis of Erg binding sites in hematopoietic progenitor cell line HPC-7 found that of the 36167 binding sites, 8646 were in promoter regions (transcription start site +/- 1 kb), 14049 were within intragenic regions and 13472 were within intergenic regions (Wilson et al., 2010). Therefore Erg binds more frequently to sites outside of promoters and there is a possibility that Erg additionally regulates ICAM-1 through interaction with enhancers or intragenic regions. ICAM-1 has been shown to be regulated by p53 through interactions with ICAM-1 intragenic regions. In sarcoma osteogenic Saos-2 cells, over-expressed p53 was found to drive ICAM-1 expression in an NF- κ B independent manner through binding to p53 responsive elements within intron 1 and 2 (Gorgoulis et al., 2003). A study published recently also suggests that the 1st intron of ICAM-1 contains thrombin-responsive regions essential for NFAT binding (Xue et al., 2009), this suggest that ICAM-1 is regulated through multiple regions of the gene.

The specificity for ETS factors to a particular binding site may also involve interaction with other transcription factors. Erg has been shown to form homodimers as well as bind other transcription factors including other ETS factors such as Ets-2, Fli-1, and Pu-1, and other transcription factors such as cJun (Camuzeaux et al., 2005). Deletion studies of the Erg protein found the ETS DNA binding domain is required but not sufficient for these interactions. It was shown that full length Erg can form homodimers with a truncated Erg

containing only the first 190 bp including the PNT domain, or with a truncated Erg containing just the ETS DNA binding domains (Carrere et al., 1998). This suggests Erg may form homodimers through the ETS domain interacting with multiple regions of Erg. As mutation of both EBS-118 and EBS-181 are required to achieve loss of repression by Erg, Erg may repress the ICAM-1 promoter through the formation of a link between the two sites. It is possible that Erg binds to itself, other ETS factors, or other transcription factors and this interaction may bring these sites in proximity to link EBS-118 with EBS-181 in the Erg-mediated repression.

To my knowledge our group is the first to study the role of ETS factors in regulating ICAM-1 expression in EC. Studies in other cell types have suggested a link between EBS -118 and EBS -96. Over-expression of Ets-2 and ERM in RK13 and human choriocarcinoma JEG-3 cells resulted in an increase in ICAM-1 promoter activity. ICAM-1 promoter constructs with mutations in EBS -118 and EBS -96 were generated. In RK13 cells basal activity of the EBS -118 mutant was approximately 45% of WT, however mutation in EBS -96 did not affect basal activity. This is in contrast to our observations in HUVEC, where EBS -96 mutation increased basal activity, and suggests a cell type specific response. ICAM-1 promoter luciferase constructs, DNase foot printing, and EMSA showed that ERM transactivated ICAM-1 through EBS -118. EBS -96 was also implicated as a weaker binding site for ERM, as it was identified to bind ERM by DNase foot printing, but not by EMSA using native protein, and the ERM-mediated response of ICAM-1 promoter luciferase activity after EBS -96 mutation was lower in RK13 cells but not JEG-3 cells (de Launoit et al., 1998). An ICAM-1 promoter construct containing mutations in EBS -118 and EBS -96 showed a similar response to that seen when EBS -118 was mutated alone, which suggest that EBS -118 is also important site involved in ETS factor-mediated transactivation of ICAM-1. One additional study used the double EBS -118/-96 mutant to investigate the repressive properties of the ETS factor FEV. FEV repressed ICAM-1 expression in RK-13 cells, and this repression was lost when both EBS-118 and EBS -96 were mutated. However these mutations were not studied separately, therefore it is possible EBS -118 is the sole EBS responsible for FEV-mediated repression. The ETS DNA binding domain of FEV shares close homology to ERG and Fli-1, however the remaining structure is non-homologous; FEV has a truncated N-terminus and does not have a pointed domain. The Alanine rich C terminus of FEV is responsible for the repression of ICAM-1 (Maurer et al., 2003; Peter et al., 1997) and Erg

does not share homology in this domain suggesting Erg represses ICAM-1 through a different mechanism.

One possible mechanism of the Erg-mediated repression of ICAM-1 is via interaction with AP-1 transcription factors. ETS factors including Erg have been shown to bind c-Jun for combinatorial control of target genes. The ICAM-1 promoter contains two AP-1:ETS repeats, however ChIP analysis shows Erg does not bind to the ICAM-1 promoter via the ETS AP-1 sites at -916 and -843 (EBS at -907 and -834) (Figure 2.6 R2). Deletion studies and the mutation of these sites did not affect basal or Erg-regulated ICAM-1 promoter activity, suggesting Erg does not repress ICAM-1 via these sites. The ETS:AP1 sites in the ICAM-1 promoter are implicated in H₂O₂-mediated ICAM-1 activity. However this was shown using deletion constructs and may not specifically involve the ETS:AP1 sites. Therefore a role for direct regulation of ICAM-1 via these sites remains to be shown.

In conclusion, we have shown a role for Erg as a regulator of basal ICAM-1. While we have shown Erg represses the ICAM-1 promoter through binding, the cause of the transcriptional activity which is repressed by Erg has not been identified. The role of EBS-181 in the repression of ICAM-1 suggests NF- κ B may play a role in Erg-mediated repression and this will be explored in the following chapters.

Chapter Three

Erg represses basal NF- κ B activity in quiescent HUVEC

3.1. Introduction

In Chapter Two we showed that Erg represses ICAM-1 expression through binding to EBS -118 and EBS -181, an EBS within the NF- κ B binding site. As discussed in Chapter One, in resting EC NF- κ B is sequestered in the cytoplasm by I κ B family members, but we and others have shown low levels of NF- κ B p65 expression in the nucleus of resting EC (Sperone et al., 2011). In the previous chapter we also showed that nuclear extracts from unstimulated HUVEC contained NF- κ B p65 capable of binding to a 38 bp oligonucleotide of the ICAM-1 promoter sequence containing both the NF- κ B binding site and EBS -181. We speculated from this that Erg may bind to the ICAM-1 promoter in resting EC and block the activity of NF- κ B p65, by interacting with NF- κ B or blocking access to the ICAM-1 promoter.

3.1.1. Erg represses the TNF- α induced activity of NF- κ B

The presence of NF- κ B in the nucleus does not necessarily mean that it is transcriptionally active. As well as nuclear translocation, other mechanisms are involved in NF- κ B activation, including post translational modifications, such as phosphorylation and acetylation, and interaction with transcriptional co-factors (Huang et al., 2010). We have previously shown that Erg inhibits TNF- α -induced ICAM-1 expression by inhibiting phosphorylation of NF- κ B serine-536 both in the cytoplasm and the nucleus (Sperone et al., 2011). This modification is required for NF- κ B transcriptional activity after pro-inflammatory stimuli; however phosphorylation of serine-536 was not seen in resting HUVEC, therefore it is not likely that this mechanism is involved in basal transactivation of ICAM-1. A link between genes regulated by Erg and those regulated by TNF- α in HUVEC has also been shown (Yuan et al., 2009), however the genes highlighted by previous analyses do not all overlap with the Erg-regulated expression data generated in our lab (Birdsey et al., 2012).

3.1.2. Erg-mediated repression of NF- κ B target genes

Microarray analysis carried out in our lab indicates that Erg represses a number of pro-inflammatory NF- κ B target genes, including ICAM-1, VCAM-1 and IL-8 (Sperone et al., 2011; Birdsey et al., 2012). Additionally previous data has shown that Erg represses IL-8 expression, and this repression is dependent on an EBS within the NF- κ B binding sites of IL-8 (Yuan et al., 2009). Our Erg-regulated expression data suggest a link between genes repressed by Erg and those transactivated by NF- κ B.

In this chapter we will investigate further the mechanisms used by Erg to repress basal ICAM-1 expression in EC.

3.1.3. Aims:

- To investigate whether Erg inhibits ICAM-1 expression through repressing NF- κ B activity in resting HUVEC.
- To investigate the mechanism involved in Erg-mediated repression of NF- κ B,
- To use bioinformatics to identify other NF- κ B target genes repressed by Erg and whether there is a correlation between genes repressed by Erg and those transactivated by NF- κ B.

3.2. Materials and Methods

3.2.1. Antibodies

Antibodies used in this chapter include: Erg-1/2/3 sc-353 (Santa Cruz), NF- κ B p65 ab7970 (AbCam), GAPDH cat MAB374 (Millipore), ICAM-1 (clone 15.2, kind gift of Prof. Nancy Hogg), IgG isotype control rabbit IgG PP64 (Millipore).

3.2.2. Cell culture

HUVEC were cultured as described in Chapter 2.2.3.

3.2.3. Erg inhibition

HUVEC were treated with siRNA for chromatin immunoprecipitation. HUVEC were seeded at a density of 1×10^6 cells per well in a gelatin coated 100 mm diameter dish in EGM2 medium (Lonza) and incubated overnight. The following day cells were treated with Allstars negative control siRNA (Qiagen) or Hs_Erg_7 siRNA (Qiagen). siRNA (final concentration 10 nM) and transfection lipid AtuFect 01 (final concentration, 1 μ g/ml (Silence Therapeutics) were mixed at 5 times concentration in OptiMEM medium (Invitrogen) at 37°C for 30 mins in polystyrene tubes. After incubation, the lipid siRNA complex was added to HUVEC to give a 1 x final concentration and incubated for 24 hours.

3.2.4. Transduction of HUVEC with I κ B α super repressor adenovirus

HUVEC (1×10^5 cells/well) were seeded onto 1% gelatin-coated 6 well plates in M199 media, supplemented with 20% foetal bovine serum, 1 U/ml penicillin/ 0.1 mg/ml

streptomycin, 2 mM L-glutamine, 3 mg/ml EC Growth Factor (ECGF, Sigma) in 10 U/ml heparin (CP Pharmaceutical). The following day, cells were transduced with 100 MOI of I κ B α Super repressor Adenovirus (AdI κ B α SR) (Miagkov et al., 1998) or AdLacZ in serum-free M199 medium for 2 hours before replacing with complete M199 medium. After 24 hours, cells were transfected with Erg or control siRNA as described above. Alternatively, after 42 hours following adenovirus transduction, cells were treated with 10 ng/ml TNF- α for 6 hours. ICAM-1 mRNA levels were assessed by quantitative RT-PCR, normalised to GAPDH.

3.2.5. Inhibition of NF- κ B in HUVEC with BAY 11-7085

HUVEC (1×10^5 cells/well) were seeded onto 1% gelatin-coated 6-well plates in EGM-2. After 39 hours, cells were treated with BAY 11-7085 (5 μ M, Sigma) diluted in DMSO and incubated for a further 24 hours. Cells were treated with 10 ng/ml TNF- α for the final 6 hours. ICAM-1 protein levels were measured by western blot using an anti-ICAM-1 (clone 15.2, kind gift of Prof. Nancy Hogg) antibody, and normalised to GAPDH (MAB374 Millipore).

3.2.6. Co-Immunoprecipitation

HUVEC were seeded (1.5×10^6 cells) on a gelatin coated 100 mm tissue culture dish (Nunc) in EGM2. The following day cells were transfected with a plasmid pBent-StrepN-RelA-Flag (gift from I. Udalova) or mock transfected. Briefly 18 μ l Genejuice (Novagen) was added to 800 μ l Optimem (Invitrogen) vortexed and incubated at room temperature for 5 minutes. 6 μ g of pBent-StrepN-RelA-Flag was then added to Optimem/Genejuice and mixed by pipetting then incubated at room temperature for 15 mins. Transfection mixture was then added to cells in fresh EGM2 and incubated for 24 hours. After 24 hours cells were fixed with 1% final concentration formaldehyde (Sigma) for 5 mins or left unfixed, then washed in 125 mM Tris pH8. Cells were washed 3 times in ice cold PBS then collected in a 15 ml falcon tube using scraping buffer of PBS containing, phenylmethylsulfonyl fluoride (PMSF) (Sigma), protease inhibitor cocktail (PIC) (Sigma), NaF and Na₃VO₄. Cells were lysed on ice for 15 mins using Farnham buffer (5 mM PIPES pH 8.0, 85 mM KCL, 0.5% Igepal, protease inhibitor cocktail) and nuclei collected by centrifugation at 3000 rpm for 5 mins at 4°C. Nuclei were lysed in 1 ml RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF, and protease inhibitor cocktail) and sonicated in the Bioruptor (Diagenode, Belgium) water bath sonicator, using 4 cycles of 30 secs on 30 secs off.

Sonicated nuclear lysate was then added to a 75 μ l Strep-Tactin beads (IBA) pre-washed in 1 ml RIPA buffer, then flow through was collected. Strep-Tactin beads were washed using 1 ml ChIP wash buffer (100 mM Tris-HCl pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid) 8 times, then bound proteins were competed off with 6x50 μ l of biotin solution. Fixed samples were heated at 65°C overnight to reverse the cross-links and both fixed and unfixed samples were analysed by western blot using anti-NF- κ B p65 and anti-Erg antibodies.

3.2.7. Chromatin immunoprecipitation

ChIP was carried out as previously described in Chapter 2.2.15.

3.2.8. Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) overlap studies were carried out using the GSEA software Version 2.0 (<http://www.broad.mit.edu/gsea>) (Broad Institute). The query dataset consisted of the 1138 genes identified as being up-regulated following 48 hours Erg inhibition in HUVEC (Birdsey et al., 2012) which were then compared against all the studies in the C2 curated genesets. GSEA correlation of microarray data from Erg GeneBloc-treated HUVEC with the following published studies: TNF- α treated pancreatic cancer cells, TNF- α treated EC, or NF- κ B p65 chIP seq data (Sana et al., 2005; Barish et al., 2010; Zhang et al., 2008) was performed using Gene Set Enrichment Analysis Version 2.0 software (<http://www.broad.mit.edu/gsea>) using 1000 data permutations.

3.2.9. Quantitative PCR

Quantitative PCR was carried out as described in Chapter 2.2.10

3.2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot

SDSPAGE was carried out as described in Chapter 2.2.11

3.2.11. Statistical Analysis

Results were expressed as mean $\pm$ SEM and analysed using GraphPad Prism 4.0 (Graph Pad Software, CA, USA). Data were analysed using Student T-Test. P values less than 0.05 were considered to be statistically significant.

3.3. Results

3.3.1. ICAM-1 basal activation by NF- κ B

The results shown in Chapter Two indicate that Erg represses ICAM-1 basal activity by binding to the ICAM-1 promoter and blocking the activity of a transcriptional activator of ICAM-1 and this repression involve EBS-118 and EBS-181. EBS-181 is part of the ICAM-1 NF- κ B binding site, suggesting that Erg-dependent repression may involve NF- κ B. To investigate whether NF- κ B is responsible for the up-regulation of ICAM-1 after Erg inhibition we used two inhibitors of NF- κ B activation, the I κ B α super-repressor adenovirus (AdI κ B α SR) and the NF- κ B inhibitory compound BAY 11-7085.

3.3.1.1. ICAM-1 induction induced by Erg inhibition is repressed by I κ B α super-repressor

AdI κ B α SR expresses recombinant I κ B α containing a double point mutation which converts the I κ B α Ser 32/36 to Ala 32/36. This I κ B α mutant cannot be phosphorylated and degraded in response to TNFR signalling (Miagkov et al., 1998), therefore it sequesters NF- κ B in the cytoplasm in the presence of inflammatory stimuli. To confirm that AdI κ B α SR inhibited NF- κ B activity, we treated HUVEC with AdLacZ control or AdI κ B α SR and stimulated cells with 10 ng/ml TNF- α for 6 hours before measuring ICAM-1 mRNA levels by qRT-PCR. As expected, TNF- α treatment of HUVEC increased ICAM-1 mRNA expression compared to control (Figure 3.1A). TNF- α -mediated induction of ICAM-1 mRNA expression in HUVEC transduced with AdLacZ control was significantly higher than un-transduced cells; however transduction with AdI κ B α SR significantly inhibited TNF- α -induced ICAM-1 mRNA levels to 30% compared to AdLacZ. This confirms that AdI κ B α SR represses the TNF- α -mediated induction of ICAM-1 expression by NF- κ B. To investigate whether NF- κ B was responsible for the up-regulation of ICAM-1 expression after Erg inhibition, HUVEC were transduced with either AdLacZ or AdI κ B α SR, and 24 hours later transfected with control or Erg siRNA for a further 24 hours before ICAM-1 mRNA levels were measured by qRT-PCR. Transduction with AdLacZ or AdI κ B α SR did not affect basal ICAM-1 mRNA levels compared to untreated; however there was a significant decrease in basal ICAM-1 mRNA expression in the untransfected HUVEC transduced with AdI κ B α SR compared to AdLacZ (Figure 3.1B). This suggests that while there is no detectable increase in basal ICAM-1 expression after adenovirus transduction, AdI κ B α SR inhibits some basal NF- κ B-mediated induction of ICAM-1 expression. Erg inhibition in AdLacZ transduced cells resulted in

approximately 2 fold higher ICAM-1 mRNA expression compared to control siRNA. Erg inhibition in AdIkB α SR treated cells resulted in an approximate 1.7 fold increase in ICAM-1 mRNA expression; however, this difference was not significant compared to control siRNA, suggesting AdIkB α SR may inhibit the increase in ICAM-1 mRNA expression after Erg inhibition (Figure 3.1B). Moreover there was a significant decrease in ICAM-1 expression after Erg inhibition in cells treated with AdIkB α SR compared to AdLacZ. As AdIkB α SR inhibits the activity of NF- κ B, these results suggests the up-regulation of ICAM-1 induced by inhibition of Erg is driven by constitutive activity of NF- κ B in resting HUVEC.

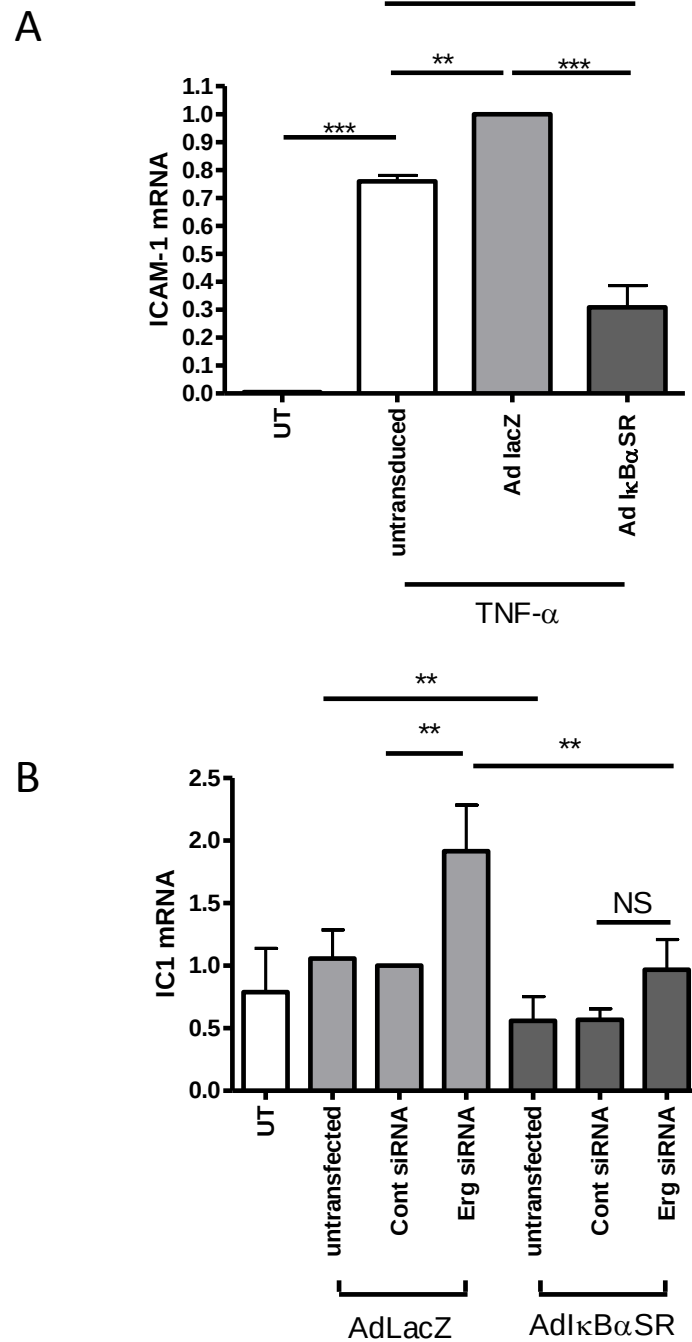


Figure 3.1 Up-regulation of ICAM-1 after Erg siRNA is repressed by AdIkBαSR. (A) HUVEC transduced with 100MOI AdIkBαSR or AdLacZ were treated with 10 ng/ml TNF-α for 6 hours. ICAM-1 mRNA levels were measured by quantitative RTPCR and results are expressed as fold change compared to control siRNA AdLacZ-treated. (B) Erg siRNA or control siRNA-treated HUVEC were transduced with 100MOI AdIkBαSR or AdLacZ. ICAM-1 mRNA levels were measured by quantitative RTPCR and results are expressed as fold change compared to control siRNA AdLacZ-treated. N=5, **P<0.01, ***P<0.001, NS = not significant.

3.3.1.2. ICAM-1 induction by Erg is repressed by BAY inhibitor

The compound BAY 11-7085 (BAY) is an inhibitor of NF- κ B activity and functions by inhibiting TNF- α -induced phosphorylation of I κ B α , therefore I κ B α is not degraded and is free to sequester NF- κ B in the cytoplasm (Pierce et al., 1997). To show that BAY inhibits NF- κ B-dependent ICAM-1 expression in HUVEC, cells were treated with either BAY or DMSO then stimulated with TNF- α (10ng/ml) for 24 hours. Western blots were carried out to quantify ICAM-1 protein levels normalised to GAPDH, and a representative image is shown in Figure 3.2A. TNF- α stimulation significantly increased ICAM-1 protein levels in DMSO treated HUVEC by approximately 28 fold (Figure 3.2B). Treatment with BAY blocked the TNF- α -mediated induction of ICAM-1. These results confirm that BAY is able to repress the TNF- α -induced up-regulation of ICAM-1 in HUVEC therefore inhibiting NF- κ B activity. To confirm that the up-regulation of ICAM-1 after Erg inhibition was due to NF- κ B activity as suggested with AdI κ B α SR, HUVEC pre-treated with Erg or control GB, were treated with either BAY or DMSO and ICAM-1 protein levels were detected by western blot (Figure 3.2 A and C). Inhibition of Erg levels in HUVEC in the presence of DMSO induced a significant increase in ICAM-1 protein levels by approximately 4 fold and 2 fold compared to both untreated and control GB respectively; however BAY treatment inhibited the increase in ICAM-1 protein levels in HUVEC treated with Erg GB compared to control GB (Figure 3.2C). Additionally, ICAM-1 protein levels were significantly decreased in Erg depleted HUVEC treated with BAY compared to DMSO (Figure 3.2 A and C) suggesting increased ICAM-1 expression after Erg inhibition is NF- κ B dependent. ICAM-1 protein was significantly increased in DMSO treated HUVEC after control GB compared to untransfected (Figure 3.2A and C), which may indicate slight activation of the cells by GB treatment, however the ICAM-1 protein induction by Erg inhibition was approximately 2 fold higher than this, confirming the role of Erg in inhibition of basal ICAM-1 expression. These results are in line with those found using AdI κ B α SR, and confirm that the increase in ICAM-1 expression after Erg inhibition is due to NF- κ B activity.

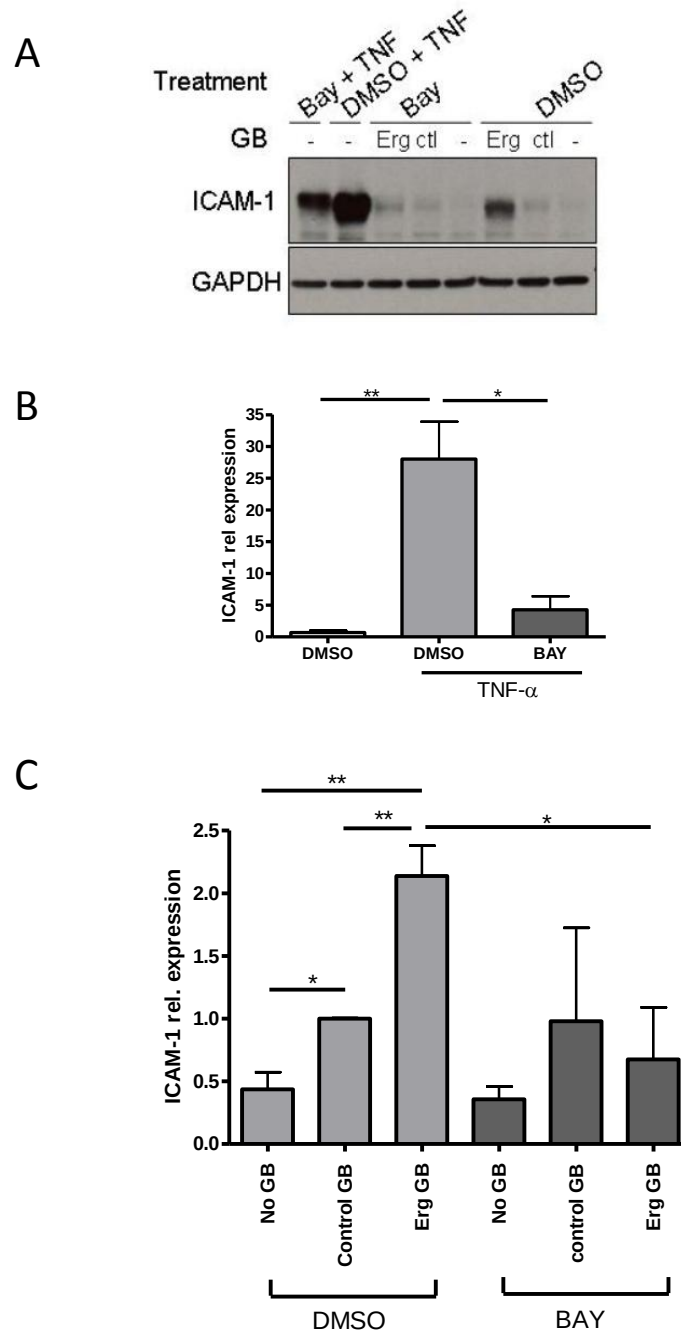


Figure 3.2 Up-regulation of ICAM-1 after Erg Genebloc is repressed by the NF- κ B inhibitor BAY-117085. Experiments carried out by Dr Andrea Sperone. A) Western blot of HUVEC lysate from cells treated for 15 hours with either Erg or control Genebloc, followed by treatment with Bay-117085 (5 μ M) or DMSO for 24 hours. For the control experiment HUVEC were pre-treated for 1 hour with Bay-117085 (5 μ M) or DMSO followed by 23 hours incubation with TNF- α (10 ng/ml). ICAM-1 protein expression is quantified relative to GAPDH and normalized against HUVEC treated with control Genebloc and DMSO. B) Quantification of western blot control experiments, N=3. $P<0.05$; $**P<0.01$ C) Quantification of Western blot from HUVEC treated with Erg or control Genebloc and DMSO or BAY, N=3. $*P<0.05$; $**P<0.01$, $***P<0.001$

3.3.2. Erg does not physically interact with NF- κ B p65

In order to dissect the mechanism behind the Erg-mediated repression of NF- κ B activity we first investigated whether Erg bound to NF- κ B p65. As discussed in Chapter One, not all NF- κ B subunits have the ability to transactivate genes, only p65 (RelA), c-Rel and RelB contain transactivation domains (See Figure 1.3). NF- κ B p65 is expressed ubiquitously while c-Rel and Rel-B expression is restricted to lymphoid and myeloid cells (Thanos and Maniatis, 1995; Liou and Hsia, 2003). Therefore we studied the possible interaction between Erg and NF- κ B p65. HUVEC were transfected with a plasmid expressing recombinant NF- κ B p65 (pBent-StrepN-RelA-Flag) (gift from I. Udalova) or mock transfected. To investigate whether interactions between Erg and p65 were dependent or independent on DNA, cells were fixed with 1% formaldehyde to crosslink protein to DNA, or left unfixed. Nuclear fractions from HUVEC transfected with recombinant NF- κ B p65 contained higher levels of NF- κ B p65, indicating that the transfection of HUVEC was successful (Figure 3.3) Mock transfection of fixed but not unfixed samples resulted in low basal levels of NF- κ B p65 in the total nuclear lysate protein sample, suggesting cross-linking may have an effect on the efficiency of the nuclear extraction, or fixation may have triggered low levels of NF- κ B p65 nuclear translocation (Figure 3.3); however, at higher western blot exposures, p65 was found in the nuclear fractions of both mock transfected samples (data not shown). Nuclear fractions of cells were immunoprecipitated using a StrepTactin column which binds the StrepN tag on recombinant p65 and immunoprecipitates were analysed by western blot with antibodies to NF- κ B p65 and Erg (Figure 3.3). As expected, no NF- κ B p65 was immunoprecipitated from the mock transfected fixed or unfixed samples; however NF- κ B p65 was immunoprecipitated from HUVEC transfected with pBent-StrepN-RelA-Flag both the fixed and unfixed samples. Erg protein was detected in all input samples, although levels appeared lower in cells transfected with pBent-StrepN-RelA-Flag compared to mock transfected for both the fixed and unfixed samples. We did not detect Erg in either of the immunoprecipitated samples suggesting Erg does not co-immunoprecipitate with recombinant p65 in either the fixed or unfixed samples. This experiment indicates Erg does not interact with NF- κ B p65 by direct binding or DNA dependent interactions, suggesting that Erg repression of NF- κ B activity is not through Erg binding to NF- κ B p65.

One unexpected finding from this experiment was the decrease in Erg protein levels in cells expressing recombinant NF- κ B p65. While this data is from a single experiment and requires repetition to find any conclusive results, it supports the hypothesis that over-expressing NF-

κ B p65 in HUVEC inhibits Erg levels and suggests an NF- κ B p65 dependent mechanism for the decrease in Erg levels after stimulation by TNF- α shown previously (McLaughlin et al., 1999).

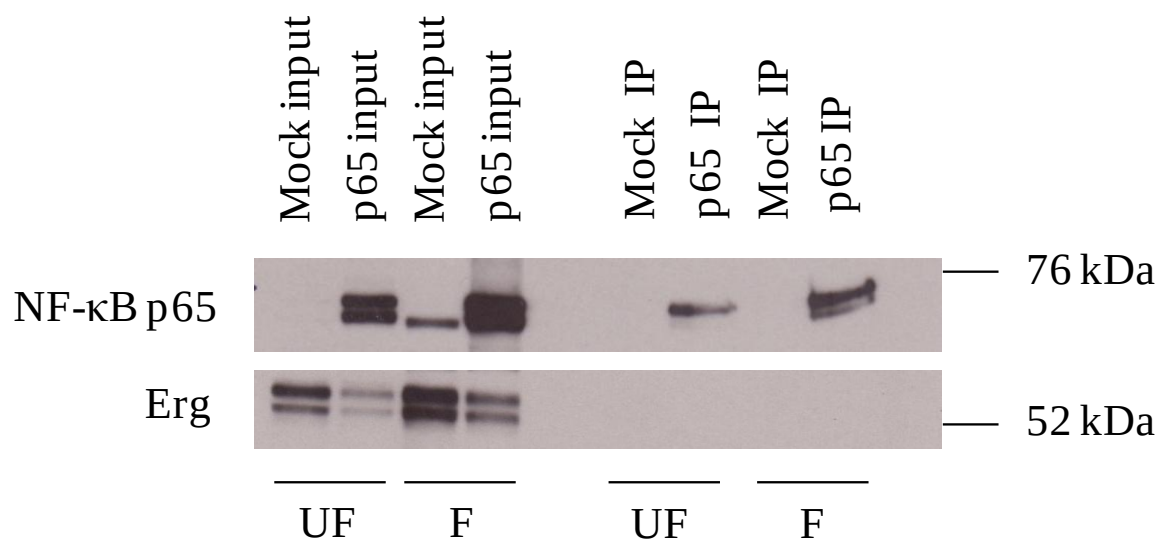


Figure 3.3 Erg does not co-immunoprecipitate with NF-κB p65. A) HUVEC were mock transfected or transfected with pBentStrepN-RelA-Flag (p65) and either fixed with 1% formaldehyde (F) or left unfixed (UF). Sonicated nuclear lysate was immunoprecipitated with a StrepTactin Sepharose column and total nuclear lysates (Input) and immunoprecipitates (IP) were analysed by western blot using anti-NF-κB p65 and anti-Erg antibodies.

3.3.3. Erg inhibits basal activation of the ICAM-1 promoter by blocking constitutive NF- κ B p65 binding to the promoter

In Chapter Two we showed that NF- κ B p65 binds an oligonucleotide from the ICAM-1 promoter sequence containing the NF- κ B binding site. Levels of most NF- κ B p65 target genes including ICAM-1 are very low in resting EC, suggesting that NF- κ B p65 has low activity in driving the endogenous ICAM-1 promoter in resting cells. We carried out ChIP to investigate whether NF- κ B p65 binds to the endogenous ICAM-1 promoter in resting HUVEC. The functional NF- κ B binding site is within the region (R)4 primer pair (Ledebur and Parks, 1995), therefore we investigated whether NF- κ B p65 binds to DNA from R4 or the flanking regions R3 and R5 in HUVEC using ChIP (Figure 3.4A). NF- κ B p65 in confluent quiescent HUVEC was not enriched on the ICAM-1 promoter at levels above those detected with an IgG control antibody in any regions tested (Figure 3.4B). To ensure that it was possible to enrich for NF- κ B p65 on the ICAM-1 promoter by ChIP, we treated HUVEC with TNF- α for 30 minutes, to induce activation and nuclear translocation of NF- κ B p65. We found p65 was significantly enriched to R4 at approximately 4.6 fold higher levels than IgG control after TNF- α stimulation. This indicates that while NF- κ B p65 is present in the nuclei of quiescent EC, and it is capable of binding to the NF- κ B binding site within an oligonucleotide, it cannot bind to the ICAM-1 promoter in the environment of endogenous chromatin in quiescent EC.

3.3.4. Erg inhibits NF- κ B p65 binding to the ICAM-1 promoter in quiescent EC

We have shown that Erg inhibition results in an NF- κ B-dependent increase in ICAM-1 expression, therefore we hypothesised that in resting HUVEC constitutively expressed Erg blocks NF- κ B p65 binding to the ICAM-1 promoter. To investigate this, HUVEC were treated with Erg or control siRNA, and ChIP was carried out with an anti-NF- κ B p65 antibody. p65 enrichment on the NF- κ B binding site of the ICAM-1 promoter was detected using R4 qPCR primers. In agreement with the ChIP results for NF- κ B p65 binding in resting cells, HUVEC treated with control siRNA showed no enrichment for NF- κ B p65 binding to R4 over control IgG; however in HUVEC treated with Erg siRNA, enrichment with an anti-NF- κ B p65 antibody was approximately 4 fold greater than with control IgG (Figure 3.4C). This data suggests the presence of Erg in resting HUVEC inhibits low levels of constitutively nuclear localised NF- κ B p65 from binding to ICAM-1 and possibly other target genes.

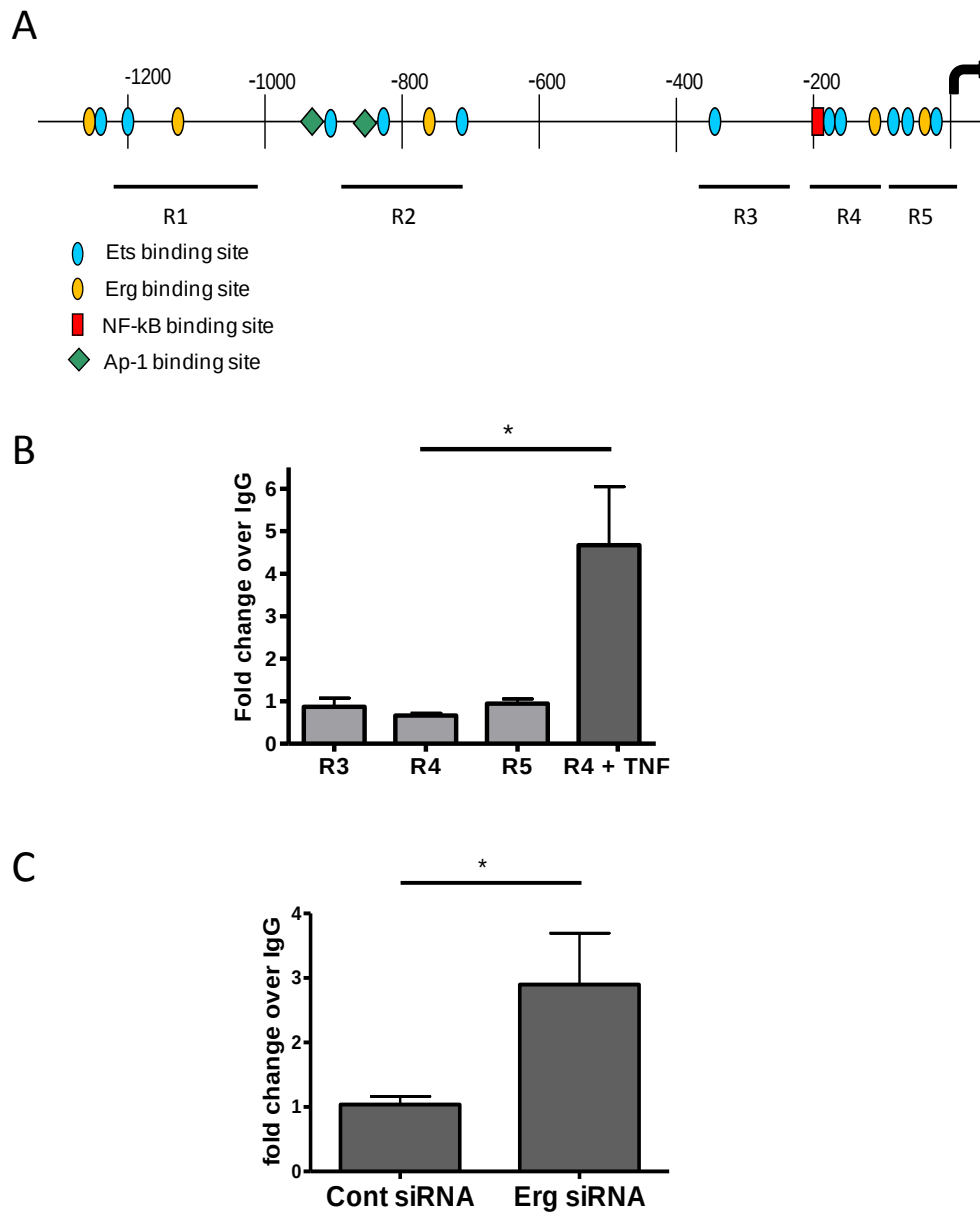


Figure 3.4 Erg inhibits NF-κB p65 binding to the ICAM-1 promoter in quiescent EC. A) Schematic diagram of the ICAM-1 promoter with transcription factor binding sites as indicated. Region 1-5 ChIP quantitative PCR amplicons indicated below. B and C) ChIP was carried out on sheared chromatin from confluent resting HUVEC +/- TNF (B), or HUVEC treated with Erg or control siRNA (C), using an anti-NF-κB p65 or control IgG antibody. Immunoprecipitated DNA was analysed by qPCR for primers covering ICAM-1 promoter regions 3-5 (B) or region 4 (C) and negative control region. Results are expressed as fold change compared to IgG normalised to input and negative control region. N=4 (B), N=6 (C), *P<0.05

3.3.5. Identifying a correlation between genes repressed by Erg and transactivated by NF- κ B using Gene Set Enrichment Analysis.

Studies from our lab and others have shown that Erg represses constitutive expression of ICAM-1, IL-8 and VCAM-1 (Sperone et al., 2011). These genes are transactivated by NF- κ B in inflammatory conditions. As we found that Erg represses ICAM-1 by inhibiting NF- κ B p65 binding to the NF- κ B binding site, we speculated that there may be a wider mechanism of Erg-mediated repression of other NF- κ B target genes. We used a genome wide bioinformatic tool, Gene Set Enrichment Analysis (GSEA), to investigate a correlation between the genes repressed by Erg and genes transactivated by NF- κ B. GSEA compared expression data from our Erg microarray analysis with expression data held on the molecular signatures database (MSigDB) which is a collection of annotated gene sets from published data for use with GSEA software.

3.3.6. GSEA overlap analysis with genes up-regulated by Erg inhibition

We first carried out an initial screen to identify if the gene set up-regulated by Erg inhibition, as identified by the microarray analysis (Birdsey et al., 2012), overlapped with other gene sets from the MSigDB. Of the top nine studies with the most significant overlaps in regulated genes, two involved the response to treatment with the NF- κ B activating cytokine TNF- α . The first gene-set is from a study in pancreatic cancer cells investigating genes up-regulated by TNF- α treatment and inhibited by an IKK inhibitor, therefore regulated by NF- κ B (Zhang et al., 2008). The second gene-set is from a study investigating the response of microvascular and macrovascular EC to TNF- α , interferon (IFN)- γ or IL-4 (Sana et al., 2005). The sets of genes regulated by TNF- α in both studies significantly overlapped with genes up-regulated by Erg inhibition in HUVEC, suggesting a specific role for Erg in NF- κ B regulatory pathways.

The analysis also highlighted that pro-inflammatory genes repressed by Erg overlapped with other studies, for example: a study of genes up-regulated in osteosarcoma U2OS cells upon inhibition of HDAC3 (Senese et al., 2007), a repressor of transcription; and a study of genes down-regulated in Ewing's sarcoma A4573 cells after knockdown of B cell-specific Moloney-MLV insertion site-1 (BMI1)(Douglas et al., 2008), also a polycomb group member and repressor of transcription. The overlap between genes regulated by these co-repressors

and Erg may indicate a possible role for these molecules in Erg-mediated repression of pro-inflammatory genes.

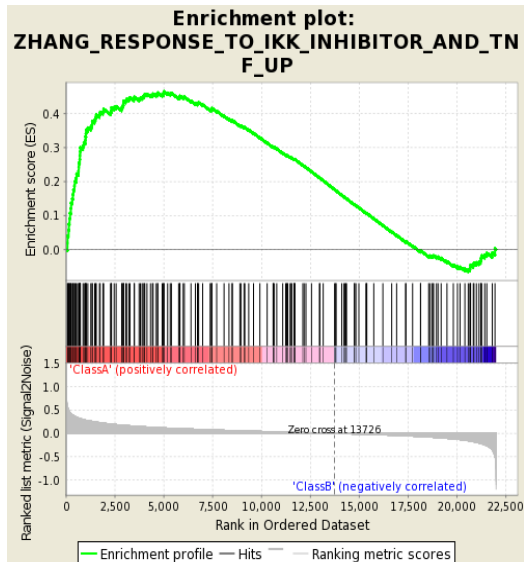
3.3.7. GSEA using complete Erg microarray data

In order to investigate whether there was a correlation between genes repressed by Erg and transactivated by NF- κ B we carried out a separate in-depth GSEA between the whole Erg microarray data set and the two studies of gene sets up-regulated after TNF- α treatment (Zhang et al., 2008; Sana et al., 2005). This is carried out by ranking raw microarray data, from the most up-regulated genes, to the most down-regulated genes after Erg inhibition in HUVEC. We then compare this with the up-regulated genes set from the two TNF- α studies, to show separate GSEA comparison with the ranked Erg microarray data. If the TNF- α -regulated gene set is distributed evenly across the Erg ranked data, there is no correlation with the Erg-regulated data, however if the TNF- α data overlaps at the top or the bottom of the Erg ranked data, it suggests there is a correlation. The level of enrichment is quantified by the enrichment score (ES), which is a reflection of the degree of correlation, represented by the green line in the graphical output (Figure 3.5A, B and C). The ES is generated by calculating a cumulative running sum along the Erg ranked data; every time a gene present in the Erg ranked data is also present in the gene set (represented by a vertical black line in the graphical output) the running sum is increased, conversely when a gene occurs in the Erg ranked data which is not in the gene set, the running sum decreases. The incremental size of the increase or decrease in the running sum is greater for genes at the extreme ends of the Erg rank, which are the most up or down-regulated. The running sum value is then normalised to correct for the size of the gene set to give the normalised enrichment score (NES). The statistical significance of the NES is calculated by the GSEA software generating a null hypothesis; the rank of the Erg microarray data is permuted and the analysis is run again. This is then compared to the actual test data. Comparison between the Erg data set and the genes up-regulated in pancreatic cancer cells after TNF- α treatment resulted in a significant NES of 1.67 (Figure 3.5A), indicating a correlation between NF- κ B target genes in pancreatic cancer cells and genes up-regulated by Erg inhibition in HUVEC. Comparison with genes up-regulated in TNF- α -treated EC resulted in a significant NES of 2.06 (Figure 3.5B). This indicates a correlation between NF- κ B target genes in EC and genes up-regulated by Erg inhibition in HUVEC. In addition, we also used GSEA to compare the Erg regulated genes with the data from a recent ChIP Seq analysis, which identified regulatory sequences bound by NF- κ B p65 in LPS-treated macrophages (Barish et al., 2010). This comparison gave a

NES of 1.11 with a p-value of 0.097 (Figure 3C), and indicates a trend towards a correlation. Analysis of the three studies together suggests a strong correlation, but not complete overlap between genes repressed by Erg and genes bound and transactivated by NF- κ B.

A

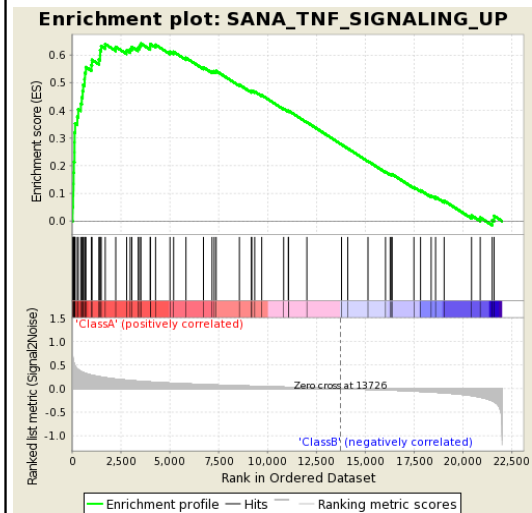
Erg data set vs. Genes up in
TNF- α treated pancreatic
cancer cells



NES = 1.6749127
P-value: < 0.001

B

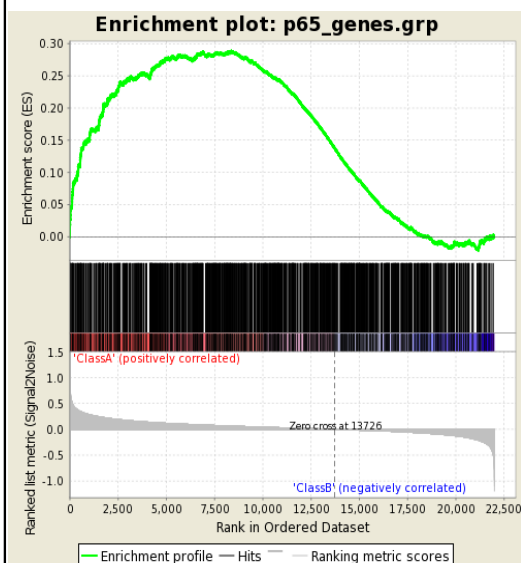
Erg dataset vs. Genes up in
TNF- α treated EC



NES = 2.0696177
P-value < 0.001

C

Erg data set vs.
NF- κ Bp65 target genes in
macrophages



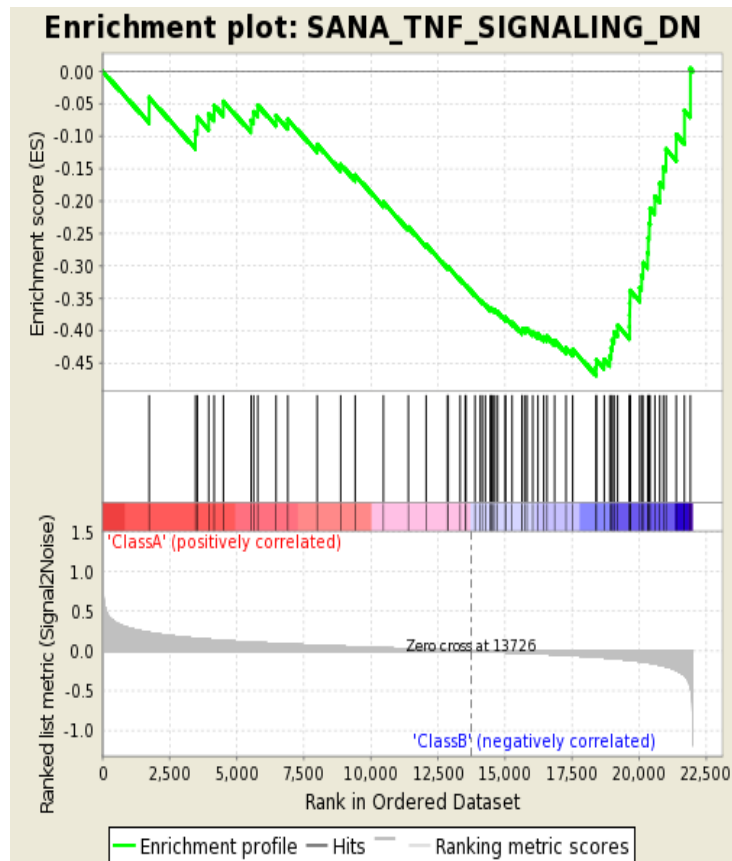
NES = 1.1068145
P-value: 0.097

Figure 3.5. Gene set enrichment analysis (GSEA) demonstrates highly significant enrichment of genes repressed by Erg with genes up-regulated by NF- κ B activating cytokine TNF- α . (A, B and C) GSEA was carried out using standard settings. The graphical outputs show enrichment (green curve) of genes up-regulated in TNF- α -treated pancreatic cancer cells (A), endothelial cells (B), or genes bound by NF- κ B p65 in macrophages after LPS stimulation (C), along a ranked list of genes up or down-regulated by 48 hours of Erg inhibition. The normalised enrichment score (NES) reflects the degree to which a gene set is over-represented at the top or bottom of a ranked list, normalized for differences in gene set size and in correlations between gene sets and the expression dataset. These experiments were carried out with the assistance of Rebecca Hannah and Professor Berthold Gottgens.

3.3.8. Erg transactivates genes repressed by TNF- α treatment

We carried out GSEA to investigate a correlation between the Erg microarray data and the gene set from the study that looked at genes down-regulated by TNF- α treatment in EC. We found a significant NES of -1.95 suggesting a negative correlation (Figure 3.6). This indicates genes down regulated by Erg inhibition, which are transactivated by Erg in resting EC, correlate with genes down regulated by TNF- α treatment. This correlation is expected as TNF- α treatment depletes Erg levels (McLaughlin et al., 1999; Yuan et al., 2009; Sperone et al., 2011), and the list of correlated genes includes characterised Erg target genes such as VWF and RhoJ (Table 3.1) (McLaughlin et al., 1999; McLaughlin et al., 2001; Yuan et al., 2011a). In contrast to the mechanisms of repression of Erg target genes by inhibiting NF- κ B binding, a decreased expression of Erg target genes after TNF- α treatment, is most likely due to TNF- α -mediated Erg depletion, rather than a repressive activity of TNF- α .

Erg dataset vs. Genes down in TNF- α treated EC



NES = -1.9514192

P-value < 0.001

Figure 3.6. Gene set enrichment analysis (GSEA) demonstrates significant enrichment of genes transactivated by Erg with genes down regulated by NF- κ B activating cytokine TNF- α . A) GSEA was carried out using standard settings. The graphical outputs show enrichment (green curve) of genes up-regulated in TNF- α -treated endothelial cells, along a ranked list of genes up or down-regulated by 48 hours of Erg inhibition. The normalised enrichment score (NES) reflects the degree to which a gene set is over-represented at the top or bottom of a ranked list, normalized for differences in gene set size and in correlations between gene sets and the expression dataset. These experiments were carried out with the assistance of Rebecca Hannah and Professor Berthold Gottgens.

Table 3.1 GSEA data enriched genes from comparison between genes up-regulated by TNF- α treatment against genes down-regulated by Erg inhibition

Gene code	Accession number	Gene Title
ACE	NM_000789.3	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
APLN	AK001855	apelin, AGTRL1 ligand
EPAS1	AF052094	endothelial PAS domain protein 1
ITGA6	NM_000210	integrin, alpha 6
LDB2	NM_001290	LIM domain binding 2
RGS4	AL514445	regulator of G-protein signalling 4
RHOJ	BE218803	ras homolog gene family, member J
VWF	NM_000552.3	von Willebrand factor
POSTN	NM_006475.2	periostin, osteoblast specific factor
MMRN1	NM_007351	multimerin 1
NTN4	AF278532	netrin 4
ATP1B3	BF059073	ATPase, Na ⁺ /K ⁺ transporting, beta 3 polypeptide
NFIB	AI700518	nuclear factor I/B
GLCE	W87398	UDP-glucuronic acid epimerase
PALMD	NM_017734.4	palmdelphin
BMX	NM_203281.2	BMX non-receptor tyrosine kinase
NOS3	NM_000603	nitric oxide synthase 3 (endothelial cell)
JAG2	AF029778	jagged 2
NT5E	AI086864	5'-nucleotidase, ecto (CD73)
GIMAP7	NM_153236.3	GTPase, IMAP family member 7
KCTD12	AI718937	potassium channel tetramerisation domain containing 12
CRIP2	NM_001312.2	cysteine-rich protein 2
ZNF521	AK021452	zinc finger protein 521
ARL5A	AW291264	ADP-ribosylation factor-like 5A
EMCN	NM_001159694.1	endomucin
CABLES1	AI889160	Cdk5 and Abl enzyme substrate 1

3.3.9. Common regulated genes from GSEA

We compared the genes in the leading-edge subset for the three comparisons of genes repressed by Erg and transactivated by NF- κ B. The leading-edge subset are those genes that appear in the ranked list at, or before, the point where the running sum reaches its maximum deviation from zero, and are significantly correlated between the two samples. Table 3.2 lists these genes for each study which are common between at least two of the studies, and those genes which are common between all three studies. As well as ICAM-1, the list of genes common amongst all three studies consists of TNF- α inducible protein (TNFAIP)-2 and 3, chemokine ligand (CXCL)2, and cellular inhibitor of apoptosis (cIAP)2.

Table 3.2. Common gene hits and accession numbers from GSEA analysis. Common genes enriched for Erg GSEA amongst all three studies are in dark grey. Common genes enriched for Erg GSEA with TNF- α treated pancreatic cancer cells and TNF- α treated EC studies are in light grey. The remaining genes were commonly enriched between genes bound by NF- κ B p65 after LPS treatment, and either TNF- α -treated pancreatic cancer cells or TNF- α -treated EC.

Gene Symbol	Accession Number	Gene Title
IFIT1	NM_001548	interferon-induced protein with tetratricopeptide repeats
CCL20	NM_001130046	chemokine (C-C motif) ligand 20
OAS1	NM_002534	2'-5'-oligoadenylate synthetase 1
TNFAIP2	NM_006291	tumor necrosis factor, alpha-induced protein 2
CCL2	S69738	chemokine (C-C motif) ligand 2
CXCL11	NM_005409	chemokine (C-X-C motif) ligand 11
ICAM1	NM_000201	Intercellular adhesion molecule 1
TNFAIP3	NM_006290	tumor necrosis factor, alpha-induced protein 3
VCAM1	NM_001078	vascular cell adhesion molecule 1
IFIH1	NM_022168	interferon induced with helicase C domain 1
IL7R	NM_002185	interleukin 7 receptor
CXCL3	NM_002090	chemokine (C-X-C motif) ligand 3
CXCL2	M57731	chemokine (C-X-C motif) ligand 2
SLC15A3	NM_016582	solute carrier family 15, member 3
BST2	NM_004335	bone marrow stromal cell antigen 2
CSF1	NM_000757	colony stimulating factor 1 (macrophage)
cIAP2	NM_001165	baculoviral IAP repeat containing 3
ATP13A3	BF218804	ATPase type 13A3
CD83	NM_004233	CD83 molecule
NFKBIZ	AB037925	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
TNFAIP6	NM_007115	tumor necrosis factor, alpha-induced protein 6
NFKB2	NM_001077493	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
ISG20	NM_002201	interferon stimulated exonuclease gene 20kDa
IL1B	NM_000576	interleukin 1, beta
IL15	NM_000585	interleukin 15
RELB	NM_006509	v-rel reticuloendotheliosis viral oncogene homolog B
RAB11FIP1	NM_025151	RAB11 family interacting protein 1 (class I)
BCL3	NM_005178.	B-cell CLL/lymphoma 3
IL1RN	NM_000577	interleukin 1 receptor antagonist
SLFN5	A1435399	schlafen family member 5
SDC4	NM_002999	syndecan 4
NFKBIE	NM_004556	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NFKB1	M55643	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
ATF3	NM_001674	activating transcription factor 3

3.3.10. Validation of common regulated genes from GSEA: cIAP2, and TNFAIP3

To validate that Erg represses the NF- κ B target genes identified by GSEA we decided to investigate two genes that were found to be correlated in all three studies, cellular inhibitor of apoptosis (cIAP)-2 and TNF- α inhibitory protein (TNFAIP)3. To first validate the Erg microarray data, we carried out qRT-PCR to measure cIAP2 and TNFAIP3 mRNA expression in HUVEC treated with Erg or control siRNA. Inhibition of Erg expression resulted in an approximate 2.9 fold increase in cIAP2 expression, and an approximate 14.1 fold increase in TNFAIP3 expression compared to control (Figure 3.7A). This confirms the microarray data and suggests Erg represses these genes. We then investigated whether Erg binds to the promoters of these genes as shown by ChIP. Like ICAM-1, cIAP2 and TNFAIP3 are both regulated by NF- κ B after TNF- α stimulation, and they contain functional NF- κ B binding sites in their promoter (Erl et al., 1999; Krikos et al., 1992) (Figure 3.9 and 3.10). We designed qPCR primers to detect binding on or around the NF- κ B sites of these two genes by ChIP (Figure 3.9 and 3.10). ChIP was carried out using sheared chromatin from resting HUVEC, and anti-Erg or IgG antibodies. Immunoprecipitated chromatin was detected by qPCR and results were normalised for total input levels, and binding to the GAPDH control gene. Erg bound to both the cIAP2 and TNFAIP3 promoters at levels approximately 3 fold or 2.5 fold higher than IgG control respectively (Figure 3.7 B). This suggests that Erg represses these pro-inflammatory genes by binding their promoters in the vicinity of NF- κ B binding sites. These data validate the correlation between genes repressed by Erg and transactivated by NF- κ B.

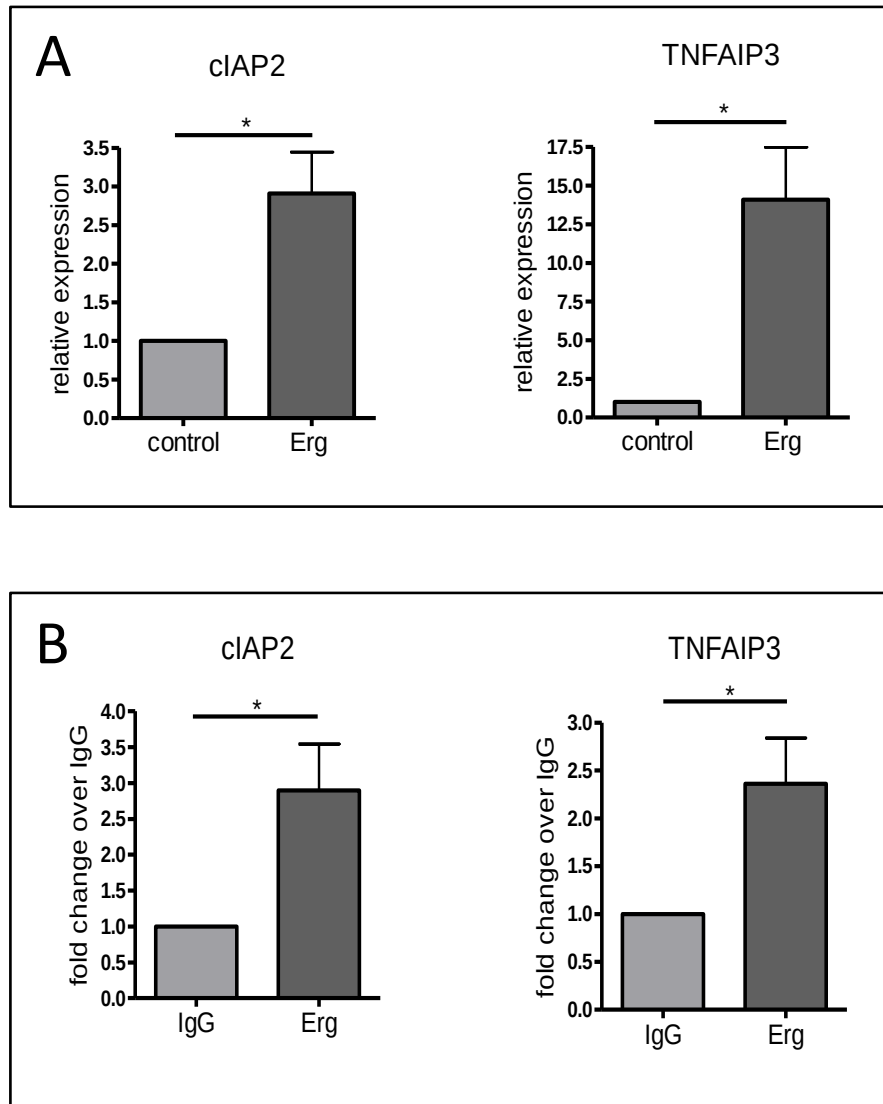


Figure 3.7 Erg represses and binds to the promoters of cIAP2 and TNFAIP3 in quiescent HUVEC A) mRNA from HUVEC treated with Erg or control siRNA was analysed by quantitative RTPCR with primers specific for cIAP2 or TNFAIP3 (N=3) *P<0.05. B) ChIP was carried out on sheared chromatin from confluent resting HUVEC using an anti Erg or control IgG antibody. Immunoprecipitated DNA was analysed by qPCR for primers covering EBS in cIAP2 or TNFAIP3 and negative control GAPDH promoter region. Data is expressed as fold change compared to IgG normalised to input and control region. N=3, *P<0.05.

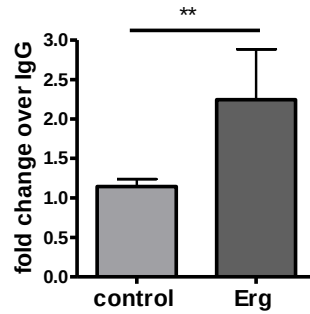
3.3.11. Erg Blocks binding of NF- κ B p65 to the cIAP2 and IL8 promoters

As inhibition of Erg in resting EC permits the binding of NF- κ B p65 to the ICAM-1 promoter, we hypothesised that a similar mechanism may be responsible for the induction of cIAP2 and TNFAIP3. ChIP was carried out on chromatin from HUVEC treated with control or Erg siRNA using an anti-NF- κ B p65 antibody. Enrichment for NF- κ B p65 binding to the cIAP2 promoter increased significantly in HUVEC treated with Erg siRNA compared to control (Figure 3.8A); however, no enrichment was found at the TNFAIP3 promoter (Figure 3.8B). This suggests that while Erg binds to the promoters of both NF- κ B target genes, the mechanism of repression may not be the same for both genes. Therefore, we examined the promoter of IL-8. IL-8 has been shown to be repressed by Erg in resting HUVEC, through an EBS (Yuan et al., 2009). Investigation of the IL-8 promoter found that as with ICAM-1, Erg binds the IL-8 promoter at an EBS within the NF- κ B binding site (Figure 3.11). Inhibition of Erg resulted in an increased enrichment of NF- κ B p65 binding to the IL-8 promoter compared with control (Figure 3.8C). This confirms that the mechanism for repression found on the ICAM-1 promoter is common amongst a set of NF- κ B target genes.

In summary of this chapter, we propose that Erg represses transcription of a subset of NF- κ B target genes. We have identified a specific subset of NF- κ B target genes that are repressed by Erg in resting EC and suggest a novel mechanism for Erg inhibition of NF- κ B DNA binding and activity.

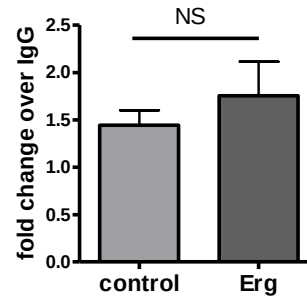
A

p65 ChIP for cIAP2 promoter



B

p65 ChIP for TNFAIP3 promoter



C

p65 ChIP for IL-8 promoter

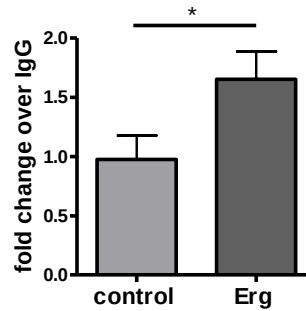


Figure 3.8 ChIP analysis of NF- κ B p65 binding to the promoters of cIAP2, TNFAIP3 and IL8 in the presence or absence of Erg ChIP was carried out on sheared chromatin from confluent resting HUVEC treated with control or Erg siRNA using an anti-NF- κ B p65 or control IgG antibody. Immunoprecipitated DNA was analysed by qPCR for primers covering NF- κ B binding sites in cIAP2 (A), TNFAIP3 (B) and IL-8 (C) and negative control GAPDH promoter region. Data is expressed as fold change compared to IgG normalised to input and control region. N=9, *P<0.05, **P<0.01.

CTCAGCTCAG TCCAATTGCG TGCAGAAATGC ACTGGATGCT GTTCCACAG AACGATGAAG TCTCCAATAA TCTTTATGGG CTCTGTCTCT -940
 GGTGGTAAAT TGTCTTIGAT CTACTGGAAG ATAGAAAATC AGATGAGCTC TCCAAATCCA TCTGTTACCC TGGTACGGAT GGCTCTCAGA -850
 TAAAGTTGCT TAAATTTACT GCTTAGAGAA CGTTGAGGGA AATATGGCAG TGCAATTAGA CTCAAAGATG TCAGGTTTCC CTGAGAATGG -760
 CACTGCAACT CAGAAACCTG CCCTGTGAGA TCAAAGGCTA TAAAGGAGG ATTTTTTTAA GTAGCCAACCT CCACCCTACT TATTAATAAA -670
 AAAAAAAAAA CAACTCACCA AACCAACAA AAGTATTCC ATACAGAAA TTTACCAAGC ATTTCAATCT TTAATAAGT AAAATAAACT -580
 GCAAAGGAGA ACTGCATGAT TTTTTCACA TACCCCTACA TTTCCTTICA CCTCTACTT TCTTGATCAG AACAAAAAGT AAAATAAAAT -490
 AGAAATATTT CACAAAGTTT CGATTTTTTT TTTTAAAT GCTGGACTTC TGCAGCTATA GTAGAAGATT GAAAAACCTA ACCTTTTAC -400
 GTGTAAAGTG TATGGCGGAT GGAGGGTGA GAACAGGGCA TATTGACCTT TTCCAGGCAG GCTAAGCAAT GATCGTCCTC TCTATATGGG -310
 TTGTTATCAA GATTTCCTCT GACCCACGAG CAATGAAGCA AATGTCTTC AGTAAATGCC GCGAAGATAT GCCACGGTTA AGAGTCATGC -220
 TTTTGGGTCA TGGAAATCCC CGAGTGGGT TGCCAGGCCA CTGATTAGA GGAAGTGTGT GTGGTTATTA CCGTGGAGT TCCCTAACT -130
 CCTAAAGGA AAGCACCAGT GCACATGCAA ACCACTGGGA GGAGTGCAGA ACGCCTGGTA CAGATAGGGG TGGGGATTG GGTGACGCAT -40
 TTAAAAGACA GCGTGAGACT CGCGCCCTCC GGCAAGGAAA AAGCCAGGCG ACAGGTGTCG CTTGAAAAGA CTGGGCTTGT CCTTGCTGGT +49
 GCATGCGTCG TCGGCCTCTG GGCAGCAGGT GGGCAAGGAA GGTGGTGTG TGTGTGTGTG TGTGTGCGTG TGTGTGTGTG TATGTGTGCG +139
 CGCGCGCGCG CCTCCCTGG TGTAAGAGAT GTGCCAGCGG CTGGCCGAGG GGCCTTAGG GCTAGAGCCC GGGGCGCTG

Figure 3.9 cIAP2 promoter sequence. Human cIAP2 promoter sequence numbering relative to transcription start site, marked by arrow. Two functional NF- κ B binding sites highlighted by red hatched boxes. Putative EBS in green, and putative ERG binding site circled in red. ChIP PCR forward and reverse primer locations indicated by blue arrows

CTGGGACCTA CGTCTCATGC AGCCTGGGGT CCAGAGCTGG CAGGAACAAT GGAGATGGGG -585
 ACCAAAGGCT GTCACCGCTG CTTTTTCTCG AAATGCCCGC CCGGGTCCTG TCTGGGGCTC -525
 CCGCCTACGC GCTCATCACG TGCACAGCCC AAACCTTTTCA GAGCCGGCCC GAGGCCTGCG -465
 GGCCCGGGCG CCAGGGTGGT TTTTAGGGTT TTCTTTCTTT CTTATTTCCTC TTCTTCTTCT -405
 CCACAGTTGA TGATTTTCTG CAGAAAAACA ACTGCGAAAA AGAACCTATT TCATTTCCAG -345
 TTCCCATCAC CGCGATTTCG ACATGGATGT GACGTGACCC CAGCTTCCGA AATGCCCAGG -285
 TGAATCACGC GGGGACACCC CGGGGCGGGG CGAGGGAGTT TCTCCGGGCG CCTGCAGGGA -225
 CCGGGCGGGG CGGGGCAGCG GGGCGGGGCA GGGAAAGGGG GCGGGGCGGG GCCCGCAGGC -165
 CCGGTCGGGC GGAGGCCGCG CGCGCCCTC GGGGCTGCG CCCTCTGGCG GCCGGCTGGA -105
 CGCACTTCGC AGCCCGACCC AGAGAGTCAC GTGACTTTGG AAAGTCCCGT GGAAATCCCG -45
 GGGCCTACAA CCCGCATACA ACTGAAACGG GGCAAAGCAG ACTGCGCAGT CTGCAGTCTT +16
 CGTGGCGGGC CAAGCGAGCT TGGAGCCCGC GGGGGCGGAG CCGTGAGAGC GGCCGCCAAG +76
 AGAGATCACA CCCCAGCCG ACCCTGCCAG CGAGCGAGCC CGACCCAGG CGTCC**ATG**GGA +136
 GCGTCGCCTC CGCCCGGTCC CTGCCCCGAC CCCC GCCTGC GGCGCGCTCC TGCCTTGACC +196
 AGGACTTGGG ACTTTGCGAA AGGATCGCGG GGCCCGGAGA GGTAACCGCC GCGCCTCCCG +256
 GAGAGGTAAC CGCCGCGCCT CCCGGAGAG

Figure 3.10 TNFAIP3 promoter sequence. Human TNFAIP3 promoter sequence numbering relative to transcription start site, marked by arrow. Translation start site ATG in bold. Two functional NF- κ B binding sites are highlighted by red hatched boxes. Putative EBS in green, and putative ERG binding site circled in red. ChIP PCR forward and reverse primer locations indicated by blue arrows

CTTTGTGCTAG TGATCATGGG TCCTCAGAGG TCAGACTTGG TGTCTTGGA TAAAGAGCAT GAAGCAACAG TGGCTGAACC -1121
 AGAGTTGGAA CCCAGATGCT CTTTCCACTA AGCATACAAC TTTCCATTAG ATAACACCTC CCTCCACCC CAACCAAGCA -1041
 GCTCCAGTGC ACCACTTTCT GGAGCATAAA CATACTTAA CTTTACAAC TGAAGTGCCT TGAATACTCT TCCATCTGG -961
 AATGTGCTGT TCTCTTTCAT CTTCTCTAT TGAAGCCCTC CTATTCCCTCA ATGCCTTGCT CCAACTGCCT TTGGAAAGATT -881
 CTGCTCTTAT GCCTCCACTG GAATTAATGT CTTAGTACCA CTTGTCTATT CTGCTATATA GTCAGTCCTT ACATTGCTTT -701
 CTTCTTCTGA TAGACCAAAC TCTTAAGGA CAAGTACCTA GTCTTATCTA TTTCTAGATC CCCACATTA CTCAGAAAGT -621
 TACTCCATAA ATGTTTGTGG AACTGATTTC TATGTGAAGC ACATGTGCC CTTCACTCTG TTAACATGCA TTAGAAAAGT -541
 AAATCTTTTG AAAAGTTGTA GTATGCCCCC TAAGAGCAGT -ACAGAAACT AAGATTCCCT AGAAACTCTC TAAAATGCTT -461
 AGAAAAAGAT TTATTTTAAA TTACCTCCCC AATAAAATGA TTGGCTGGCT TATCTTCACC ATCATGATAG CATCTGTAAT -381
 TAACTGAAAA AAAATAATTA TGCCATTAAA AGAAAATCAT CCATGATCTT GTTCTAACAC CTGCCACTCT AGTACTATAT -301
 CTGTCACATG GTACTATGAT AAAGTTATCT AGAAATAAAA AAGCATACAA TTGATAATTC ACCAAATTGT GGAGCTTCAG -221
 TATTTTAAAT GTATATTAAA ATTAAATTAT TTAAAGATC AAAGAAAAGT TTCGTCATAC TCCGTATTTG ATAAGGAAACA -141
 AATAGGAAAT GTGATGACTC AGGTTTGCCC TGAGGGGATG GGCCATCAGT TGCAAATCGT GGAATTTCTT CTGACATAAT -61
 GAAAAGATGA GGGTGCATAA GTTCTCTAGT AGGGTGATGA TATAAAAAGC CACCGGAGCA CTCCATAAGG CACAAACTTT +20
 CAGAGACAGC AGAGCACACA AGCTTCTAGG ACAAGAGCCA GGAAAGAAACC ACCGGAAAGGA ACCATCTCAC TGTGTGTAAA +100
 CATGACTTCC AAGCTGGCCG

Figure 3.11 IL8 promoter sequence. The Human IL8 promoter sequence with numbering relative to transcription start site, which is marked by arrow. Translation start site ATG in bold. Functional NF- κ B binding site highlighted by red hatched boxes. Putative EBS in green and putative ERG binding site circled in red. Functional Erg binding site located at base pair -77 located within NF- κ B site. ChIP PCR primers sites indicated by blue arrows.

3.4. Discussion

Erg expression in EC is inhibited by TNF- α and LPS treatment, two stimuli important in the transactivation of pro-inflammatory genes. We have previously shown that Erg inhibition in resting EC in the absence of any pro-inflammatory stimuli results in the increased expression of pro-inflammatory genes including ICAM-1, VCAM-1, and IL8 (Sperone et al., 2011). The functional importance of this is shown by the increased leukocyte adhesion in Erg depleted HUVEC in the absence of any other inflammatory stimuli (Yuan et al., 2009). The importance of Erg in maintaining EC homeostasis is also demonstrated in the expression pattern of Erg in the endothelium of human coronary arteries containing eccentric atherosclerotic plaques. We have previously shown that while Erg is expressed in healthy endothelium, it is absent from EC over the shoulder region of the plaque, a site of active leukocyte recruitment where EC are activated, suggesting Erg is required to inhibit leukocyte recruitment *in vivo* (Sperone et al., 2011). Leukocyte adhesion requires the co-operation of multiple adhesion molecules and cytokines, including ICAM-1 and IL-8. Therefore it is possible that constitutive Erg expression maintains a homeostatic and quiescent endothelium by inhibiting the basal expression of NF- κ B target genes to suppress inflammation.

In the current chapter we have shown that Erg represses ICAM-1 through inhibition of NF- κ B p65 binding to DNA. We then identified a correlation between genes repressed by Erg and genes transactivated by the NF- κ B regulating cytokine TNF- α in both EC and pancreatic cancer cells. Moreover, we showed a trend towards correlation between genes repressed by Erg and genes bound by NF- κ B p65 in macrophages. This correlation was validated with the analysis of Erg-mediated repression and Erg binding to the cIAP2 and TNFAIP3. These results confirmed that while Erg does not repress all NF- κ B target genes, there is a strong correlation between genes repressed by Erg and genes bound and transactivated by NF- κ B. In agreement with the mechanism of Erg inhibiting NF- κ B p65 binding to the ICAM-1 promoter, Erg also inhibits the binding of NF- κ B p65 to the promoters of cIAP2 and IL-8 in resting EC. However it does not inhibit the binding of NF- κ B p65 to the TNFAIP3 promoter. This suggests Erg represses TNFAIP3 using an alternative mechanism.

IAPs were initially identified as inhibitors of caspase activity; however recent evidence suggests that cIAP1, cIAP2, and XIAP facilitate ubiquitin-dependent signalling activated by acting as ubiquitin E3 ligase to mediate activation of NF- κ B. cIAP2 forms part of the TNFR1 signalling complex and ubiquitylates members of this complex which results in a signalling

cascade to induce acetylation of the IKKs and phosphorylation of I κ B (Mahoney et al., 2008). cIAP2 expression is up-regulated by TNF- α treatment, and this was shown to be via NF- κ B p65 binding to two NF- κ B binding sites (Hong et al., 2000). In resting EC there is a low level of cIAP2 expression.

TNFAIP3 also known as A20 is an inhibitor of apoptosis, protecting cells from TNF- α induced cytotoxicity and is also an inhibitor of inflammation. The expression of TNFAIP3 is triggered by inflammatory stimuli which induce binding of NF- κ B to two NF- κ B sites within the promoter (Krikos et al., 1992). The role and mechanism of TNFAIP3 actions are cell and stimulus specific. TNFAIP3 inhibits apoptosis through blocking the cleavage of apical caspases 8 and 2, executioner caspases 3 and 6, bid cleavage, and release of cytochrome-c, possibly through acting as a deubiquitinase (Krikos et al., 1992). TNFAIP3 expression is only transiently induced by pro-inflammatory stimuli; TNFAIP3 represses NF- κ B in an auto-regulatory manner which in turn inhibits TNFAIP3 transactivation. TNFAIP3 inhibits NF- κ B activity through inhibiting the activation of the IKK complex which leads to the re-synthesis of I κ B α . TNF- α induced endogenous TNFAIP3 has been shown to bind the TNFR1 complex. Again TNFAIP3 is thought to inhibit IKK activity by acting as a deubiquitinase; however the exact mechanism has not been identified. The role of TNFAIP3 is cell and stimuli specific; for example TNFAIP3 inhibits TNF- α induced apoptosis in EC, but not in HeLa Cells (Zhang et al., 2002).

We and others have shown the presence of NF- κ B p65 in the nucleus of resting cells (Sperone et al., 2011; Collard et al., 1999); however no role for nuclear localised constitutive NF- κ B p65 has been identified. The activity of NF- κ B family members is implicated in the basal expression of ICAM-2 (McLaughlin et al., 1999) and P-selectin (Pan and McEver, 1995) in EC although NF- κ B p65 was not found to play a role in basal P-selectin. The role of NF- κ B p65 in quiescent EC needs to be investigated further, possibly through the use of p65 siRNA.

Recently a distinct role for NF- κ B p50 homodimers, which do not have transactivating domains, as homeostatic repressors in macrophages was described. NF- κ B p50 homodimers were shown to bind to a subset of interferon response elements (IRE) characterised by their guanine-rich central domain. NF- κ B p50 homodimers were shown to act as gatekeepers by binding to these IRE and blocking the inappropriate expression of interferon regulatory factor (IRF) target genes exposed to low but detectable levels of IRF3 (Cheng et al., 2011). We

propose that Erg may act as a gatekeeper by providing a checkpoint to pass before induction of NF- κ B target genes occurs in EC.

NF- κ B activity is repressed by multiple factors. The most characterised repressive mechanism is that of the I κ B family of proteins which bind NF- κ B and sequester it in the cytoplasm. Each I κ B family member has different specificities and kinetics of regulation. The cytoplasmic localization of NF- κ B by I κ B α is a dynamic process, with I κ B α -NF- κ B complexes shuttling between the nucleus and the cytoplasm (Birbach et al., 2002). This is thought to be because I κ B- α only masks one of the NF- κ B dimers' nuclear localisation signals (NLS). This constant shuttling is demonstrated through inhibition of nuclear export in resting cells which results in nuclear localisation of NF- κ B and I κ B α (Birbach et al., 2002). This is in contrast to I κ B β and δ which can mask both NLS within an NF- κ B dimer (Tam et al., 2001). NF- κ B activity is also repressed by other transcription factors. The transcription factor Homeobox protein A9 (HOXA9), like Erg, is down regulated after TNF- α stimulation. HOXA9 has been found to inhibit NF- κ B activity by blocking DNA binding, however this was not through HOXA9 binding to DNA. (Trivedi et al., 2008). Kruppel-like factor (KLF)-4 also repressed NF- κ B activity however it does not inhibit nuclear translocation or DNA binding, but through an unknown mechanism affects transactivation of the promoters of target genes (Hamik et al., 2007). In addition, ligand bound-nuclear receptors such as the glucocorticoid receptor (GR) inhibit NF- κ B activity by binding to p65 and repressing activity in the nucleus through recruitment of repressive co-factors (Ito et al., 2006), or inhibiting DNA binding. To our knowledge Erg is the first transcription factor identified that inhibits NF- κ B activity by binding to the promoters of NF- κ B target genes and blocking DNA binding of NF- κ B p65.

In this study we showed that two EBS were required for Erg-mediated repression of ICAM-1 expression; one EBS within the NF- κ B motif, and another EBS at -118 bp relative to the transcription start site. In the ICAM-1 promoter, these two EBS are 69 bp apart, a distance equivalent to approximately half a nucleosome. The proximity of these two sites may be important to allow Erg-mediated repression through modification of the chromatin structure of the ICAM-1 promoter. Analysis of the promoter sequences of other Erg repressed NF- κ B target genes from the GSEA, also identified putative Erg binding sites within a distance equivalent to 1-2 nucleosomes from an NF- κ B site. We suggest that the ETS:NF- κ B motif and its interaction with other proximal EBS may play an important part in the repressive mechanism. Recently BCL6 was shown to repress a subset of NF- κ B target genes in

macrophages by binding the promoters at a distance equivalent to approximately one nucleosome or 200 bp away from NF- κ B binding sites (Barish et al., 2010). In the absence of inflammatory stimuli, BCL6 recruited HDAC3 to promoters of NF- κ B target genes inhibiting binding of NF- κ B and repressing transcription. Erg may similarly repress NF- κ B p65 binding by recruiting co-repressors to the promoters of NF- κ B target genes. Erg interacts with the Histone-3 lysine-9 specific-methyltransferase ESET, and ESET binds the co-repressors HDAC-1 and 2, and mSin3A and B (Yang et al., 2002; Yang et al., 2003a). Whether Erg represses NF- κ B target genes through recruiting these or other co-repressors is being investigated and will be discussed in Chapter Four.

In addition to the ETS:NF- κ B motif described here, there are a number of other examples where ETS transcription factors regulate specific target genes through combinatorial promoter motifs and interaction with other transcription factors. The enhancers of many endothelial specific genes contain Forkhead (FOX):ETS motifs and are synergistically activated by FOX and ETS transcription factors (De Val et al., 2008). Additionally, analysis of the genome-wide binding sites of ten key regulators of blood stem/progenitor cells identified a combinatorial interaction between a heptad of transcription factors including Erg (Wilson et al., 2010). Other ETS combinatorial transcription factor motifs include the serum response elements, which bind ternary complex ETS factors and the serum response factor (Buchwalter et al., 2004), and ETS:AP1 binding motifs (Wasylyk et al., 1998). Our data highlights for the first time a mechanism of combinatorial repression involving Erg and NF- κ B, controlling basal repression of ICAM-1 and other pro-inflammatory endothelial genes.

In EC, Erg expression is down regulated by inflammatory stimuli (McLaughlin et al., 1999; Yuan et al., 2009); this is in contrast with other ETS factors including Ets-1, Ets-2 and ESE-1, whose expression is increased after treatment with factors such as IL-1 β TNF- α , angiotensin II or thrombin (McLaughlin et al., 1999; Zhang et al., 2008; Goetze et al., 2001; Hultgardh-Nilsson et al., 1996; Cheng et al., 2011; Redlich et al., 2001; Rudders et al., 2001; Grall et al., 2003). These ETS factors have been shown to drive the expression of pro-inflammatory genes such as VCAM-1, NOS2, cyclooxygenase-2 (COX-2), and MCP-1. In Chapter Two of this study we showed that Ets-2 and Fli-1, although constitutively expressed in EC, do not repress ICAM-1 expression. The contrast in expression levels and activities highlights the important role of ETS factors in regulating inflammation, and the opposing role of Erg as a repressor of inflammatory pathways in EC. Additionally the role of Erg as a repressor of NF- κ B is in contrast to other ETS factors. ETS factors have previously been

shown to act synergistically with NF- κ B in inflammatory gene expression. The occurrence of overlapping ETS and NF- κ B binding sites have been identified in the regulatory regions of inflammatory genes such as IL-3, IL-12, IL-2, IL-2 receptor α and granulocyte-macrophage colony stimulating factor (GM-CSF) (Gottschalk et al., 1993; Thomas et al., 1997; Gri et al., 1998; John et al., 1995). Inhibition of the synergy between ETS factors and NF- κ B has been targeted for the development of therapeutics. Chimeric decoy oligonucleotides containing EBS and NF- κ B consensus sequences in combination were able to inhibit inflammation in abdominal aortic aneurysms to a greater level than either site alone, and this involved inhibition of ICAM-1, VCAM-1 and CCL2 levels (Shiraya et al., 2006).

GSEA analysis is a powerful technique for the identification of correlation between NF- κ B and Erg target genes; however, there are limitations in identifying all genes repressed. For example we have previously shown that Erg represses VCAM-1 (Sperone et al., 2011), and VCAM-1 was identified in our Erg microarray data, and correlated with the gene set from EC treated with TNF- α , however it was not identified in the other correlations because VCAM-1 is not expressed in pancreatic cancer cells or macrophages. This cell specificity may have been the reason we could not find a significant correlation between genes regulated by Erg in HUVEC and those bound by NF- κ B p65 in macrophages, and this is reflected in the higher NES between Erg microarray data and TNF- α treated EC compared to pancreatic cancer cells; therefore cell type specificity should be considered when analysing this data.

In addition to the gene regulation by TNF- α in various EC, Sana *et al* (Sana et al., 2005) also studied genes regulated by IFN- γ and IL4. Of the three stimuli, only those genes regulated by TNF- α - treatment correlated with our data. This suggests that IFN- γ and IL4 do not regulate the same genes as Erg. In previous studies IFN- γ has been shown to regulate an ICAM-1 promoter construct in EC through the activity of STAT-1, while in Chapter Three we showed that IFN- γ regulates the ICAM-1 promoter in HUVEC. However Sana and others have shown IFN- γ does not transactivate endogenous ICAM-1 expression in EC (Look et al., 1994; Sana et al., 2005). This suggests a different mechanism of transactivation of the ICAM-1 promoter construct by IFN- γ in HUVEC, than the endogenous ICAM-1 promoter, and highlights the limitations in promoter construct studies, and the importance of examining endogenous gene regulation of native chromatin in specific cells.

While previous studies have identified a link between genes repressed by Erg and genes transactivated by TNF- α in HUVEC (Yuan et al., 2009), many of the genes previously

suggested to be regulated by Erg and TNF- α were not identified in our microarray or GSEA analysis. Only IL-8 and IL-7R were also found to be regulated in our GSEA analysis. Similarly, previous studies did not identify a number of well known NF- κ B target genes which we have found were also regulated by Erg, including TNFAIP3 and cIAP2. We investigated whether there was a correlation to one extreme of the ranked Erg microarray data. In contrast, Yuan et al (Yuan et al., 2009) investigated whether there were any genes differentially regulated, both activated and repressed, between the two studies and pooled all the results together. This would be appropriate if the mechanisms of Erg-mediated transactivation and repression were the same, but our data suggests this is not the case. For example, ICAM-1 is repressed by Erg directly through DNA binding and inhibition of NF- κ B DNA binding. In contrast VWF is transactivated by Erg, but not repressed by NF- κ B binding to the VWF promoter; the decreases in VWF levels after TNF- α treatment is likely to be a consequence of a depletion of Erg levels by TNF- α treatment (McLaughlin et al., 1999).

Previously our group has shown that Erg over-expression in EC does not inhibit TNF- α -induced NF- κ B p65 binding to an oligonucleotide containing the ICAM-1 promoter NF- κ B binding site (Sperone et al., 2011). This is similar to our observations from EMSA analysis in Chapter Two, which shows NF- κ B p65 from resting EC is able to bind an oligonucleotide containing the ICAM-1 NF- κ B binding site. However we found in this chapter that NF- κ B p65 could not bind the endogenous ICAM-1 promoter in the context of native chromatin in resting HUVEC. Therefore there is a possibility that over-expressed Erg may also inhibit TNF- α -induced NF- κ B binding to the endogenous ICAM-1 promoter in EC and the repression may also be caused by competition for promoter sites.

Another potential mechanism of Erg-mediated basal repression which has been identified in previous studies from our group is through Erg-mediated post-translational modification of NF- κ B. Erg represses TNF- α -induced phosphorylation of NF- κ B p65 on serine residue-536 (Sperone et al., 2011). This post-translational modification of p65 is required for transcriptional activity, has been shown to increase NF- κ B binding to p300, and lowers the affinity for I κ B α . As the level of NF- κ B serine-536-phosphorylation in resting EC was too low to detect, Erg repression of basal ICAM-1 via this mechanism in resting cells was not found (Sperone et al., 2011). However there is a possibility that Erg may also repress pro-inflammatory gene expression in basal conditions via post-translational modifications of NF- κ B p65. Our group also found that AdErg over-expression enhanced the re-synthesis of the NF- κ B inhibitor I κ B α after TNF- α treatment (Sperone et al., 2011). While these repressive

mechanisms were all detected in HUVEC treated with TNF- α , they may also play a role in the basal repression of ICAM-1.

The GSEA overlap analysis also showed that Erg-regulated inflammatory genes, including ICAM-1, were also regulated by two co-repressors, BMI1 a member of the polycomb family of transcriptional repressors, and HDAC3 a histone deacetylase that has been shown to have a role in inhibition of inflammation (Ghisletti et al., 2009; Barish et al., 2010). Erg may co-operate with these co-repressors possibly by recruiting them to the promoters of Erg-repressed genes. The role of co-repressors in Erg-mediated repression will be explored in Chapter Four.

In summary, in the current chapter we have shown a correlation between the genes repressed by Erg and genes transactivated by NF- κ B. We have shown that Erg represses the basal activity of constitutive low levels of nuclear NF- κ B p65, and that Erg inhibits p65 binding to promoters of pro-inflammatory target genes in quiescent EC. We suggest that Erg maintains EC homeostasis by acting as a gatekeeper to by repress low levels of nuclear localised NF- κ B which would otherwise induce pro-inflammatory molecule expression, and leukocyte recruitment. Thus Erg provides an important barrier to protect against inappropriate EC activation, which if left unchecked, could result in chronic inflammatory diseases such as atherosclerosis.

Chapter Four

Erg represses ICAM-1 by recruiting co-repressors

4.1. Introduction

4.1.1. Epigenetic regulation of inflammation.

As discussed in Chapter One epigenetics is the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states (Bird, 2007). The understanding of epigenetic mechanisms and in particular the acetylation and deacetylation of histone molecules in inflammation is a growing field of investigation. In general acetylation of histone molecules induces a transcriptionally active state, and conversely deacetylation of histones induces a transcriptionally repressed state (Kouzarides, 2007). Histone methylation is able to induce an active or a repressed state depending on the specific residue on the histone tail that is affected (Bannister and Kouzarides, 2005).

4.1.2. Erg and epigenetics

Previous investigations have shown that Erg associates with epigenetic co-factors (Yang et al., 2002). A yeast two-hybrid screen using the N-terminus of Erg as bait identified the interaction between Erg and the histone methyltransferase which was named Erg associated protein with a Set domain (ESET). ESET is a Histone 3 lysine 9 (H3K9) specific trimethyltransferase, and methylation of H3K9 is a modification associated with repression of transcription. ESET also binds to HDAC1 and HDAC2 and the scaffolding proteins mSin3A and mSin3B (Yang et al., 2003a). These proteins form a repressive complex. As Erg binds to ESET, it is possible that the repression of inflammatory genes by Erg is induced by the recruitment of ESET and the other co-repressors to the promoters of these genes.

The role of epigenetic co-factors in the regulation of inflammation is complicated due to HDACs deacetylating not only histone molecules but other proteins including transcription factors, a process which has been demonstrated in a number of studies (Ito et al., 2000; Ashburner et al., 2001; Chen et al., 2005; Zhong et al., 2002).

4.1.3. Erg repression of NF- κ B activity may involve histone modification

In the previous chapter we showed that Erg represses a subset of target genes in resting HUVEC. We also showed that the presence of Erg inhibited NF- κ B p65 binding to the ICAM-1 promoter. The nuclei of resting EC contain low but detectable levels of NF- κ B p65 (Sperone et al., 2011; Collard et al., 1999), and expression of pro-inflammatory genes is low.

We have shown that the depletion of Erg is sufficient to allow NF- κ B-dependent transactivation of pro-inflammatory genes. Erg represses ICAM-1 through binding to the promoter of ICAM-1 in resting EC and inhibiting the binding of NF- κ B. This inhibition of binding involved two ETS sites 68 bp apart, a distance of approximately half a nucleosome. Transcription factors binding to these sites may recruit chromatin modifying co-factors which alter the structure of DNA to make it inaccessible to transcriptional machinery. This may involve the recruitment of HDAC, or HMT which respectively deacetylate or methylate histones within nucleosomes causing them to form a closed structure. Erg may regulate the activity of NF- κ B target genes in an epigenetic manner by binding their promoters and altering the chromatin conformation to an inaccessible state. ETS factor mediated recruitment of co-factors has been shown previously. The ETS factor Tel represses pro-inflammatory genes in macrophages by recruitment of co-repressors. Tel binds to the promoters of pro-inflammatory genes such as CCL2 and IL-12b and recruits a repressive complex containing the co-repressor SMRT which binds mSin3A and HDAC1 and 2 (Chakrabarti and Nucifora, 1999; Ghisletti et al., 2009).

4.1.4. Epigenetic regulation of NF- κ B activity

Epigenetic mechanisms also have an important role in the regulation of inflammation. NF- κ B target genes triggered by the same pro-inflammatory stimuli show different kinetics, resulting, for example, in immediate expression of cytokines, and late gene expression of adhesion molecules. ChIP analysis in murine macrophages has shown that exposure to pro-inflammatory stimuli resulted in different rates of NF- κ B p65 binding to promoters of target genes and this rate was dependent on histone H4 acetylation (Saccani et al., 2001). In resting murine cells, genes such as MnSOD, that were associated with H4Ac were immediately bound by NF- κ B p65 upon LPS stimulation, while in genes that lacked basal association with H4Ac, such as IL-6, NF- κ B p65 binding was delayed and required H4Ac induced by pro-inflammatory stimuli. The lack of constitutive H4Ac associated with these late genes suggest they may be actively repressed by co-repressor recruitment, and Erg may be responsible for this repression in EC.

The above data suggests that transactivation of some pro-inflammatory genes first requires de-repression through loss of co-repressors. An example of this mechanism is the activity of BCL-6 in the repression of inflammatory genes in macrophages. Chromatin immunoprecipitation sequencing (ChIP-seq) has been used to investigate the NF- κ B cistrome

in macrophages treated with LPS. There are approximately 31,000 binding sites for NF- κ B p65 in LPS treated macrophages. In quiescent macrophages the transcriptional repressor BCL-6 binds to a subset of these sites within 200 bp or approximately one nucleosome. Like Erg-mediated repression in EC, BCL-6 represses the basal expression of pro-inflammatory genes in macrophages. ChIP-seq data showed that HDAC3 was also associated with the areas of the genome bound by BCL6 and NF- κ B in a BCL-6 dependent manner. It was suggested that HDAC3 is recruited by BCL6 to these sites to confer a repressed chromatin conformation (Barish et al., 2010).

Another example of de-repression of NF- κ B target genes was found in the prostate cancer cell line, DU145. The promoters of NF- κ B target genes, cIAP2 and IL-8, were bound by the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and HDAC3, which were associated with repressive NF- κ B p50 homodimers. Stimulation by laminin was associated with loss of SMRT and HDAC3 on these promoters and an increase in NF- κ B p65 binding (Hoberg et al., 2006).

Finally, the nuclear receptor co-repressor (NCoR) has been shown to be involved in epigenetic repression of pro-inflammatory genes. NCoR binds to a number of pro-inflammatory gene promoters in resting HEK293 cells (Baek et al., 2002). NCoR in turn binds TGF- β -activated kinase 1 (TAB2) which binds HDAC3 and this complex mediates repression of IL1 β target genes such as KAI1. Stimulation with IL-1 β results in a decrease in NCoR, TAB2, and HDAC3 binding, and an increase in NF- κ B p50 binding and transcriptional activation (Baek et al., 2002).

The ability of transcription factors to bind DNA may be dependent on nucleosomal structure surrounding binding sites. Some transcription factors can bind to DNA when wrapped around a nucleosome while others require the opening up of DNA around binding sites before they bind. It has been deduced from crystal structure analysis that NF- κ B does not bind to DNA packaged in nucleosomes (Natoli et al., 2005), possibly because too little space is left on DNA after an NF- κ B dimer has bound for the DNA to be packaged into a nucleosome. Therefore if the promoter of an NF- κ B target gene is wrapped around a nucleosome, steric hindrance should block NF- κ B binding. In contrast ETS factors have been shown to be able to bind to DNA packaged in nucleosomes (Lu et al., 2004). This supports our model in which Erg binds to nucleosomal DNA and represses transcription by recruiting repressive co-factors and inhibiting NF- κ B p65 binding.

These studies all show that basal repression of NF- κ B-mediated transcription involves the recruitment of epigenetic co-repressors including HDACs to promoters of target genes. We suggest that Erg may also repress NF- κ B activity by recruiting epigenetic co-repressors to the promoters of pro-inflammatory genes. In this chapter we investigated the epigenetic regulatory mechanisms associated with the repression of ICAM-1 by Erg. As Erg has been shown to bind to the co-repressor ESET, and ESET in turn binds to HDAC1, HDAC2, mSin3A and mSin3B, we investigate whether these co-repressors are present at the ICAM-1 promoter in resting HUVEC. In addition we investigate the role of histone deacetylases in the repression of ICAM-1 by Erg, by attempting to inhibit the Erg-mediated repression using an HDAC inhibitor, trichostatin A (TSA). Finally we utilise *in silico* regulation data held on the ENCODE database to investigate the difference between genes repressed and activated by Erg.

4.1.5. Aims

- To investigate the role of epigenetic mechanism in Erg-mediated repression.
- To investigate whether the histone methyltransferase ESET is involved in Erg-mediated repression.
- To study the role of HDAC in Erg mediated repression.
- To investigate genome wide *in silico* data associated with Erg target genes.

4.2. Methods

4.2.1. Antibodies

Antibodies used in this chapter include: Polyclonal antibodies Erg-1/2/3 sc-353 (Santa Cruz Biotechnology), Histone H3 Trimethyl Lys9 Rabbit 39161 (Active motif), ESET sc-66884 (Santa Cruz Biotechnology), HDAC1 ca 06-720 (Millipore), HDAC2 sc6296 (Santa Cruz Biotechnology), mSin3A sc-768 (Santa Cruz Biotechnology), mSin3B sc-994 (Santa Cruz Biotechnology), IgG isotype control rabbit IgG PP64 (Millipore).

4.2.2. Cell culture

Cell Culture was carried out as previously described in Chapter 2.2.3

4.2.3. Adenovirus transduction

Adenovirus transduction was carried out as described in Chapter 2.2.7 with the exception that HUVEC were transduced with 100 MOI AdIkB α SR and AdLacZ for 24 hours.

4.2.4. siRNA transfection

HUVEC were transfected with control and Erg siRNA as previously described in Chapter 2.2.5.

4.2.5. ChIP

Chromatin was immunoprecipitated using the ChIP-ITTM express kit (Active Motif), as described in Chapter 2.2.15 with the exception that the following antibodies were used anti-ESET, anti-HDAC1, anti-HDAC2, and Anti- H3K9Me3 and IgG Rabbit isotype control.

4.2.6. Inhibition of HDACs with Trichostatin A

HUVEC were transduced with AdErg, AdLacZ or untreated (See Chapter 2.2.7). After 42 hours of incubation 100 ng/ml of trichostatin A (TSA) (Sigma) was added to each well of a 6 well plate for 6 hours. Total RNA was extracted as described in Chapter 2.2.8.

4.2.7. Analysis of in silico data associated with Erg target genes

Regulatory features of ICAM-1 and VE-Cadherin were analysed using the Ensembl server www.ensembl.org.

4.3. Results

4.3.1. Members of the ESET repressive complex are expressed endogenously in HUVEC

As shown before, Erg represses NF- κ B p65 binding to DNA in native chromatin (Figure 3.4), but while Erg binds to an oligonucleotide containing EBS181 and an NF- κ B site, p65 is also binds this oligonucleotide (Figure 2.14); this suggests that repression may be dependent on a mechanism not identifiable by EMSA, Erg may mediate modification of the chromatin structure around the promoter of Erg repressed genes, to inhibit NF- κ B p65 binding. Since Erg was shown to interact with ESET we first investigated whether the components of the ESET repressive complex were present in HUVEC. These proteins are reported to be ubiquitously expressed (Dodge et al., 2004); to confirm their expression in HUVEC we carried out a western blotting analysis. Figure 4.1 shows representative images from western blots using antibodies to Erg, ESET, HDAC1, HDAC2, mSin3A and mSin3B. These indicate that each of these co-factors is expressed in resting endothelial cells in culture. Therefore there is a possibility that endogenous Erg binds to these co-factors in endothelial cells and recruits them to the ICAM-1 promoter for transcriptional repression.

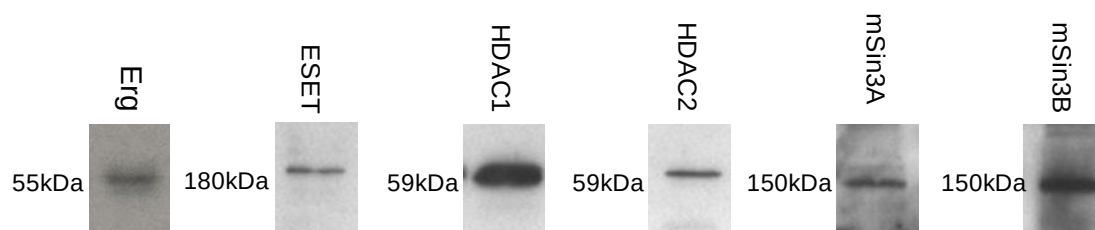


Figure 4.1. Repressive co-factors are expressed in HUVEC under basal conditions. Total protein lysates of HUVEC were separated by SDS-PAGE and analysed by western blotting for each co-factor and Erg.

4.3.2. The co-repressor ESET is associated with the ICAM-1 promoter in resting HUVEC

We next decided to investigate whether ESET was bound to the ICAM-1 promoter in the same region as Erg using ChIP. Initially this was carried out using standard PCR with primers covering ICAM-1 proximal promoter region (Figure 2.4), and PCR products were detected using ethidium bromide gel electrophoresis. Enrichment for ICAM-1 promoter using an anti-ESET antibody was greater than enrichment with an IgG control antibody and enrichment in a non-specific control region was similar between IgG and ESET (Fig 4.2A). This suggests that ESET binds to the ICAM-1 promoter in resting cells in the same region as Erg.

In order to investigate whether ESET is enriched on the ICAM-1 promoter at R4, we carried out quantitative PCR using primers covering R1-5 (Figure 2.6 A). While there was significant enrichment at regions 1 and 2, the greatest significant enrichment was found at R4 (Fig 4.2B). This suggests that ESET is bound to the ICAM-1 promoter in the same region as Erg.

4.3.3. The presence of ESET at the ICAM-1 promoter is not dependent on Erg

ESET contains a domain capable of binding methylated CpG islands, which are a mark of heterochromatin (Yang et al., 2002); however as the ICAM-1 proximal promoter region does not contain methylated CpG islands (Wawryk et al., 1991), this suggests that ESET may be binding to the ICAM-1 promoter through interaction with a DNA binding factor. We investigated whether the presence of ESET at the ICAM-1 promoter is dependent on Erg, by inhibiting Erg expression using siRNA and studying ESET DNA binding via ChIP. In HUVEC treated with control siRNA, ESET was enriched on the ICAM-1 promoter at levels similar to those seen in the untreated sample. In HUVEC treated with Erg siRNA, ESET was still significantly enriched at the ICAM-1 promoter (Figure 4.2C). In addition, there was no significant difference between the ICAM-1 promoter enrichment for ESET in the Erg siRNA treated compared to control siRNA treated samples. This suggests that the presence of ESET at the ICAM-1 promoter in resting EC may not depend exclusively on Erg. Therefore ESET binding to the ICAM-1 promoter is not Erg dependent.

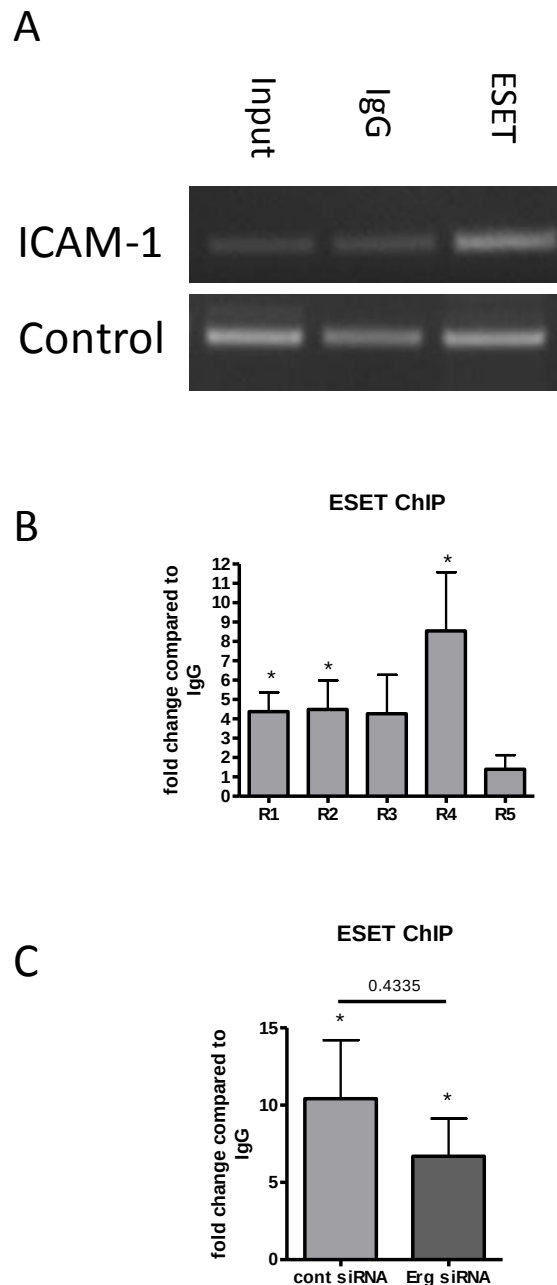


Figure 4.2 ESET is associated with the ICAM-1 promoter. Chromatin immunoprecipitation was carried out on confluent HUVEC monolayer using an anti-ESET antibody or IgG control. A) Enriched chromatin was amplified by PCR for ICAM-1 promoter or control region and input and enriched samples were analysed on an ethidium bromide gel. Image is representative of 3 separate experiments. B) Enriched chromatin was analysed by quantitative PCR using primers to R1-5 and control, data is expressed as fold change compared to IgG normalised to input and the control region. N=3. * P=<0.05. C) ESET ChIP was carried out on HUVEC treated with control or Erg siRNA using anti ESET antibody or IgG control. Enriched chromatin was analysed by q-PCR using primers to R4 and the control region. Data expressed as fold change compared to IgG, normalised to input and control region. N=5.* P=<0.05

4.3.4. Erg-mediated repression of ICAM-1 involves histone deacetylases

HDAC are recruited to DNA through their interaction with other proteins. Their effect can be modulated through the use of HDAC inhibitors. Compounds that act as HDAC inhibitors interact in a similar manner to the acetyl lysine substrate of HDAC, and chelate the metal ion within the HDAC active site. This leaves the HDAC active site blocked and unable to deacetylate lysine residues (Bieliauskas and Pflum, 2008). To find out whether HDAC are involved in Erg-mediated repression of ICAM-1 we used the HDAC inhibitor Trichostatin-A (TSA). HUVEC were transduced with AdErg for 42 hours then treated with TSA for 6 hours before Erg and ICAM-1 mRNA levels were measured by qRT-PCR. As expected, transduction of AdErg resulted in a significant 72-fold induction in Erg mRNA compared to untreated cells, while AdLacZ transduction did not affect Erg mRNA expression (Figure 4.3A). Treatment of HUVEC with TSA did not affect basal Erg mRNA levels, and TSA treatment did not significantly modify Erg mRNA levels in HUVEC transduced with AdErg or AdLacZ. As shown before, AdErg transduction resulted in a significant repression of ICAM-1 mRNA levels, while there was no significant effect of AdLacZ transduction (Figure 4.3B). TSA-induced a significant increase in ICAM-1 mRNA levels in un-transduced HUVEC, suggesting some basal repression is released by treatment with TSA. In addition, TSA treatment of AdErg-transduced HUVEC resulted in a significant increase in ICAM-1 mRNA levels, back to baseline levels, compared to no TSA treatment. These data indicate that Erg-mediated repression of ICAM-1 is inhibited by the HDAC inhibitor TSA, and suggest HDACs are involved in the mechanism of Erg-mediated repression.

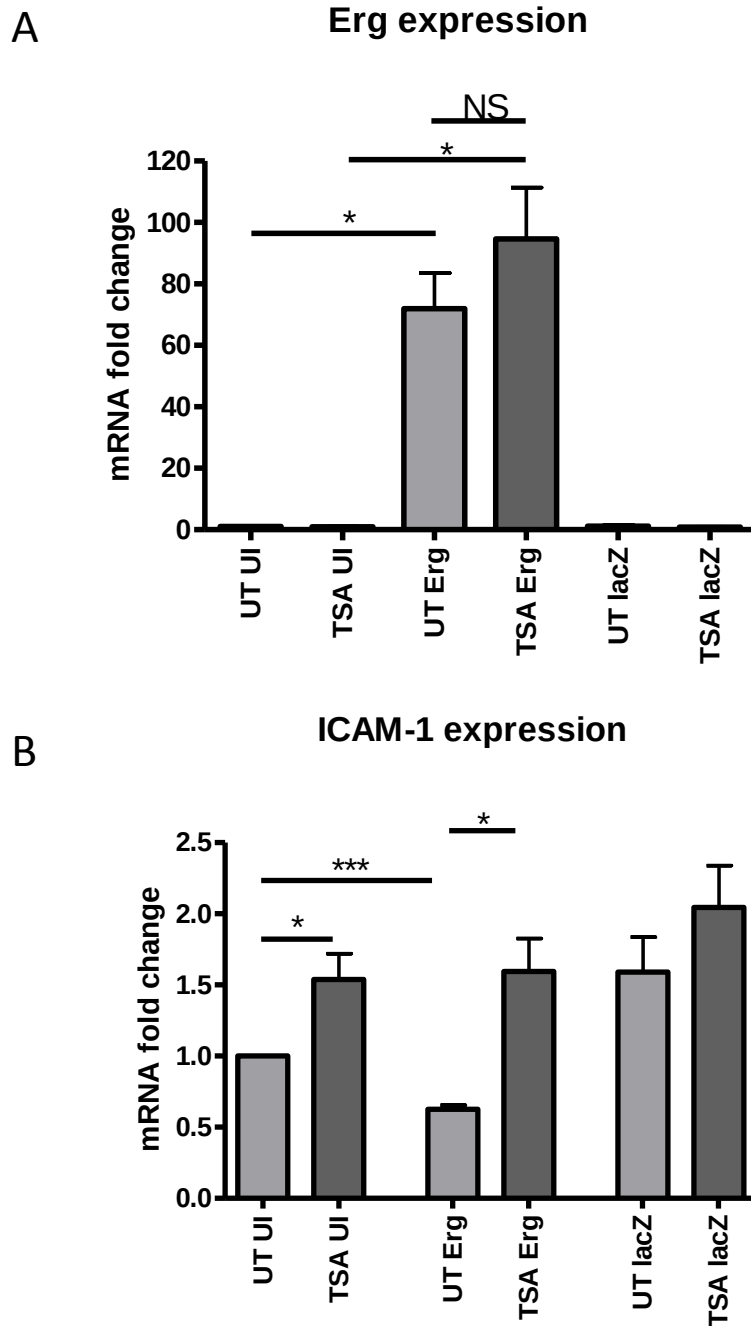


Figure 4.3 Erg mediated repression of ICAM-1 is inhibited by trichostatin A (TSA). HUVEC were transduced with AdErg or AdLacZ for 42 hours before treatment with TSA for 6 hours. mRNA was analysed by quantitative RTPCR. A) Erg mRNA expression B) ICAM-1 mRNA expression. Data is normalised to GAPDH and expressed as relative to untreated and uninfected. NS = not significant. N=3 *P<0.05, *** P<0.001

4.3.5. HDAC1 is associated with the ICAM-1 promoter in quiescent HUVEC

As TSA inhibits the activity of HDAC we decided to investigate whether HDAC bound to the ICAM-1 promoter in the same region as Erg. Because HDAC1 is part of the ESET repressive complex, we investigated by ChIP whether HDAC1 bound to the ICAM-1 promoter. We initially carried out ChIP on sheared chromatin from a confluent HUVEC monolayer using anti-HDAC1 and an IgG control antibody. Enriched chromatin was amplified using PCR primers for ICAM-1 proximal promoter (Figure 2.4) and PCR products were detected by ethidium bromide gel electrophoresis. Enrichment with an anti-HDAC1 antibody produced a band with a greater intensity than that produced by enrichment with the IgG control antibody, indicating HDAC1 binds to the ICAM-1 promoter (Figure 4.4A). To ensure this was due to specific enrichment and not due to a higher amount of non-specific chromatin in this sample, immunoprecipitated chromatin was also amplified using PCR primers for GAPDH. The chromatin enriched with HDAC1 antibody produced a band with slightly greater intensity than the chromatin enriched with IgG, suggesting the enrichment shown with the ICAM-1 PCR primers may be due to non-specific background chromatin. To quantify the HDAC1 enrichment on the ICAM-1 promoter we used quantitative PCR using primers the R4 primer pair. Enriched chromatin was expressed as relative to the IgG control, normalised to input levels and the control region (Figure 4.4B). This showed that HDAC is significantly enriched at the ICAM-1 promoter approximately 2-fold more than control. Therefore, in resting EC the ICAM-1 promoter is bound by the repressive factor HDAC1.

4.3.6. Association of HDAC1 on ICAM-1 promoter does not depend on Erg

HDACs do not bind DNA directly and are recruited to genes through their interaction with transcription factors or co-factors. We wanted to investigate whether HDAC1 binding to the ICAM-1 promoter was dependent on Erg. Chromatin from HUVEC treated with control or Erg siRNA was immunoprecipitated using an anti-HDAC1 antibody or IgG control and enriched chromatin was analysed by qPCR using the R4 and control primer pairs (Figure 2.6A). HDAC1 was significantly enriched at R4 of the ICAM-1 promoter in chromatin from HUVEC treated with control siRNA (Figure 4.4C). Inhibition of Erg with siRNA resulted in a decrease in HDAC1 enrichment at R4; however this was not significant with a p value of 0.0588. This suggest that HDAC1 does bind to the ICAM-1 promoter in HUVEC in the

presence of Erg, but further experiments are required to confirm whether the presence of HDAC1 is dependent on Erg.

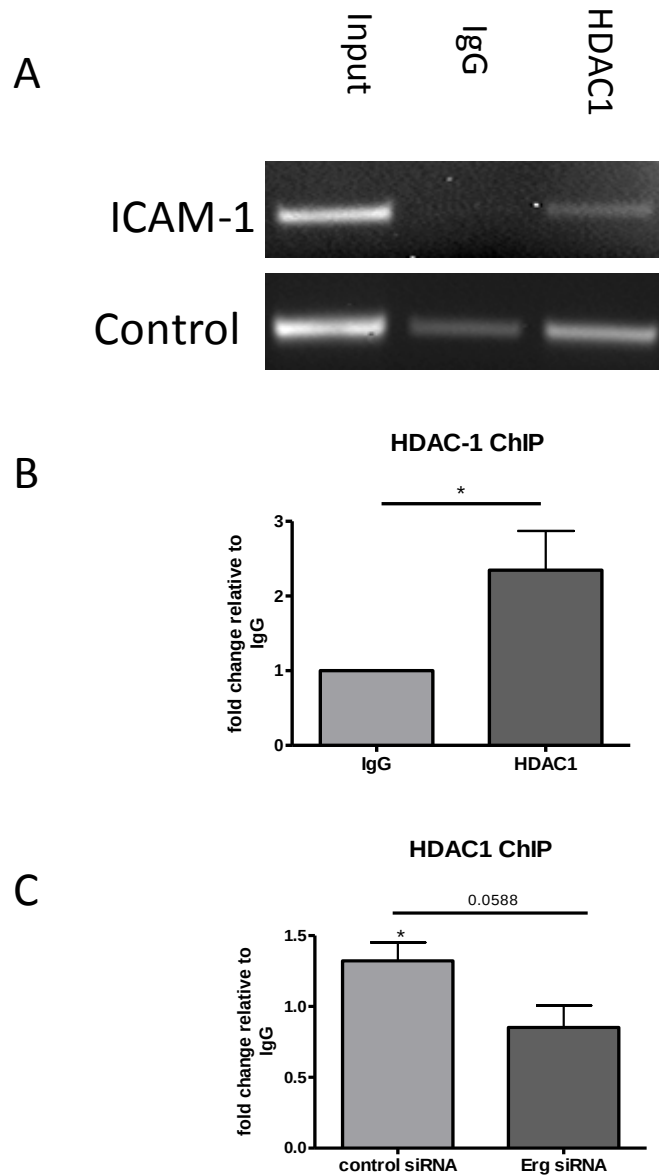


Figure 4.4 HDAC1 is associated with the ICAM-1 promoter. Chromatin immunoprecipitation was carried out on a confluent HUVEC monolayer using an anti-HDAC1 antibody or IgG control. A) Enriched chromatin was amplified by PCR for ICAM-1 promoter or control region and input and enriched samples were analysed on an ethidium bromide gel. Image is representative of 3 separate experiments B) Enriched chromatin was analysed by quantitative PCR using primers to R4 and control, data is expressed as fold change compared to IgG normalised to input and the control region, N=4. * $P < 0.05$. C) HDAC1 ChIP was carried out on HUVEC treated with control or Erg siRNA using anti HDAC1 antibody or IgG control. Enriched chromatin was analysed by q-PCR using primers to R4 and the control region. Data expressed as fold change compared to IgG, normalised to input and control region N=4. * $P < 0.05$.

4.3.7. Investigation of the association of HDAC2 with the ICAM-1 promoter

HDAC2 is part of the ESET repressive complex; in addition HDAC2 has been shown to have an anti-inflammatory role, with HDAC2 deficiencies implicated in inflammatory diseases such as chronic obstructive pulmonary disease (COPD) (Ito et al., 2000). Therefore we used ChIP to investigate whether HDAC2 binds to the ICAM-1 promoter. Sheared chromatin from a confluent HUVEC monolayer was immunoprecipitated using an anti-HDAC2 antibody or IgG control. Enriched chromatin was amplified by PCR using primers to the proximal promoter region of ICAM-1 and PCR products were detected by ethidium bromide gel electrophoresis. More ICAM-1 specific chromatin was enriched with an anti-HDAC2 antibody compared to IgG; however, there was also greater enrichment of the control region with the anti-HDAC2 antibody compared to control IgG (Figure 4.5A). This suggests that the enrichment of HDAC2 at the ICAM-1 promoter may be due to non-specific chromatin in the sample immunoprecipitated with HDAC2 antibody. Therefore we cannot conclude that HDAC2 binds to the ICAM-1 promoter.

4.3.8. Nucleosomes associated with the ICAM-1 promoter contain Tri-methylated Histone3 lysine-9

ESET is a Histone 3 lysine 9 specific methyl-transferase which tri-methylates lysine 9. We hypothesised that the presence of ESET at the ICAM-1 promoter would tri-methylate H3 on nucleosomes associated with ESET at the ICAM-1 promoter to greater levels than in regions where ESET does not bind. ChIP was carried out using an anti-tri-methylated-histone 3 lysine 9 (H3K9me3) antibody on sheared chromatin from a confluent HUVEC monolayer. Enriched chromatin was amplified using PCR primers for ICAM-1 proximal promoter or a control region and visualised by ethidium bromide gel electrophoresis. Enrichment of the ICAM-1 promoter using H3K9me3 was greater than enrichment with the IgG control antibody, and the enrichment for the control region was equal between H3K9me3 and IgG (Figure 4.5B). This suggests that the nucleosomes associated with the ICAM-1 promoter in resting HUVEC contain tri-methylated H3K9, and indicate a repressive chromatin structure at this promoter. This is in agreement with our observations that the ICAM-1 promoter is actively repressed in quiescent endothelial cells.

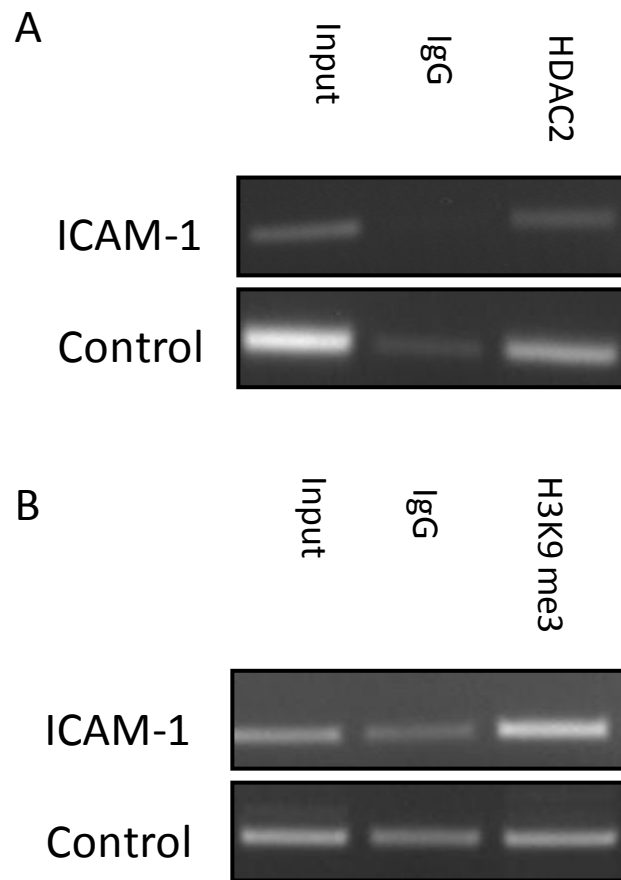


Figure 4.5 HDAC2 and H3K9me3 modifications are associated with the ICAM-1 promoter. Chromatin immunoprecipitation was carried out on confluent HUVEC monolayer using an anti-HDAC2 (A) or H3K9me3 (B) antibodies or IgG control. A and B) enriched chromatin was amplified by PCR for ICAM-1 promoter or control region and input and enriched samples were analysed on an ethidium bromide gel. Images are representative of 3 separate experiments.

4.3.9. In silico analysis of the ICAM-1 promoter suggests little transcriptional activity

The encyclopaedia of DNA elements (ENCODE) is an online database containing information on genes, transcripts, and transcriptional regulatory regions, as well as the DNA binding proteins that interact with regions of the genome, such as histone modifications, and transcription factor binding sites. Encode contains genome wide data on many different species and cell types. Data from genome-wide analysis of HUVEC is available, and we used the ENCODE database through the Ensembl server (www.ensembl.org) to investigate the regulatory features of the ICAM-1 gene. As no data was available from cells in which ICAM-1 has known high expression, we compared the regulatory features surrounding ICAM-1 with those surrounding VE-Cadherin, a gene constitutively transactivated by Erg in HUVEC (Birdsey et al., 2008). We configured the settings to present DNaseI hypersensitivity sites. DNaseI digest preferentially at sites of open chromatin, typically at promoters (Auerbach et al., 2009). The ICAM-1 gene has DNaseI hypersensitive sites within the 2kb surrounding the promoter, confirming this is a promoter region (Figure 4.6A). In contrast the constitutively expressed VE-Cadherin gene has many more DNaseI hypersensitive sites spread across the gene, indicating the whole gene has a more active and open chromatin structure (Figure 4.6B). We also examined the data for RNA-polII (polII) binding sites that confer an active state. The polII ChIP-seq data suggest polII binds to the ICAM-1 promoter in HUVEC (Figure 4.6A), but polII is not distributed across the gene suggesting it may be poised at the transcription start site, a characteristic of many inducible genes (Brookes and Pombo, 2009) (Adelman et al., 2009). In comparison polII on the VE-Cadherin gene does not have such a large peak within the promoter region, but its distribution is spread across the gene, indicating active transcription (Figure 4.6B). The ENCODE database does not contain H3K9me3 analysis for HUVEC, therefore we could not confirm our H3K9me3 results. Data from ChIP-seq experiments for other histone modifications and transcription factors is available; therefore we also investigated the H3K4me3, and H3K9ac modifications, indicative of open chromatin and active transcription, and H3K27me3, a modification indicative of silenced heterochromatin (Bannister and Kouzarides, 2011). There were no peaks for any of these selected histone modifications within the ICAM-1 gene in HUVEC (Figure 4.6A). In contrast, the VE-Cadherin promoter showed peaks for H3K9ac and H3K4me3 (Figure 4.6B), both modifications of active chromatin (Bannister and Kouzarides, 2011), in agreement with the constitutive expression of VE-Cadherin in EC. This analysis confirms that in resting

HUVEC, the histone modifications associated with ICAM-1 are not conducive to active transcription. Therefore we suggest Erg binding to the ICAM-1 promoter and the associated histone modifications confer a repressed state.

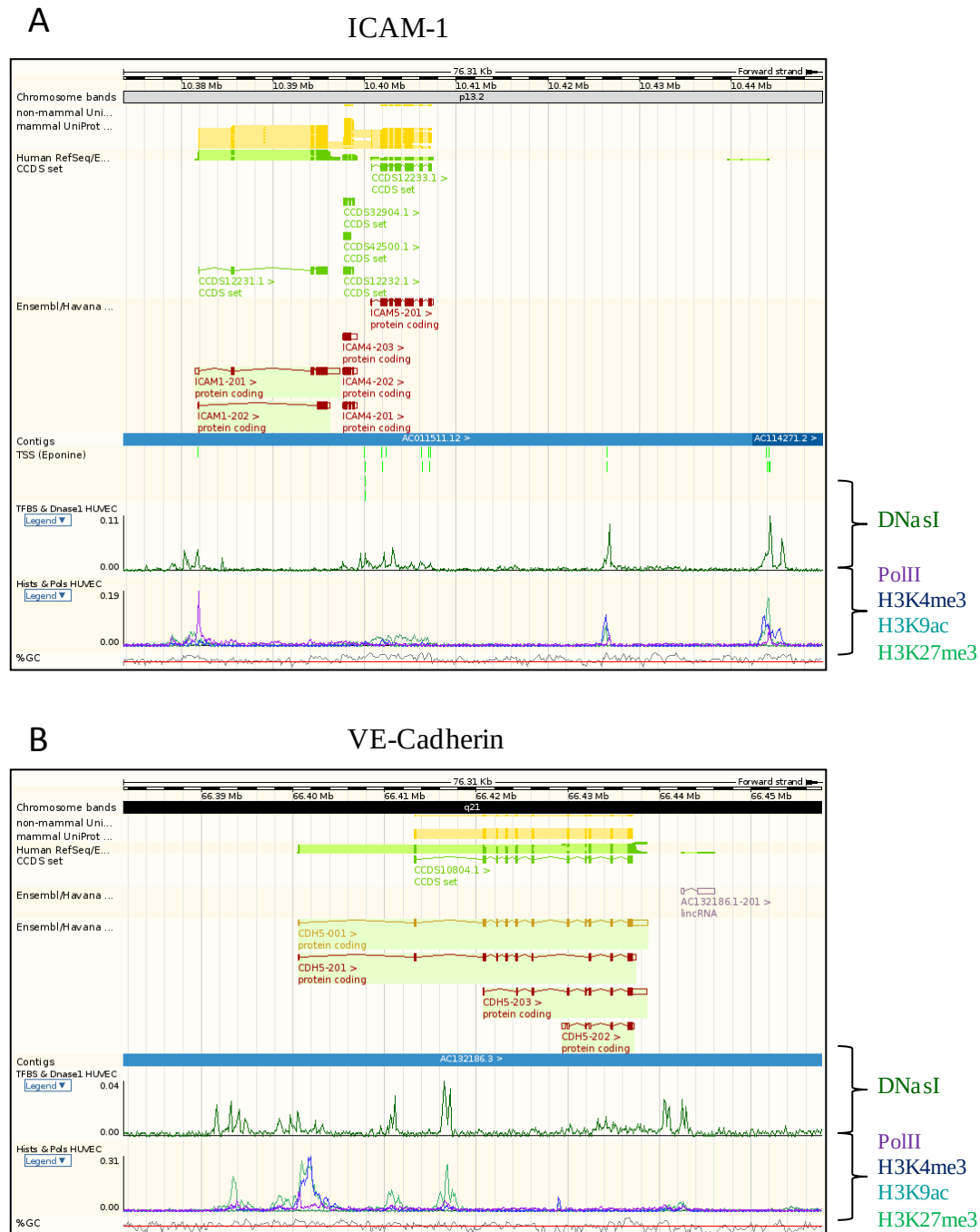


Figure 4.6 Extracted ENCODE data for ICAM-1 and VE-Cadherin from the Ensembl browser. Expression data from HUVEC showing isoforms and intron and exon structure. DNaseI hypersensitive peaks in dark green, RNA polII peaks in purple, H3K4me3 peaks in dark blue, H3K9ac peaks in blue green and H3K27me3 peaks in green.

4.4. Discussion

An important mechanism transcription factors use to repress target genes is through the recruitment of histone modifying co-factors to the promoters of genes. The most characterised histone modifications include deacetylation and methylation. Erg has been previously found to bind the histone methyltransferase ESET which specifically trimethylates H3K9, a modification that induces a repressive chromatin structure. In addition ESET has been shown to bind the repressive co-factors mSin3A and B and HDAC1 and 2, to form a repressive complex. We have shown that ESET is associated with the ICAM-1 promoter in resting endothelial cells in the same region that Erg binds and inhibits the binding of NF- κ B p65. As ESET does not bind DNA directly, we investigated whether the presence of ESET on the ICAM-1 promoter was Erg-dependent. We were unable to show a decrease in ESET binding by Erg inhibition; therefore we were unable to conclusively show that the association of ESET with the ICAM-1 promoter was dependent on the presence of Erg. Erg inhibition by siRNA is not complete, and the residual endogenous Erg may still facilitate association of ESET to the ICAM-1 promoter. Therefore an additional method to investigate the recruitment of ESET by Erg would be through studying the recruitment of ESET in endothelial cells from Erg knockout mice. It is also possible that ESET may be associated with the ICAM-1 promoter via other unidentified DNA binding proteins. Recently ESET has been shown to be involved in the TGF-beta mediated repression of IL-2 in T-lymphocytes. ESET was recruited to the IL-2 promoter by SMAD3, and induced H3K9me3 modification of chromatin associated with the IL-2 promoter (Wakabayashi et al., 2011). Therefore ESET may bind other unidentified transcription factors which are associated with the ICAM-1 promoter in resting endothelial cells, and Erg-mediated repression of ICAM-1 may be independent of ESET.

HDAC inhibitors have been used to show the role of HDAC in regulation of target genes (Marks, 2010). The HDAC catalytic domain is common in HDACs of class I IIa and IIb. It forms a small pocket for a transition metal ion, for example Zn^{2+} , which is targeted by HDAC inhibitors. We showed that the pan-HDAC inhibitor TSA could reverse the Erg-mediated repression of ICAM-1 mRNA levels, suggesting that HDACs are involved in Erg mediated repression. As TSA has the ability to inhibit a large number of HDACs, we investigated the association of two specific HDACs with the ICAM-1 promoter. HDAC1 was chosen as it is part of the ESET repressor complex. In addition, other ETS factors have been found to

repress target genes by recruiting HDAC1 to promoters of target genes (Kihara-Negishi et al., 2001; Yang et al., 2001). Our results suggest that HDAC1 is associated with the ICAM-1 promoter in resting EC at the same region bound by Erg and ESET, but we could not conclusively determine whether this was due to the presence of Erg. ChIP analysis suggests that HDAC2 binds to the ICAM-1 promoter, but more work needs to be carried out to quantify this interaction and identify whether Erg is involved in HDAC2 recruitment to this site.

HDACs have been found to play an important role in inflammatory mechanisms. HDAC inhibitors have both positive and negative effects on inflammatory pathways. For example in macrophages, treatment with HDAC inhibitors increased the response to LPS. This resulted in increased induction of pro-inflammatory genes including COX2 and CXCL2. Additionally treatment of resting macrophages with the HDAC inhibitor TSA decreased basal levels of a number of pro-inflammatory genes including CCL2 (Aung et al., 2006; Halili et al., 2010). HDACs and HDAC inhibitors have pleiotropic effects, and without in depth investigation it is difficult to identify molecular target of HDAC regulation. An example of the importance of HDACs in controlling inflammation was shown in COPD patients. Decreased expression of HDAC2 in alveolar macrophages is associated with glucocorticoid insensitive COPD. Glucocorticoid sensitivity was rescued by over-expression of HDAC2, suggesting an important role for HDAC2 in repression of pro-inflammatory gene expression (Ito et al., 2006). TSA acts as a broad spectrum HDAC inhibitor, inhibiting HDACs 1-9 with roughly equal efficacy (Bieliauskas and Pflum, 2008), therefore more specific HDAC inhibitors, such as the HDAC1 specific SB-429201 (Hu et al., 2003) or the class I HDAC specific Azumamide E (Maulucci et al., 2007), should be used to identify which HDACs are involved in Erg mediated repression of ICAM-1.

In addition to HDAC binding, we showed that the ICAM-1 promoter is associated with histones with the H3K9me3 mark. This is the modification induced by ESET and is associated with a repressed chromatin structure. Detection of histone modifications by ChIP is an important tool in characterising the regulation of genes. To gain a broad understanding of the general mechanisms used by Erg, analysis of the histone modifications surrounding Erg target genes will need to be further studied. It has been widely suggested that H3 lysine 9 methylation is linked to formation of heterochromatin and long term repression (Mosch et al., 2011). However in dendritic cells the chromatin of a subset of LPS-inducible inflammatory genes was found to be modified by H3K9 methylation in resting conditions; this modification

was lost upon pro-inflammatory stimulation and returned after resolution of inflammation (Saccani and Natoli, 2002). It was suggested that methylation and de-methylation add an additional layer to inducible gene expression, preventing early induction.

There is evidence suggesting expression of ICAM-1 and other NF- κ B target genes is inhibited by the polycomb group member and histone deacetylase SIRT1. The endothelium of ApoE^{-/-} mice expressed low levels of ICAM-1 and VCAM-1 which were both up-regulated after the knockout of one SIRT1 allele (Stein et al., 2010). Therefore, investigation of the association of SIRT1 and Erg may identify to a novel inflammatory repressive complex.

A number of ETS factors act as repressors of transcription through interaction with epigenetic co-repressors. The ETS factor Elk-1 acts as both a transcriptional activator and repressor on the same promoters, and repression is dependent of sumoylation of Elk-1. Sumoylation facilitates the Elk-1-dependent recruitment of HDAC2 to repressed target genes such as EGR-1 (Yang and Sharrocks, 2006). Elk-1 also interacts with the co-repressor mSin3A and HDAC1 (Yang et al., 2001). Pu-1 is another ETS factor that acts as an activator or a repressor, depending on the associate epigenetic co-factors. Using murine erythroleukaemia (MEL) extracts, Pu-1 was found to bind to the co-repressor MeCP2, a methyl CPG binding protein, via the Pu-1 ETS domain (Suzuki et al., 2003). MeCP2 in turn recruits mSin3A and HDAC to Pu-1, to form a repressive complex. Tel, an ETS factor that acts as transcriptional repressor, has been found to interact with the co-repressors mSin3A and NCoR through its PNT and central domains (Wang and Hiebert, 2001). HDAC3 also binds the central domain of Tel independently of other co-repressors. Tel represses the activity of the stromelysin promoter in NIH3T3 cells and this is inhibited by TSA treatment (Wang and Hiebert, 2001).

While we have identified a potential role for ESET and HDAC in the Erg-mediated repression of ICAM-1, in order to identify potential therapeutic targets it is important to dissect the mechanism further. Novel Erg interacting targets could be identified through immunoprecipitation of endogenous Erg and mass spectroscopy of co-precipitated proteins. A similar experiment was carried out to investigate co-repressors that interact with the BCL6 repressive transcription factor (Miles et al., 2005). As Erg acts as an activator and a repressor in the same pool of EC, this approach could identify co-activators and co-repressors of Erg transcriptional activity.

We have shown that Erg and several co-repressors bind to the ICAM-1 promoter; however an appreciation of the significance of these results would be gained by analysing the genome wide binding patterns of Erg. ChIP-seq to identify Erg binding sites has been carried out in the prostate cancer cell line VCap (Wei et al., 2010), Jurkat cells (Mochmann et al., 2011) and haematopoietic precursor HPC-7 cells (Wilson et al., 2010). However, Erg is an important regulator of a number of endothelial specific genes, and Erg binding to these genes may be undetectable in non-endothelial cells; therefore ChIP-seq in EC will provide important information about the direct regulatory network of Erg target genes in EC. In addition to Erg, ChIP-seq should also be carried out to investigate patterns of histone modifications, and co-occupation of epigenetic co-factors. While *in silico* data is available on some transcription factor binding and histone modifications in HUVEC, it would be beneficial to study the effects of the presence and absence of Erg on the chromatin landscape in endothelial cells.

We have shown that Erg, ESET and HDAC1 all bind to the ICAM-1 promoter in resting endothelial cells. However at any one time a population of HUVEC will show heterogeneous gene regulation of genes. Each cell will be in a different stage of transcription, some cells with a gene being actively transcribed, and in other cells the same gene may have little or no activity. This heterogeneity of activity will also result in differences in transcription factor binding between cells at any one time. To identify that Erg and other co-factors are binding at the same time, to the same promoter, in the same cell and therefore truly identified as co-factors, requires ChIP re-ChIP. This is carried out by first immunoprecipitating chromatin using an anti-Erg antibody, and then probing that immunoprecipitated sample for the presence of a co-factor. This will enable us to identify which repressive complexes Erg is involved in.

In summary, the data in this chapter has shown that Erg repression of ICAM-1 is associated with the binding of repressive chromatin modifying co-factors to the ICAM-1 promoter in the same region as Erg. More investigations need to be carried out to confirm that Erg recruits these repressive co-factors to the promoters of repressed target genes. In addition, genome wide analysis of the possible components of the Erg repressive complex is needed to show that Erg-mediated repression of a subset of NF- κ B target genes involves modifying the chromatin around NF- κ B binding sites leaving it inaccessible to low levels of nuclear localised NF- κ B.

Chapter Five

Summary and future work

In this Thesis I aimed to investigate the role of Erg in maintaining endothelial homeostasis by repressing inflammatory genes expression. The role for Erg as a transcriptional activator of genes involved in endothelial homeostasis is well characterised but little is known about the role of Erg as a transcriptional repressor. The loss of endothelial cell homeostasis is a characteristic of many inflammatory diseases including atherosclerosis. By understanding more about the regulators of this homeostasis and the mechanisms of their actions it may be possible to identify novel therapeutic targets to treat inflammatory diseases.

Microarray data generated in our lab suggested Erg represses a number of pro-inflammatory genes. To investigate the mechanisms behind this repression we initially decided to focus on the repression of ICAM-1 by Erg. We found that Erg repressed ICAM-1 protein and mRNA expression levels and that in resting EC, Erg bound to the promoter of ICAM-1. Detailed analysis of the ETS binding sites within the ICAM-1 promoter using a luciferase reporter system showed that the repression of ICAM-1 by Erg was dependent on two ETS binding sites located 118 and 181 bp upstream of the transcription start site. One of these ETS binding sites, EBS-181, is located within a functional NF- κ B binding site. While the other ETS binding site, EBS-118, has previously been shown to be responsible for transactivation of ICAM-1 by ETS factors including Ets-2 and ERM in non-endothelial cells, our study found that inhibition of a number of ETS factors which are endogenously expressed in EC did not affect ICAM-1 expression, suggesting a specific and non-redundant role for Erg in the repression of ICAM-1.

We showed through ChIP analysis that Erg binds to the ICAM-1 promoter; however, this analysis does not have high enough resolution to identify the specific EBS bound by Erg. We therefore investigated whether the two EBS responsible for Erg mediated repression were bound by Erg using EMSA. We showed that nuclear lysate from resting HUVEC contained proteins which bound an oligonucleotide containing EBS-118 and the addition of an anti-Erg antibody resulted in the appearance of a super-shift band, suggesting that Erg binds to EBS-118 *in vitro*. We also investigated whether Erg binds to the EBS-181 by EMSA. Nuclear lysate from resting HUVEC contained proteins which bound to an oligonucleotide containing EBS-181, also including the NF- κ B binding site. The addition of an anti-Erg antibody decreased the intensity of these complexes suggesting that Erg was present in these complexes. Likewise, an anti-NF- κ B p65 antibody was also able to decrease the intensity of the complex, suggesting p65 is present in the nuclear lysate from resting HUVEC and capable of binding the NF- κ B binding site in resting HUVEC.

These results lead us to hypothesise that Erg may bind to the promoter of ICAM-1 in quiescent HUVEC, and inhibit ICAM-1 transactivation by NF- κ B p65. We showed, using inhibitors of NF- κ B activity, that the induction of ICAM-1 by Erg inhibition was NF- κ B dependent, suggesting that in resting EC there is NF- κ B activity which if left uninhibited may induce a dysregulated inflammatory response. We showed that the presence of Erg in resting EC inhibits the binding of NF- κ B p65 to the ICAM-1 promoter as inhibition of Erg expression resulted in an enrichment of NF- κ B p65 to the ICAM-1 promoter.

As genome wide gene expression data generated in our lab suggests that Erg inhibits a number of pro-inflammatory genes, we used bioinformatic analysis (GSEA) to investigate whether there was a correlation between genes repressed by Erg and genes transactivated by NF- κ B. We compared our data with two separate microarray studies, one study in EC investigating genes transactivated by TNF- α , an activator of NF- κ B pathways, and the other in pancreatic cancer cells investigating genes transactivated by TNF- α and inhibited by IKK inhibitor. Both comparisons showed a significant correlation between genes repressed by Erg and genes transactivated by NF- κ B. Comparison between our Erg microarray data and ChIP-Seq analysis of NF- κ B p65 binding sites in LPS treated macrophages, showed a trend towards correlation although this was not significant.

The GSEA data suggested that there may be a common mechanism of Erg mediated repression amongst a number of NF- κ B target genes. The GSEA analysis was validated on the NF- κ B target genes cIAP-2, TNFAIP3 and IL-8. We showed that Erg repressed cIAP-1 and TNFAIP3 mRNA expression, and bound to the promoter of cIAP-2 and TNFAIP3 in the vicinity of the functional NF- κ B binding sites. As seen with ICAM-1, inhibition of Erg induced an enrichment of NF- κ B p65 binding to the cIAP-2 promoter but not TNFAIP3. IL-8 has previously been shown to be repressed by Erg in HUVEC, through an EBS within the functional NF- κ B site (Yuan et al., 2009). We showed that inhibition of Erg by siRNA also induced NF- κ B p65 binding to the IL-8 promoter. This confirms that Erg represses a number of NF- κ B target genes through a common mechanism.

Many transcription factors act as repressors of transcription through the recruitment of repressive co-factors which enable modification of the chromatin environment resulting in an open or closed chromatin structure. Erg has been found to bind the HMT ESET which in turn binds the co-repressors HDAC1, HDAC2, mSin3A and mSin3B. This lead us to hypothesise that Erg may repress ICAM-1 expression through the recruitment of co-repressors and

inhibiting transactivating factors. We showed that ESET bound to the ICAM-1 promoter in EC at the same region as Erg. However, this binding did not appear to be dependent on the presence of Erg.

We utilised the HDACi TSA to investigate whether HDAC played a role in Erg mediated repression of ICAM-1. We found that the inhibition of ICAM-1 induced by Erg over-expression could be rescued by treatment with TSA, which suggested HDAC may be involved in Erg mediated repression of ICAM-1. HDAC1 was found to bind to the ICAM-1 promoter in resting EC, and while Erg inhibition reduced the level of HDAC-1 bound to the ICAM-1 promoter, the p-value was 0.0588 therefore this decrease was not significant. ChIP analysis also showed HDAC2 bound to the ICAM-1 promoter in resting HUVEC; however, the role of Erg in this binding was not shown.

Repressive co-factors modify histone molecules on nucleosomes to confer a closed chromatin structure which inhibits transcription. If ESET was bound to the ICAM-1 promoter on the same region as Erg then histones in the vicinity of this binding site should have the corresponding modification. We investigated whether the post translational modification induced by ESET, H3K9me3, was enriched at the ICAM-1 promoter in resting HUVEC. H3K9me3 levels were enriched at the ICAM-1 promoter, suggesting the chromatin at this site is in a repressed conformation. Further work needs to be carried out to investigate whether ESET or Erg are responsible for this modification of the histone molecules at the ICAM-1 promoter. Figure 5.1 depicts our mechanistic hypothesis of Erg-mediated repression of the ICAM-1 promoter through compaction of chromatin and inhibition of NF- κ B p65 binding.

The ENCODE project contains data from analysis of a number of regulatory features in HUVEC. This allows *in silico* analysis of the ICAM-1 promoter to complement our work. While there is no data currently on repressive epigenetic marks, we were able to show the absence of activating modifications on the ICAM-1 gene compared to VE-Cadherin, an Erg target gene that is constitutively active in resting EC. We found that ICAM-1 contained far less DNaseI hypersensitivity sites suggesting a more closed chromatin structure; moreover, ICAM-1 gene did not show high levels of histone modifications H3K4Me3 and H3K9Ac associated with active transcription, as seen in VE-Cadherin. While RNA-polII was enriched on the ICAM-1 gene, this enrichment was localised at the transcription start site rather than across the gene as seen with VE-Cadherin, suggesting RNA-polII is poised on the ICAM-1 promoter, and not facilitating active transcription. These data are in agreement with our

hypothesis that Erg binds to the ICAM-1 promoter and confers a repressed chromatin state through the recruitment of repressive epigenetic co-factors.

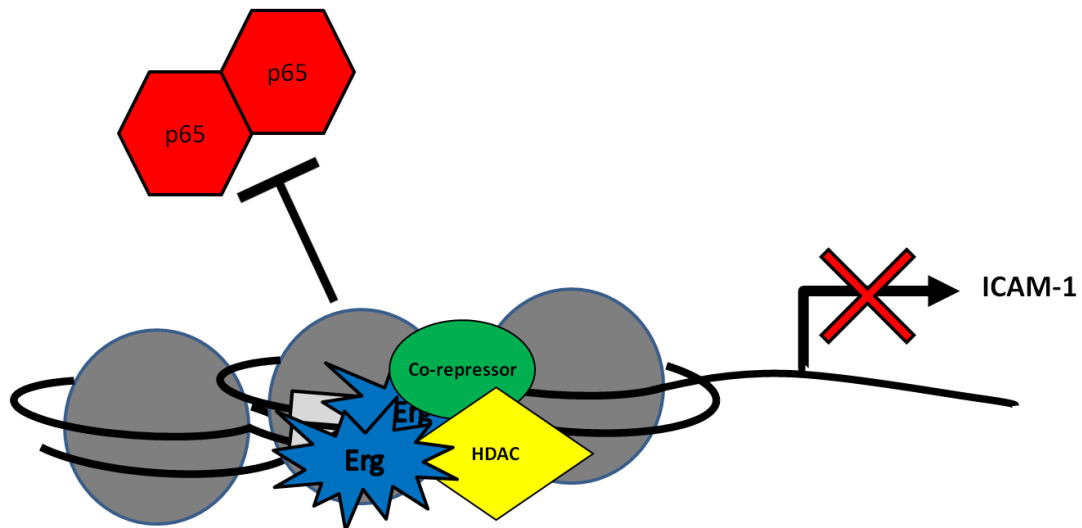


Figure 5.1 Cartoon representing possible mechanism of Erg mediated repression. In quiescent EC Erg binds to the ICAM-1 promoter via EBS-118 and EBS-181 and inhibits binding of NF- κ B p65. This may be the result of Erg-mediated chromatin compaction through the recruitment of co-repressors and HDAC.

While we have identified a mechanism for Erg mediated repression of pro-inflammatory genes, further investigation of this mechanism needs to be carried out. We do not know what determines whether Erg acts as an activator or a repressor. Microscopic analysis of Erg expression in HUVEC shows a homogeneous expression pattern of Erg and its target genes; this suggest that in EC Erg acts as an activator and a repressor of different target genes in the same cells at the same time. There are various mechanisms that may dictate whether Erg represses or activates a gene such as promoter binding site motifs, interaction with other transcription factors or regulatory co-factors, or through post-translational modification of Erg.

We have shown that Erg acts as a repressor by binding to the promoters of target genes; however, genome wide analysis of Erg targets through ChIP-Seq would enable us to confirm whether Erg binds to the promoters of many NF- κ B target genes, as shown with ICAM-1, cIAP2 and IL-8. Analysing Erg ChIP-Seq data in combination with Erg microarray data would enable us to identify Erg-target genes bound directly by Erg, or those that are regulated indirectly. ETS factors have been shown to act in synergy with other transcription factors bound to common promoters through composite binding motifs, and analysis of genome wide Erg binding sites might allow us to identify more of these. ChIP-Seq analysis to investigate Erg binding sites in haematopoietic progenitor cells has shown that the majority of Erg binding sites were intergenic or intragenic rather than within promoters (Wilson et al., 2010). This indicates that Erg may affect transcription through binding enhancer regions. In order to investigate this, Chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) could be carried out (Li et al., 2010). This would allow analysis of Erg binding sites, and identify sites where Erg binds to distant enhancers which then interact with promoters of target genes through a DNA or chromatin loop. This would enable us to more precisely identify genes directly targeted by Erg.

The activity of Erg as a transcriptional activator or repressor may depend on interaction with other proteins; Erg may interact with other transcription factors resulting in activation, inhibition or synergy. Alternatively, Erg may interact with co-factors which may induce changes in the chromatin environment or post-translationally modify Erg. Analysis of proteins that bind Erg has previously been carried out using a yeast two-hybrid assay as described to identify the interaction between Erg and ESET. Another possible way to investigate Erg interacting partners is through mass spectroscopic analysis of protein complexes immunoprecipitated with a tagged recombinant Erg, such as the tandem affinity

purification (TAP) system (Li et al., 2011). This would allow analysis of Erg-interacting partners in mammalian cells, and aid in the elucidation of binding complexes on Erg target genes. If interacting partners are identified which may be involved in Erg-mediated repression or activation at Erg target genes, ChIP-reChIP could be carried out to show that the same binding sites in the same cell are bound by the complex at the same time.

As discussed in the introduction, the activity of ETS factors has been found to be altered by modifications such as phosphorylation, glycosylation, ubiquitylation, and sumoylation. Data from the yeast two hybrid screen suggested that Erg interacted with Ubc9, a sumo-conjugating enzyme which also interacts with other ETS factors such as Ets-1, Fli-1 and Tel (Ji et al., 2007; van den Akker et al., 2005; Chakrabarti et al., 2000). The interaction between Erg and Ubc9 may facilitate sumoylation of Erg to alter the activity of Erg by affecting its DNA binding, or affecting its interaction with other transcription factors or epigenetic co-factors. Identification of residues within Erg involved post translational modification and interactions with modifying enzymes would allow us to dissect the roles for these interactions in the transcriptional activity of Erg.

Studying Erg in a monoculture of HUVEC allows easy manipulation to identify mechanisms of Erg activity; however, EC do not live in isolation and they are affected by signals produced by the surrounding cells and environment. Therefore, validation of the importance of Erg as a mediator of EC homeostasis needs to be carried out in a model organism. Our group has commenced the characterisation of an endothelial specific Erg knockout mouse (Birdsey GM. and Randi AM. unpublished data). In order to confirm the role for Erg in EC homeostasis, analysis of susceptibility to model inflammatory diseases in normal and stressful environments could be carried out. Once interactions with co-factors are identified, further investigation of the role of these interactions *in vivo* should be carried out.

In summary, we have provided data which indicates Erg maintains EC homeostasis by acting as a gatekeeper in the presence of low levels of nuclear localised pro-inflammatory NF- κ B p65, providing a checkpoint to uncontrolled activation of pro-inflammatory genes

Appendix 1: Abbreviations

aa: amino acid
AAA: abdominal aortic aneurism
AD: Anno Domini
AdErg: adenovirus encoding for p55 Erg
AdLacZ: adenovirus encoding for β -galactosidase
Ang: angiotensin
ANK: ankyrin repeats
AP-1: activator protein-1
Apo-E: apolipoprotein E
BCL: B-cell CLL/lymphoma
BMI1: B-cell specific Moloney-MLV insert site-1
bp: base pairs
C/EBP: CCAAT-enhancer-binding proteins
CBP: CREB-binding protein
CCL: chemokine (C-C motif) ligand
ChIP: chromatin immunoprecipitation
cIAP2: cellular inhibitor of apoptosis protein 2
CLDN: claudin
Cont: control
COPD: chronic obstructive pulmonary disease
COX: cyclo-oxygenase
CpG: cytosine-phosphate-guanine
CREB: cyclic AMP-response element binding protein
CTA: carboxy terminal transcriptional activation
CTCL: cutaneous T-cell lymphoma
CXCL: chemokine (C-X-C motif) ligand
DAMP: damage associated molecular pattern
DD: death domain
DMSO: dimethyl sulfoxide
DTT: dithiothreitol
EBS: ETS binding site
EC: endothelial cells

ECGF: endothelial cell growth factor
ECM: extra cellular matrix
EDB: ETS DNA binding domain
Elk: ETS like gene
EMSA: electrophoretic mobility shift assay
ENCODE: encyclopaedia of DNA elements
ER: endoplasmic reticulum
ERF:Ets-2 repressor factor
Erg: ETS related gene
Erk: extracellular signal-regulated kinase
ERM: ETS related molecule
ER α : oestrogen receptor alpha
ESET: Erg associated protein with a SET domain
ESL: E-selectin ligand
ETA: ETS transcriptional activation domain
ETS: E26 transformation specific
EWS: Ewing's sarcoma
FBS: foetal bovine serum
FDA: food and drug administration
FEV: fifth Ewing's variant
Fli-1: friend leukaemia virus integration 1
FOX: forkhead
FUS: fused in sarcoma
g: gravity
GABP α : GA binding protein alpha
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GAS: gamma interferon activation sequence
GB: genebloc
GM-CSF: granulocyte-macrophage colony stimulating factor
GPCR: G protein-coupled receptors
GR: glucocorticoid receptor
GRR: glycine-rich region
GSEA: gene set enrichment analysis
GTP: guanosine triphosphate

H: histone
HAT: histone acetyltransferase
HBSS: hanks' balanced salt solution
HDAC: histone deacetylase
HDACi: HDAC inhibitor
HMT: histone methyltransferases
HO-1: hemeoxygenase-1
HOX: homeobox protein
HRP: horseradish peroxidase
HUVEC: human umbilical vein endothelial cells
ICAM: intercellular adhesion molecule
IFN: interferon
IFR: interferon receptor
Ig: immunoglobulin
IKK: inhibitor of κ B kinase
IL: interleukin
IL-1R: IL-1 receptors
IP: immunoprecipitation
IRAK: interleukin 1 receptor-associated kinase
IRE: interferon response element
IRF: interferon regulatory factor
I κ B: inhibitor of κ B
JAK: Janus kinase
JAM: junctional adhesion molecule
JNK: cJun N-terminal kinase
kDa: kilodalton
KLF: kruppel-like factor
LDL: low-density lipoproteins
LFA-1: lymphocyte function-associated antigen 1
LPS: lipopolysaccharide
LZ: leucine-zipper
mAbs: monoclonal antibodies
MAC1: macrophage antigen 1
MadCAM: mucosal vascular addressin cell adhesion molecule

MAK1: macrophage antigen 1
 MAPK: mitogen activated protein kinase
 MCP-1: monocyte chemoattractant protein-1
 M-CSF: macrophage colony-stimulating factor
 MHC: major histocompatibility complex
 MMP: matrix metalloproteinase
 MnSOD: manganese containing superoxide dismutase
 MSR: macrophage scavenging receptor
 MYD88: myeloid differentiation primary response gene 88
 NAD⁺: nicotinamide adenine dinucleotide
 NCoR: nuclear receptor co-repressor
 NES: normalised enrichment score
 NF- κ B: nuclear factor kappa B
 NK: natural killer
 NLS: nuclear localization sequence
 NO: nitric oxide
 NOS: NO synthase
 NRT: negative regulatory transcriptional activation domain
 OST: On-SighT
 pAbs: polyclonal antibodies
 PAH: pulmonary arterial hypertension
 PAMP: pathogen associated molecular pattern
 PCR: polymerase chain reaction
 PDTC: pyrrolidine dithiocarbamate
 PECAM: platelet endothelial cell adhesion molecule
 PEST: proline, glutamic acid, serine and threonine-rich region
 PFU: plaque forming units
 PMA: phorbol 12-myristate 13-acetate
 PNT: pointed domain
 PSGL: P-selectin glycosylated ligand
 PTP: protein tyrosine phosphatase
 RHD: rel homology domain
 ROS: reactive oxygen species
 RUNX: runt-related transcription factor

SAHA: suberoylanilide hydroxamic acid
SAM: S-adenosylmethionine
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
Seq: sequencing
SIRT: sirtuins
SMC: smooth muscle cell
SMRT: silencing mediator for retinoic acid and thyroid hormone receptor
SRE: serum response element
SRF: serum response factor
STAT: signal transducer and activator of transcription
TAB2: TGF- β -activated kinase 1
TACE: TNF converting enzyme
TAD: transactivation domain
TCF: ternary complex factor
TCR: T-cell receptors
TF: tissue factor
TFPI: tissue factor pathway inhibitors
TLR: Toll-like receptors
TMPRSS2: transmembrane protease, serine 2
TNF: tumour necrosis factor
TNFAIP3: TNF- α inducible protein-3
TNFR: TNF receptor
TPA: tissue plasminogen activator
TRADD: TNFR-associated death domain
TSA: trichostatin-A
TSS: transcription start site
UTR: untranslated region
VCAM-1: vascular cell-adhesion molecule
VEGFR: vascular endothelial growth factor receptor
VLA4: very late antigen-4
VSMC: vascular smooth muscle cells
VWF: von Willebrand factor
WPB: Weible pallade bodies

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