Polarised Secretion of Leukaemia Inhibitory Factor

Eric James Hill

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Thesis Summary

Leukaemia inhibitory factor (LIF) is a cytokine that is active on a wide variety of cells. Multiple LIF transcripts have been described. The transcripts LIF-D and LIF-M encode different signal peptides, which in mouse have been associated with differential localisation of the mature protein. LIF-D is associated with a freely diffusible protein, whereas the LIF-M is associated with the extracellular matrix.

The polarity of LIF secretion has yet to be described and could illuminate the mechanisms of LIF localisation. Here the polarised endogenous secretion of human LIF and IL-6 in Caco-2 cells was characterised under normal culture conditions and following induction with IL-1β. Whether the apical or basolateral membrane was stimulated influenced the pattern of secretion (LIF: Unstimulated, 59% basolateral. Dual stimulation, 68% basolateral. Basolateral stimulation, 79% basolateral. Apical stimulation, 53% basolateral.). IL-6 displayed a similar dependence on the site of stimulation but was predominantly secreted at the membrane that was stimulated.

To determine the effect of the alternate signal peptides on the polarity of LIF secretion, LIF was epitope tagged with FLAG. Epitope-tagging with FLAG was used to separate endogenous from exogenous protein expression. However, despite the normal biological activity of LIF-FLAG and detection of the FLAG in a western blot, detection of the LIF-FLAG under non-reducing conditions was not observed, and therefore it was unsuitable for secretion studies.

Untagged LIF was expressed exogenously in Madin-Darby canine kidney (MDCK) cells under the control of a tetracycline responsive promoter that allowed a variety of LIF expression levels to be tested. Exogenous murine LIF was secreted predominantly from the apical (60%) membrane of MDCK cells irrespective of the signal peptide expressed.

Key words: Leukaemia Inhibitory Factor, Caco-2 cells, MDCK cells, polarised cells, secretion.

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Abbreviations

AMP	Adenosine monophosphate
APP	Acute phase protein
ATP	Adenosine triphosphate
bFGF	basic Fibroblast growth factor
cDNA	Complementary DNA
CBD	Cytokine binding domain
CIMPR	Calcium independent mannose 6-phosphate receptor
CLC	Cardiotrophin like cytokine
CLF	Cytokine like factor
CMV	Cytomegalovirus
CNTF	Ciliary neurotrophic factor
CT-1	Cardiotrophin
DNA	Deoxyribonucleic acid
Dox	Doxycycline
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ES cell	Embryonic stem cell
FNT	Fibronectin
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-6	Interleukin-6
Jaks	Janus kinases
K_D	Dissociation constant

kDa	Kilodalton
LIF	Leukaemia inhibitory factor
LIFR	LIF receptor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
mRNA	messenger Ribonucleic acid
OSM	Oncostatin M
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
rtTA	Reverse tetracycline controlled transacitvator
sLIFR	Soluble LIF receptor
Stats	Signal transducers and activators of transcription factors
SEM	Standard error of the mean
SHP2	SH2 containing protein-tyrosine phosphatase
Tet	Tetracycline
TGFβ	Transforming growth factor β
TGN	*Trans* Golgi Network
TIMPs	Tissue inhibitors of matrix metalloproteinases
TRE	Tetracycline response element
tTA	Tetracycline controlled transactivator

Chapter 1: Introduction.

1.1 Cell signalling.

Mechanisms enabling one cell to influence the behaviour of another are diverse and in animals, cells communicate by utilising hundreds of signalling molecules. They can include gasses such as nitric oxide, fatty acid derivatives, nucleotides, amino acids, small peptides and proteins. These molecules can act over long or short distances. There are essentially four main forms of intercellular signalling: 1) Contact dependent signalling relies upon molecules that remain bound to the surface of the signalling cell and can therefore only influence cells which directly contact the signalling cell. 2) Paracrine signalling relies on signals that are released into the extracellular spaces and act on neighbouring cells. 3) Neuronal signalling relies on the transmission of electrical signals along the axons and the release of neurotransmitters at synapses, which can be located far from the cell body. 4) Endocrine signalling relies on the secretion of hormones into the bloodstream, which are capable of communicating widely throughout the body.

Responses to extracellular signals in general, are determined as much by the nature of the target cells as by the nature of the signal molecule. Each cell is programmed to respond to a specific combination of extracellular signalling molecules. In order to accomplish this, each cell type displays a set of receptors that enables it to respond to a corresponding set of signal molecules. In addition, different cells are able to respond differently to the same extracellular signal molecules as a result of progressive cell specialisation in the course of development. Such specialisation can influence the set of receptors a cell possesses and the intracellular

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signalling proteins to which the receptors are coupled. Many types of cell use the same intracellular signalling proteins, but the set of genes that they regulate may be different between cell types. This is because, usually, more than one type of gene regulatory protein must bind to a gene, to activate its transcription. The activation of a gene can only occur if the right combinations of gene regulatory proteins are present. As a result the way in which two different cells interpret a particular signal can vary enormously.

Cytokines are defined as extracellular signalling proteins (~20kDa) that usually act as local mediators in cell-to-cell communication. Most are secreted and are freely diffusible, some can be expressed on the cell membrane, whilst others are held in the extracellular matrix. Cytokines are potent molecules, which act as regulators in inflammatory and immune reactions. They can stimulate changes in cell proliferation, differentiation, migration and repair processes at nanomolar to picomolar concentrations. Release is generally localised within specific microenvironments and is usually not systemic. Indeed systemic injection into animals can have profound effects resulting in illness or death. The potency of cytokines and the wide expression of their receptors have required the development of elaborate control mechanisms for the production and localisation of these proteins.

1.2   General features of Leukaemia inhibitory factor (LIF)

LIF is a secreted cytokine, of the interleukin-6 (IL-6) family that exhibits pleiotropic activities in a wide range of tissues and cell types. LIF plays a role in haematopoiesis (Smith *et al.* 1988), neuronal survival (Schweizer *et al.* 2002)

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endometrial decidualisation, blastocyst implantation (Vogiagis and Salamonsen 1999), hypothalamus-pituitary-adrenal axis (HPA) activation (Wang *et al.* 1996), pituitary development (Shimon *et al.* 1997), osteoblast and osteoclast function (Heymann and Rousselle 2000) and lung inflammatory processes (Knight *et al.* 1999).

Experiments undertaken to examine the consequences of sustained excess LIF levels demonstrated a range of pathologies. Mice injected with cells overexpressing LIF developed a cachectic like appearance and became hypermotile and ultimately died after two months. Following an autopsy, the mice displayed loss of all fatty tissue, thymus atrophy, calcification of liver, heart and skeletal muscle and depletion of spermatogonia in the seminiferous tubules of the testes (Metcalf and Gearing 1989).

LIF knock out mice may offer more insight into LIF function than the LIFR activation due to overexpression, as the release of endogenous LIF is temporally and spatially restricted. However, LIF-/- mice may suffer limitations, as the *in vivo* functions can often be masked by redundancy with other gp130 cytokines (cytokines which share the common receptor subunit gp130). Although LIF-/- mice may behave normally enough, multiple functional deficiencies may not be revealed unless the mice receive appropriate stress stimuli. Experiments with LIF-/- mice demonstrate an essential role of LIF in generation of a normal stem cell compartment (Escary *et al.* 1993), repair of nerve injury (Cafferty *et al.* 2001), control of inflammation (Zhu *et al.* 2001), regulation of the HPA (Hypothalamo-Pituitary-Adrenal) axis (Chesnokova and Melmed 2000) and in implantation (Stewart *et al.* 1992). Interestingly LIF -/- females are infertile. The defect in implantation appears to be maternal as LIF-/- blastocysts

implant successfully when transplanted into wild type females (Stewart *et al.* 1992). Providing LIF-/- females with exogenous LIF during this period restores implantation (Stewart *et al.* 1992). The temporal and spatial expression of LIF (Bhatt *et al.* 1991), LIFR and gp130 (LIF receptor components) (Yang *et al.* 1994), has been defined during the establishment of pregnancy. It appears that despite all of the other signalling molecules that are of known importance for implantation, LIF is irreplaceable (Vogiagis and Salamonsen 1999).

LIF is expressed in a wide variety of tissues. Cellular sources include: stromal cells, muscle cells, epithelial cells, astrocytes, osteoblasts, chondrocytes, neurons and immune cells such as macrophages, and T-cells albeit at very low levels. High expression is observed, however, in pregnant females prior to implantation, during infection, inflammation and cancer (Auernhammer and Melmed 2000).

Production of LIF is tightly controlled; indeed in normal non-inflamed tissue outside of the uterus LIF mRNA is only detected at low levels (Waring *et al.* 1992). However, LIF mRNA and protein production is readily induced by a number of pro-inflammatory cytokines such as interleukin-1 (IL-1) (Rathjen *et al.* 1990), as well as exposure to lipopolysaccharide (LPS) (Wang *et al.* 1996), and growth factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and transforming growth factor (TGFβ) (Rathjen *et al.* 1990). In contrast, the cellular production of LIF is inhibited by anti-inflammatory agents, such as glucocorticoids and estradiol (Bamberger *et al.* 1997).

X-ray crystallography has shown LIF to be a four-α-helix bundle cytokine, showing a high degree of homology with the structures of IL-6 and CNTF (Ciliary neurotrophic factor);(Robinson *et al.* 1994); (Fig 1.1). LIF is variably and heavily glycosylated producing a protein with an observed molecular mass of 25-60kDa (Hilton *et al.* 1988), which can be de-glycosylated to produce a 20kDa protein (Gough *et al.* 1992). Although LIF proteins show a high degree of homology across species, it has been observed that although human LIF is able to act upon both human and murine cells, murine LIF is not active on human cells (Layton *et al.* 1994).

Three isoforms have been described, which are derived from a single copy of the LIF gene. Two of the isoforms, corresponding to transcripts LIF-D and LIF-M, encode different signal peptides, which in mice appear to affect the localisation of LIF protein. Consequently, the mature proteins have exactly the same polypeptide sequence but show different localisation. The LIF-D protein is secreted and is diffusible; LIF-M on the other hand is secreted but is associated with the extracellular matrix (ECM) (Rathjen *et al.* 1990). The mechanism by which matrix localisation is achieved and the capacity of signal peptides to direct proteins to the ECM are core topics within this thesis and will be discussed in greater detail in subsequent sections.

1.3   Gp130 Family of cytokines.

The IL-6 family is comprised of a growing number of cytokines, including LIF, viral IL-6, Oncostatin M (OSM), IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), cardiotrophin-like-cytokine (CLC) which is secreted when bound to the soluble receptor cytokine like factor-1 (CLF), IL-27 (Pflanz *et al.* 2002) and neuropoietin (Derouet *et al.* 2004) (Fig 1.2). Each IL-6 type cytokine can be

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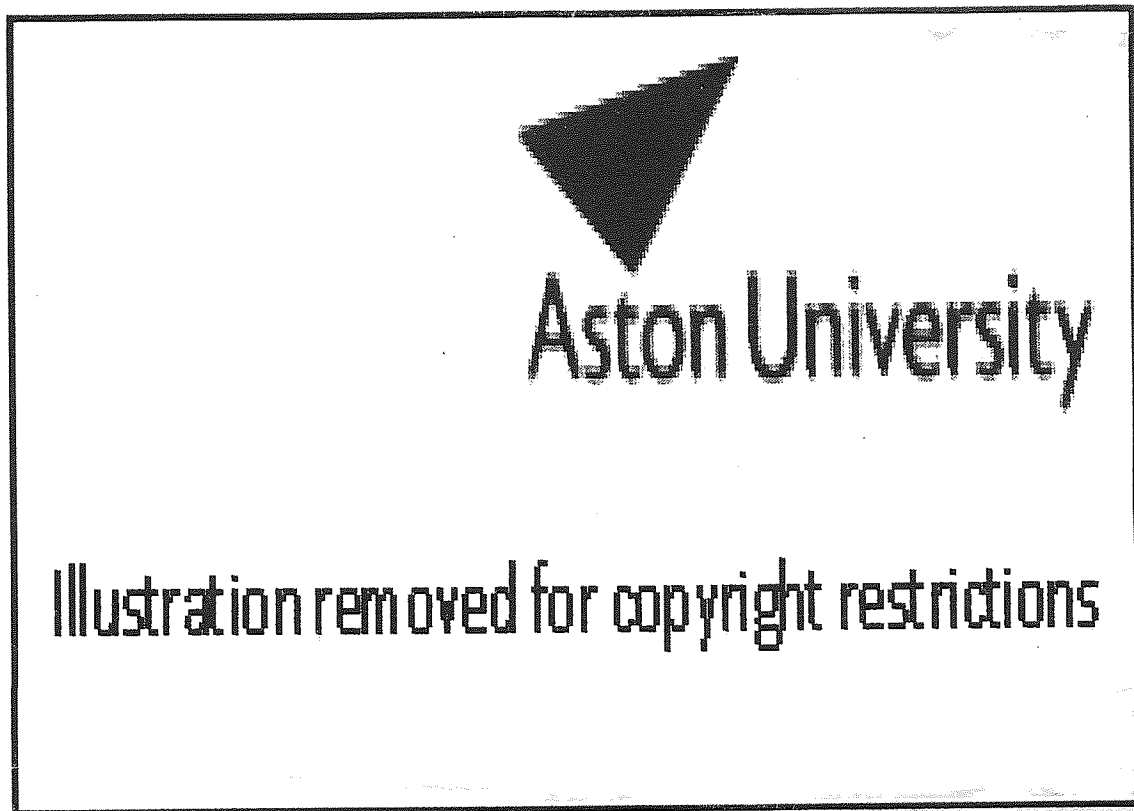
Figure 1.1 Three-dimensional structure of murine LIF. Diagram of the crystal structure of murine LIF showing binding sites I to III. LIF is a four-α-helical bundle. The tertiary structure of LIF, from the N-C-terminus consists of helices A,B,C and D, linked by two long loops AB and CD as well as the short loop BC. Reproduced with kind permission of Ann Vernallis.

characterised by a profile of receptor recruitment that involves at least one molecule of gp130. Gp130 is a single-pass transmembrane protein, which does not possess an intrinsic tyrosine kinase domain. IL-6 family cytokines assemble receptor complexes into homo- or heterodimers that lead to the activation of associated tyrosine kinases and subsequent activation of transcription factors.

1.3.1 IL-6 family receptor structure/usage.

The receptors that are involved in the signalling of IL-6 type cytokines can be subdivided into signalling (gp130, LIFR, OSMR and WSX-1) and non-signalling (CLF, IL-6Rα, IL-11Rα and CNTFRα) (Fig 1.2). IL-6, IL-11 and CNTF must bind specifically to their α receptor subunits before they can effectively recruit the signalling receptor subunits. CNTFRα does not have a transmembrane domain instead it is attached to the membrane via a glycosyl-phosphatidyl-inositol linkage. IL-6 and IL-11 signal via gp130 homodimers. Although the remaining (LIF, CNTF, CT-1 and CLC) signal via heterodimers of gp130 and LIFR, human OSM has demonstrated the ability to signal via either LIFR-gp130 or OSMR-gp130 heterodimers. In mice, murine OSM will only signal via OSMR-gp130 (Lindberg *et al.* 1998). In addition, IL-27 signals via heterodimers of WSX-1 and gp130. (Pflanz *et al.* 2004).

There are numerous overlapping activities between IL-6 type cytokines. However, each factor has demonstrated a unique repertoire of biological properties, indicating that strict functional compensation between family members is unlikely. Differences exhibited in signal transduction are most likely a consequence of unique groupings of cytoplasmic receptor domains. For example, LIFR, gp130, and OSMR recruit common and unique sets of intracellular signalling molecules, which may

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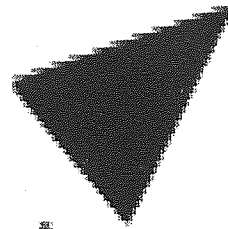
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Figure 1.2 Schematic diagram of subunit sharing for IL-6 type cytokines. LIFR-chains (gp190); (purple) are present in LIF, OSM, CNTF, NP, CLF and CT-1. The gp130 receptor subunit (green) is used by all family members. Additional ligand specific chains are present in the receptors for IL-6, IL-11, IL-27, CNTF, CT-1 and an alternate OSM receptor. Reproduced with kind permission from C. Piquet-Pellorce.

LIFR and gp130 are structurally related. The ectodomain of gp130 contains one cytokine-binding domain (CBD). The CBD contains conserved structural features including a distinct pattern of four cysteine residues in the N-terminal domain and a WSXWS (single letter amino acid designation) motif in the C-terminal domain. The CBD is located between the N-terminal Ig-like domain and three C-terminal fibronectin type III domains, which are adjacent to the single transmembrane domain. The ectodomain of LIFR is almost identical to gp130, except that it contains an additional CBD on the N-terminal side of the Ig-like domain (Auernhammer and Melmed 2000; Heinrich *et al.* 2003) (Fig 1.3).

Intracellularly, the receptors are more divergent, compared with the α - receptors, the signal transducing receptors have characteristically longer cytoplasmic domains. In the membrane proximal region, these cytokine receptors contain so called box1 and box 2 motifs. Box 1 is proline rich and box 2 is dominated by hydrophobic amino acids followed by charged ones. It is these regions that are involved with the recruitment of Janus kinases (Jaks). In addition, the cytoplasmic domains also contain several phosphotyrosine residues, which upon phosphorylation act as docking sites for SH2-domain containing proteins (Heinrich *et al.* 2003).

1.3.2 LIF receptor complexes and binding sites.

The LIF receptor complex (LIFR-gp130) is composed of both high and low affinity receptors. The gene for human LIFR is located on chromosome 5p 12-13 (Gearing *et al.* 1993) and produces a 110kDa protein which can be variably
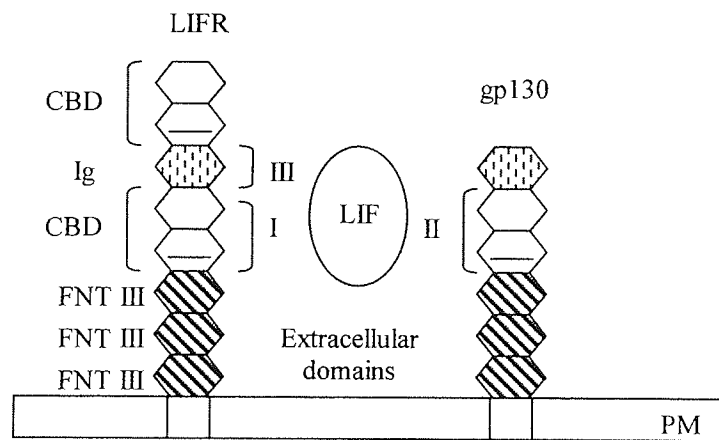
Figure 1.3   Schematic diagram of the LIF-LIFR-gp130 complex.   The extracellular domain of gp130 possesses one cytokine binding domain (CBD) located between the N-terminal Ig-like domain and three fibronectin type III (FNTIII) domains, which are adjacent to a single transmembrane domain. The extracellular domain of LIFR is identical except it contains a CBD on the N-terminal side of the Ig-like domain. LIF possesses three binding regions. Site I has been proposed to contact the membrane proximal CBD of LIFR. Site II binds to the CBD of gp130 while site III is thought to bind to the Ig domain of LIFR. Adapted from Auernhammer and Melmed 2000.

glycosylated to produce a 190kDa protein (Gearing *et al.* 1991). Human gp130 is located on chromosome 5q 11 (Kidd *et al.* 1992) produces a 100kDa protein, which can be variably glycosylated to produce a 150kDa protein (Hibi *et al.* 1990). LIF binds to the low affinity receptor LIFR, (gp190) with an affinity of K_D 10^{-9} M. The intrinsic affinity of LIF for gp130 is much lower than for LIF-R and has not been rigorously determined. However, for binding gp130 in displacement assays with OSM it can displace OSM at 100-fold higher concentrations than OSM itself, which is known to bind gp130 with a K_D of 1nM (Gearing *et al.* 1992; Hudson *et al.* 1996). Association of the LIF-LIFR complex with gp130 results in the conversion to a high affinity complex of K_D 10^{-11}M capable of transducing signals into the cell (Gearing *et al.* 1991; Gearing *et al.* 1992). The increase in affinity as a result of clamping by gp130, reflects a reduction in the off rate of the ligand from the receptor complex.

In addition to the membrane bound form, murine LIFR also exists in a soluble form that lacks the transmembrane and cytoplasmic domains (sLIFR). Both receptor forms are derived from a single gene by alternative splicing. Serum levels of sLIFR are highest during pregnancy in the liver and uterus (Chambers *et al.* 1997). It has been shown that sLIFR inhibits LIF action and as a result may act to counter any undesirable biological affects of LIF (Hui *et al.* 1998). A human form of sLIFR has been identified and is thought to act in a similar manner.

The calcium independent Mannose 6-phosphate Receptor (CIMPR) also acts as a binding site for LIF, and has been found to have nanomolar affinity for glycosylated human LIF binding via its N-linked carbohydrate moieties. It has been suggested that CIMPR could function in two biological processes i.e. protein

trafficking/turnover and transmembrane signalling. Binding of LIF to CIMPR however, has not been observed to cause downstream functional effects, but mediates a rapid internalisation and degradation of LIF thereby affecting its bioavailability (Blanchard *et al.* 1999). Binding to the CIMPR is of interest since two of the cell lines used in this thesis express this receptor.

1.3.3 Gp130/LIFR cytokine-receptor interactions.

Mutagenesis studies have identified distinct areas on the surface of gp130 cytokines that specifically interact with their respective receptors. All IL-6 type cytokines bind to the CBD of gp130, via site II of the cytokines in the middle of the A- and C-helices. The second signalling receptor (either a second gp130, LIFR or OSMR) is recruited to site III at the N-terminus of the D-helix. Site III is recognised by the Ig-like domain of gp130, LIFR or OSMR. Thus, in a homodimer, two different binding epitopes of gp130 are involved in ligand recognition. When a non-signalling α-receptor is involved in the receptor complex, it binds with its CBD to site I on the C-terminus of the D-helix (Heinrich *et al.* 2003). LIF binding to LIFR via site I has been proposed on the basis of molecular modelling, but has not been clearly demonstrated by mutagenesis studies (Auernhammer and Melmed 2000). As the two receptor recognition sites on LIF (II and III) are topologically separate, disruption of site II creates a LIFR antagonist that occupies LIFR in a non-productive complex that fails to recruit gp130 (Hudson *et al.* 1996) (Fig 1.3).

The crystal structure of LIFR has not been solved. However, LIFR mutagenesis studies, have proposed that LIF binds to LIFR via the Ig-like domain of LIFR (Owczarek *et al.* 1997).

1.3.4 IL-6 family cytokine signalling via Jak/Stat pathway.

Gp130 cytokine family receptors lack intrinsic kinase activity. Jaks are intracellular tyrosine kinases that constitutively bind the membrane proximal domains of the cytokine receptors in the absence of ligand. They are the major initiators of signalling induced by cytokines that use these receptors. Ligand induced receptor dimerisation is thought to bring the associated Jaks into close proximity, leading to their activation and receptor autophosphorylation (Fig 1.4). The phosphorylation of tyrosine residues on gp130 and LIFR signal transducing chains creates docking sites for signal transducers and activators of transcription (Stats) factors (mainly Stat1 and Stat 3). Subsequently, Stats become phosphorylated by receptor associated Janus tyrosine Kinases (Jaks), which triggers the release of Stats from the receptors. Stats are activated in the cytoplasm, but they exert their function in the nucleus. Activated Stats are able to form homo- and heterodimers, which are a prerequisite for DNA binding. The mechanism by which Stats enter the nucleus is not fully understood, but the translocation is transient and is thought to be dependent upon tyrosine phosphorylation (Heinrich *et al.* 2003).

Following translocation, Stats bind to specific enhancer sequences and stimulate and/or repress transcription of target genes. The sequences of the preferred DNA elements depend upon the composition of the Stat dimer complex, e.g. Stat 1 and Stat 3 preferentially bind $TTCN_3GAA$ present in the promoter regions of Acute phase proteins (APP) (Heinrich *et al.* 2003). Responses to receptor activation include: APP genes, e.g. C-reactive protein, α antichymotrypsin, lipopolysaccharide
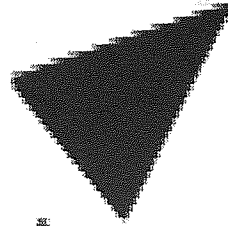
Figure 1.4 Schematic of LIFR-gp130 complex and its molecular signalling pathways. Heterodimerisation of LIFR-gp130 complex by LIF activates Jak kinase activity, followed by phosphorylation of gp130 and LIFR. Phosphorylated tyrosine residues on LIFR and gp130 provide specific docking sites for the SH2 domains of Stat proteins, causing receptor association and subsequent phosphorylation of Stats, which translocate to the nucleus. LIF also stimulates the Ras-MAPK pathway. Reproduced with kind permission of Ann Vernallis.

binding protein and tissue inhibitor of metalloproteinases (TIMPs). In addition, transcription factors such as Jun B and C-Fos are upregulated. Interestingly, LIF has been shown to support the growth of embryonic stem (ES) cells in an undifferentiated state via STAT3 activation (Boeuf *et al.* 2001). This has led to the widespread use of LIF as an essential factor for the *in vitro* maintenance and growth of pluripotent embryonic stem cells.

Upon stimulation, not only are Jaks and Stats activated, but also the mitogen-activated protein kinase (MAPK) pathway, possibly via SHP2 (SH2 domain containing protein tyrosine phosphatase-2). SHP2 interaction with Grb2-SOS complex at the membrane results in the activation of the Ras-Raf-MAPK pathway (Heinrich *et al.* 2003). Stat3 activation and MAPK activation appear to work together to support the proliferation of cells. In BAF cells transfected with LIFR-gp130, stat3 provides an anti-apoptotic signal and MAPK provides a mitogenic signal (Fukada *et al.* 1996).

A mechanism that down regulates the Jak/Stat pathway via the inhibition of tyrosine phosphorylation of gp130, Stat 1 and Stat 3 has been identified. Suppressor of cytokine signalling 1 and 3 (SOCS-1 and SOCS-3) proteins are induced via the Jak/STAT pathway and lead to feedback inhibition of this pathway (Heinrich *et al.* 2003).

1.4 LIF Gene organisation.

The LIF gene shows a complex organisation, which is widely conserved amongst eutherian (placental) mammals (Haines *et al.* 1999). The human gene is 6.2kb, and maps to chromosome 22q.12. The gene encoding OSM lies approximately 16kb away suggesting both products are a result of recent gene duplication. The murine LIF gene maps to a region on chromosome 11, which is more or less syntenic to the human chromosome 22q. Both genes show a sequence identity of around 80%. The human and murine genes consist of three exons and two introns (Fig 1.5). Exons two and three contribute to the mature protein consisting of one hundred and eighty amino acids, whereas exon one determines the nature of the signal sequence. Analysis of LIF gene transcription has identified three alternate LIF transcripts, which contain alternate first exons spliced onto common second and third exons (Haines *et al.* 1999). In mice, translation from AUG initiation codons upstream of the signal peptidase cleavage site, in two of the alternate first exons, yields secreted proteins. One of the transcripts, LIF-D, generates a protein that is associated with a freely diffusible form, whereas the LIF-M transcript is associated with a product that is bound to the extracellular matrix (Rathjen *et al.* 1990). The evidence for this association is discussed in section 1.4.2.

A large number of growth factors have been found to associate with extracellular matrix proteins or heparin sulphate. These include fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and transforming growth factor (TGF-β) (Taipale and Keski-Oja 1997).

Race PCR cloning of the cDNA encoding the murine LIF-M (mLIF-M) transcript has revealed that the cDNA differed from that of the mLIF-D, such that the
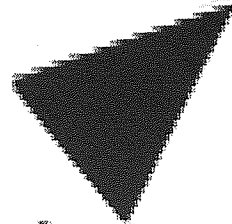
Figure 1.5 LIF gene structure. The first exon determines the nature of the signal peptide and is alternatively spliced to give rise to three transcripts. Exon two and three encode the mature protein. The LIF-D transcript produces a diffusible protein, the LIF-M protein is associated with the ECM, and the LIF-T protein remains intracellular. Reproduced with kind permission of Ann Vernallis.

first part of the signal peptide MKVLAAG, was replaced with the sequence MRCR. The entire leader sequences upstream of amino acid twenty-four are removed in both mature forms of LIF. As the M transcript encodes a biologically active protein that is specifically targeted to the ECM, it has been suggested that the short N-terminal peptide sequence of MRCR may be a protein-sorting signal capable of targeting polypeptides to the ECM (Rathjen *et al.* 1990).

The third transcript for murine LIF (mLIF-T), is initiated by an in-frame initiation codon in exon two, and completely lacks a signal sequence and the amino terminus of the mature peptide. This truncated protein is retained intracellularly. Human LIF (hLIF) transcripts have also been shown to encode both intracellular (T-form) and extracellular (D- and M-forms) proteins. However, there is no in frame ATG initiation codon in the first exon of hLIF-M. As for the mLIF-M transcript, hLIF-M also encodes secreted proteins, however, high levels of intracellular proteins are also translated from the hLIF-M transcript (Voyle *et al.* 1999). This indicates that translation of hLIF-M proteins must sometimes initiate at a non-AUG codon in exon one, thus giving rise to the full mature protein, whilst other hLIF-M transcripts behave as hLIF-T transcripts and are translated at an ATG located in exon two. It is currently unknown if proteins translated from hLIF-M transcripts preferentially associate with the ECM.

1.4.1 Differential Expression of LIF-D and LIF-M.

LIF transcripts are expressed in a temporally and spatially regulated manner by many murine tissues, during both embryogenesis and adult life (Haines *et al.*

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2000). The extent and form of regulation of LIF expression are highly specific, depending on both the phenotype of the responsive cells secreting LIF and the nature of the agonist inducing LIF (Rathjen *et al.* 1990). Using ribonuclease protection analysis, both LIF-D and LIF-M were shown to be expressed during early embryogenesis. Moreover, there was both temporal and tissue-specific regulation of expression of the two transcripts (Rathjen *et al.* 1990). Interestingly, the pattern of transcription changes during development in murine brain tissue. Adult brains produced predominantly high levels of the mLIF-M transcript, whereas embryonic and neonatal brains expressed low amounts of both LIF-D and LIF-M. In addition, it has been shown that neonatal intestine expresses only mLIF-D whereas adult intestine produces both mLIF-D and mLIF-M (Robertson *et al.* 1993). Consistently, human LIF-M (hLIF-M) transcripts have been observed in embryonal carcinoma cell lines (Voyle *et al.* 1999). Differential induction of both murine and human LIF-M and LIF-D was demonstrated in the presence of a variety of specific inducers (IL-1α, bFGF, TGF-β) and repressors (dexamethasone) in a variety of cells (Rathjen *et al.* 1990). LIF mRNA has been detected in the hypothalamus and pituitary. In normal animals, the hypothalamic and pituitary mLIF-M form is more predominant than the mLIF-D form. However, after LPS treatment, the mLIF-D form increased significantly, along with an increase in the mLIF-M form (Wang *et al.* 1996). The apparent fine control over the production of the alternate LIF transcripts would suggest that each serves a biologically distinct role.

1.4.2 Biological activity of alternate LIF isoforms.

The existence of alternate LIF transcripts was first established as a result of using feeder cells to condition culture media in order to maintain embryonic stem (ES) cells as pure populations of stem cells. Stem cells were often grown in medium that had been conditioned by buffalo rat liver (BRL) cells. It was subsequently discovered that maintenance of ES cells was a result of secretion of soluble LIF protein into the medium by BRL cells (Smith *et al.* 1988). In contrast, it was discovered that medium conditioned by other feeder cell lines, i.e. C3H 10T1/2, STO and 3T3 embryonic fibroblasts was relatively inefficient at maintaining ES cells, thus indicating little soluble LIF activity. However, it was found that they could be used to maintain ES cells in direct co-culture arguing in favour of an immobilised form of the LIF protein (Rathjen *et al.* 1990).

To demonstrate the existence of an immobilised form of LIF protein, cell free preparations of ECM were generated from C3H 10T1/2, STO and PYS-2 (parietal yolk sac) feeder layers and were found to significantly inhibit the differentiation of ES cells. This demonstrated the existence of a matrix associated biological activity functionally equivalent to LIF (Rathjen *et al.* 1990). In contrast, ECM preparations from MRC-5 (human fibroblast), which secrete high levels of soluble LIF into their culture medium, did not significantly inhibit the differentiation of ES cells. Subsequent purification and biochemical characterisation of the ECM obtained from feeder cell layers, revealed that ECM associated LIF activity resided in a factor biochemically related to diffusible LIF protein, that is likely to be LIF itself (Rathjen *et al.* 1990).

Investigation into LIF transcription revealed two transcripts. One transcript, named D, encodes the diffusible form of LIF, and high levels correlate with the ability of particular cells to condition media i.e. MRC-5 human fibroblasts. The other transcript, named M, encodes the matrix-associated form of the protein, and was stably expressed by cells that required contact in order to act as feeder cells, i.e. C3H 10T1/2 cells and STO embryonic fibroblasts.

HT1080 (human fibrosarcoma) cells were shown to express relatively low amounts of matrix-bound LIF, however it was possible to stably transfect these cells to obtain clones that expressed significant levels of murine LIF-D and LIF-M. Extracellular matrix preparations from cells expressing the former had marginal effects in suppressing ES cell differentiation, whereas ECM preparations from LIF-M transfectants suppressed the differentiation of ES cells. This finding demonstrated that the proteins encoded by the murine LIF-M transcript could be specifically targeted to the ECM of human HT1080 cells and were biologically active (Rathjen *et al.* 1990). This is one of the key experiments supporting a matrix-localisation for the LIF protein derived from mLIF-M.

Overexpressions of the alternate LIF-D and LIF-M transcripts, in chimeric mouse embryos demonstrated distinct phenotypes during gastrulation. Embryos overexpressing the LIF-D form appeared normal compared to controls, whereas those overexpressing the LIF-M form showed abnormal proliferation of tissues and the absence of differentiated mesoderm (Conquet *et al.* 1992). It would, therefore, suggest that the physiological significance of the alternative transcripts is a result of the distinct physical localization of their products.

The association of LIF with the extracellular matrix (ECM) has been suggested to involve the formation of a specific complex between LIF and an ECM binding protein. Mereau *et al.* (1993) demonstrated that association of LIF with the ECM is an inherent property of the mature LIF protein, implying that diffusible (LIF-D) could also associate with the ECM if it has access to the ECM binding protein. They observed that LIF interacts with two classes of high affinity binding sites on rat UMR cells cultured in monolayers. Using chemical cross-linking studies, they demonstrated that LIF interacts with a 200kD cell associated protein and a 140kD ECM localised protein. The 200kDa protein is likely to be the transmembrane LIFR. The identity of the 140kDa protein is uncertain. The specific association of LIF to the 140kD ECM localised protein was shown to be biologically active, due to its ability to support the growth of ES cells in an undifferentiated state. The molecular mass of the ECM localised binding protein was found to be very similar to those of the secretable form of the LIF receptor (sLIFR). Thus, the ECM binding protein has been suggested to be a secreted form of the LIFR (Mereau *et al.* 1993). Gearing *et al.*(1991) demonstrated the existence of alternative cDNA clones encoding a truncated (140kD) form of the LIFR. In addition, recombinant sLIFR was found to remain physically associated to the cells, possibly via interaction of the FNIII domains with the ECM.

The Mereau *et al.*(1993) study argues that the association of LIF with the ECM is an inherent property of the mature LIF protein. This association is brought about by an interaction with mLIF and the ECM binding protein. Rathjen (Rathjen *et al.* 1990) suggested that cells expressing mLIF-D do not incorporate LIF into their ECM, whereas those expressing mLIF-M are able to do so. In view of these findings, the potential role of the alternate amino termini may be to allow preferential access of

mLIF-M to the ECM binding protein during biosynthesis. However, a prerequisite for LIF binding must be the expression of LIF binding proteins. The phenomenon of ECM localization may extend beyond the expression of the alternate transcripts and the ECM binding protein by individual cells when heterologous cell types are in close proximity. It is possible that, LIF-binding proteins secreted into the matrix by one cell type, could bind LIF secreted by an adjacent heterologous cell type.

1.5   Signal peptides and protein sorting.

In order to determine the localisation of the mature LIF protein, the alternate LIF signal peptides would need to exert their influence within the endoplasmic reticulum since this is where signal peptides are cleaved.

All proteins are synthesised on ribosomes within the cytosol, their fate depends on sorting signals, which are encoded within their amino acid sequence, and directs their delivery to locations throughout the cell. Proteins that do not have sorting signals remain in the cytosol. Those that have sorting signals can be directed from the cytosol to the mitochondria, endoplasmic reticulum (ER) and nucleus. There are two types of sorting signal; signal peptides consist of a stretch of amino acids that are often cleaved once sorting is complete. The other consists of the three dimensional arrangement of amino acids that comprise a signal patch that persists in the finished protein. Although the amino acid sequence of sorting signals can vary significantly, it appears that physical properties e.g. hydrophobicity can be more important than the exact amino acid sequence.

N-terminal signal sequences that direct nascent polypeptides to the ER show little conservation in their sequences. However they can usually be defined by a specific set of characteristics. They are usually characterised by an N-terminal side containing a positively charged stretch of polar residues, a hydrophobic core with a marked preference for leucines or alanines and by polar C-terminal region (Stroud and Walter 1999).

When ribosomes translate proteins destined for secretion or plasma membrane integration, they are directed to pores in the ER by an RNA-multiprotein complex, the signal recognition particle (SRP). Once formed, the SRP-ribosome complex binds to the SRP receptor, which is expressed on the cytosolic face of the ER. Binding of the SRP complex brings about interaction with the translocon (Sec61 complex). The subsequent step, which is also dependant upon a functional signal sequence, is opening of the translocon toward the ER lumen to allow passage of the nascent chain (Kim *et al.* 2002). Once through the translocon, the signal sequence is cleaved by a membrane associated signal peptidase in the lumenal side (Stroud and Walter 1999). Signal sequences have post-targeting roles beyond initiating translocation. Processing of signal sequences can occur either co- or post-translationally, and there exists a time window when the presence of the signal sequence can affect the maturation of the mature portion of the polypeptide (Stevens and Argon 1999). Information encoded in the hydrophobic domain of a number of signal peptides influences the timing and efficiency of at least two steps in maturation, namely N-linked glycosylation and signal sequence cleavage (Rutkowski *et al.* 2003). In addition, ER associated degradation, transport kinetics, protein-protein interactions, or even final protein conformation may be affected (Rutkowski *et al.* 2001).

Whilst minimal differences are seen in their targeting function, signal sequences display a remarkable degree of variation in initiating nascent chain access to the lumenal environment. N-terminal signal sequences mediate targeting of nascent chains to the ER, but also encode critical differences in translocon gating that are coordinated with their respective mature domains to facilitate efficient translocation. Thus a mature domain maybe matched to contain specific sequence elements, i.e. some mature domains may contain structural features that allow it to be efficiently translocated by a weakly gating signal sequence, while other mature domains may absolutely require an effective gating signal sequence for effective translocation (Kim *et al.* 2002).

Rutkowski *et al.* (2003) proposed that prior to its cleavage, the signal sequence keeps the nascent chain in a conformation that is refractory to efficient glycosylation. Cleavage allows glycosylation to occur efficiently. If cleavage takes place after a substantial amount of chain elongation into the lumen has occurred, then glycosylation will be incomplete.

Li *et al.*(2000) examined the kinetics of signal peptide cleavage of HIV envelope protein gp160 and gp120 in several mammalian cell lines, in relation to protein folding, association with chaperones and glycosylation. They demonstrated that cleavage of the signal sequence of gp160 and gp120 is highly inefficient, probably due to the unusually high number of positively charged amino acids within the signal peptide that precedes the hydrophobic core. The majority of newly synthesised but signal sequence retained gp160 and gp120 remained as an unfolded or incompletely folded form in the ER, with extended chaperone binding and slow

transport from the ER-golgi and secretion. Replacement of the natural signal sequence with alternative signal sequences resulted in rapid folding, glycosylation and presentation of gp120 at the cell surface (Li *et al.* 1994). Although, folding of this glycoprotein is slow and as a result it resides in the ER longer, the yield of properly folded molecules is high and degradation is undetectable (Land *et al.* 2003). Since the murine LIF-M signal peptide has two arginines, instead of a single lysine in the mLIF-D form, the mLIF-M-form may have effects on its rate of signal peptide cleavage, which may have subsequent effects on protein maturation.

Changes in naturally occurring signal peptides further demonstrate the importance of the relationship between a signal peptide and its effect on protein maturation. A common T17A polymorphism is found to occur in the signal sequence of the cytotoxic T-lymphocyte Antigen 4 (CTLA4) receptor, on T-cells. CTLA4 is a receptor that regulates immune responses. The T17A polymorphism is associated with an increased risk for autoimmune disease. As the polymorphism is absent from the mature protein, it is hypothesised that its biological effect must involve early stages of protein processing prior to signal peptide cleavage. A threonine-alanine substitution introduces a hydrophobic and α-helix propensity. Although both alleles translocate to the ER and are completely and correctly cleaved, they differ in the fact that up to 1/3 of the CTLA4 proteins that are secreted by the alanine-containing signal sequence are glycosylated on only one of two possible sites that are normally glycosylated. The resulting molecules show altered trafficking in that cell surface expression is decreased, as a result of retro-translocation into the cytosol followed by proteasomal degradation (Anjos *et al.* 2002). These findings demonstrate how signal

peptides can determine the efficiency of post-translational modifications, other than cleavage and suggest inefficient processing can affect the function of mature proteins.

In summary, signal peptides are essential for targeting nascent polypeptide chains to the translocon, and for their subsequent translocation across the ER membrane. In addition, the signal peptides may also influence the post-translational modification of the nascent polypeptide chain. Since LIF isoforms exhibit alternate signal peptides, it is possible that they may influence the folding, post-translational modification and transport of the mature LIF protein.

1.6 Cell polarity.

The polarity of LIF secretion is, as yet, not described and could shed light into how LIF-M transcripts gain access to ECM binding sites. Epithelial cells have demonstrated the ability to preferentially secrete matrix-associated proteins from the basolateral side of the cell. As LIF-M has been shown to associate with the ECM, it is conceivable that LIF-M may also be preferentially secreted in a basolateral direction. This thesis sets out to test this hypothesis.

Most cells in tissues are polarised and have two or more distinct plasma membrane domains, to which different types of vesicles must be directed. Cells that exhibit polarised transport pathways include epithelial cells, endothelial cells, migrating fibroblasts, hepatocytes and neurons. Epithelial cells have been widely used to study the apical and basolateral routes that deliver newly synthesised proteins to the cell surface. A typical epithelial cell has an apical domain, which faces the

lumen: it also has a basolateral domain, which covers the rest of the cell. These routes are not restricted to polarised cells such as epithelial cells, but are present in non-overtly polarised cells such as fibroblasts. Yoshimori *et al.*(1996) compared the membrane trafficking routes of BHK and CHO fibroblasts with those of polarised epithelial cells. Their results suggested that fibroblasts have apical and basolateral cognate routes from the *trans*-Golgi network (TGN) to the plasma membrane. In addition, it has been shown that fibroblasts often transiently polarise in response to acute stimuli such as migrating to close a wound. Migration might require polarised exocytosis at the leading edge to facilitate the building of extracellular matrix, membrane protrusions and adhesion plaques (Schmoranzer *et al.* 2003).

In epithelial cells, extrinsic cues from cell-cell adhesion, result in the formation of cytoskeletal and signalling networks at cell contacts, resulting in partial reorganisation of the cell. However, full establishment of epithelial cell polarity requires both cell-cell and cell-ECM adhesion (Drubin and Nelson 1996). This polarised organisation is the basis for the function of these cells in vectorial transport of ions and solutes across the epithelium, as well as secretion of numerous bioregulatory proteins (Drubin and Nelson 1996). In polarised cells, the transport pathways from the *trans* Golgi network to the plasma membrane operate selectively to ensure that different sets of membrane proteins, secreted proteins and lipids are delivered to the different domains of the plasma membrane.

Transport of transmembrane proteins to the basolateral surface is mediated by cytoplasmic sorting signals. A first set of signals relies on a critical tyrosine residue placed in the context of at least one large hydrophobic amino acid YXXΦ (where X is

any amino acid and Φ is an amino acid with a bulky hydrophobic group). In some case these motifs are predicted to form a structure known as a right β-turn. The second type consists of a motif grouped around a leucine/leucine or leucine/isoleucine pair, as well as other unrelated motifs (Keller and Simons 1997). The YXXΦ and the di-hydrophobic based sorting signals selectively bind to clathrin adaptor protein complexes AP-1 and AP-2. Tyrosine based signals specifically bind to the medium (μ) chains of these complexes. Some evidence suggests that di–hydrophobic signals bind to the β-chains instead. Interactions between tyrosine- and di-hydrophobic signals and AP-1/AP-2 complexes are responsible for cargo-selective sorting into TGN- and plasma membrane-derived clathrin coated vesicles (Nelson and Yeaman 2001). The role of clathrin coated vesicles in transport from the TGN to the plasma membrane has been less certain. More recently AP-1B has been proposed to mediate transport from the TGN to the basolateral membrane. Expression of AP-1B is sufficient to confer correct basolateral sorting of LDL-receptor to the basolateral domain in cells that lack AP-1B (Mostov *et al.* 2003).

The apical pathway is suggested to be fundamentally different, relying upon association with glycosylphingolipids in lipid rafts that form in the membrane of the *trans* Golgi network. These rafts act as sorting platforms for inclusion of proteins destined for the apical membrane (Keller and Simons 1997). Membrane proteins with unusually long transmembrane domains accumulate in the rafts, as well as glycosylphosphatidylinositol (GPI)-anchored proteins. It has also been suggested that N-glycans (Rodriguez-Boulan and Gonzalez 1999), and O-glycans (Yeaman *et al.* 1997) can act as sorting determinants for entry into rafts and apical transporting vesicles. However, apical sorting of many proteins occurs in the absence of N-

glycosylation. Although much is known at present concerning the polarised distribution of transmembrane proteins, relatively little is known about secreted proteins.

1.7   The polarity of LIF secretion.

Whether LIF secretion is polarised is an important question that has been raised in a number of areas. LIF is produced by numerous epithelial cells, including the intestine (Rockman *et al.* 2001), the uterus (Vogiagis and Salamonsen 1999) and the lung (Knight *et al.* 1997). Since the polarised secretion of IL-6 has been described (Mascarenhas *et al.* 1996; Moon *et al.* 2000) it is likely that LIF secretion is also polarised. Basolateral secretion may be physiologically important for some epithelia since it allows communication with stromal cells.

LIF protein and mRNA are present in the luminal endometrium during the luteal phase, and are mainly restricted to the glandular epithelium (Shen and Leder 1992). There is strong evidence that locally secreted cytokines control the implantation process and can cause implantation failure. Murine LIFR-/- gp130-/- embryos undergo normal implantation, suggesting that epithelially derived LIF does not act directly via the blastocyst to allow implantation (Stewart *et al.* 1992). It may regulate implantation by acting on the uterine epithelium in an autocrine/paracrine manner preparing the uterus for implantation. The polarity of LIF secretion from polarised uterine epithelium has not been explored; although its detection in human uterine washings suggests it is released, at least apically from endometrial glands.

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Should it be released bi-directionally, actions on the uterine stroma could also be postulated (Vogiagis and Salamonsen 1999).

Ledee-Bataille *et al.*(2002) analysed uterine flushings from women, to determine the concentrations of LIF at the time of implantation. They proposed that as LIF-T remains intracellular and LIF-M is associated with the ECM, the LIF they detected in uterine flushings was derived from LIF-D (Ledee-Bataille *et al.* 2002). They suggested that a high secretion of LIF in the apical direction towards the uterine lumen is harmful for embryo implantation. Their logic is that an excess of potentially pro-inflammatory cytokines at this stage could have the same detrimental effects on embryo survival as it does on foetal survival in an established pregnancy. Secretion towards the basal cells, on the other hand, seems necessary for implantation: LIF action on the uterine stroma, particularly in controlling trophoblast invasion and placental development is well documented (Salamonsen *et al.* 1997). Stroma also contains migratory cells of lymphoid origin including macrophages, neutrophils, eosinophils, and mast cells and the possibility of endometrial LIF acting on these cells also needs consideration (Vogiagis and Salamonsen 1999).

Another tissue where the polarity of LIF secretion is likely to be important is the lung. The greatest levels of LIF expression in the lungs was observed in fibroblasts, with lower levels of expression in epithelial cells. Once released from epithelial cells LIF may exert a number of effects. There is increasing evidence to support the hypothesis that LIF and related cytokines play a role in the proliferation of epithelial and mesenchymal cells. When the localisation of LIF was investigated the profile of immunoreactivity in human airways showed that LIF is localised to distinct

populations of basally situated cells. It has been suggested that these are possibly primary stem cells from which mucus secreting and cilliary cells are derived. It is not known whether the source of LIF is from the primary stem cells themselves or other cell types. LIF may also exhibit other effects, which may be important during inflammation. LIF is barely detectable in the lung of non-asthmatics, but dramatically increases in mild asthmatics. Evidence suggests that LIF may act to prime and activate eosinophils, which could have roles in inflammation. In diseases such as asthma, the epithelium of the conducting airway is in a chronically activated state with increased turnover of the superficial layers of the epithelium with likely cross-talk between epithelial cells and cells immediately beneath the basement membrane. In this situation, the basolateral secretion of LIF has the potential to interact with myofibroblasts, which may lead to collagen deposition and thickening of the basement membrane, which is a common characteristic of asthma (Knight 2001). The multiple roles of LIF in the lung suggest that the polarity of its secretion will determine its targets and may contribute to disease in chronically activated cells.

Secretion of LIF from the intestinal epithelium has been demonstrated and may have important roles within the intestine and may inform our understanding of the trafficking of LIF. This secretion of LIF by intestinal epithelial cells is a core topic of this thesis and will be described in Chapter 3.

1.8   Glycosylation

Many of the soluble and membrane bound proteins that are processed in the endoplasmic reticulum (ER) are glycoproteins. In a process known as glycosylation

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one or more oligosaccharide chains are covalently linked to an amino acid side chain. Most commonly, sugars are attached to a protein through the NH_2 group of the side chain of an asparagine residue (N-linked) in the endoplasmic reticulum. Less frequently an oligosaccharide chain is added to a protein through the OH- group of a serine or threonine in the Golgi-apparatus (O-linked glycosylation). In the ER, a precursor oligosaccharide composed of N-acetylglucosamine, mannose and glucose, is transferred to the NH_2 group of an asparagine residue by the enzyme oligosacchyrl transferase. Whilst still in the ER modification of the original precursor oligosaccharide occurs and continues in the Golgi, to produce a diverse number of N-linked oligosaccharide structures. As N-linked oligosaccharides are present on most proteins transported through the ER and Golgi, it is perhaps not surprising that glycosylation functions to aid protein folding in the ER and in transport processes guiding ER-Golgi transport, as well as protein sorting in the *trans*-Golgi network. Sugars are also important for protein function; since they can mediate protein-protein interactions. The presence of oligosaccharides tends to make a glycoprotein more resistant to proteases; therefore glycosylation is also important to the stability of proteins.

1.8.1 LIF glycosylation

Since it has been shown that signal sequences can influence many aspects of protein maturation including glycosylation, LIF provides an opportunity to examine whether differences in signal sequences are naturally exploited to regulate protein maturation. The key question for LIF is how does a signal sequence that is cleaved in the ER confer ECM localisation. The alternate signal peptides of LIF may affect

many aspects or protein maturation, such as the rate of signal peptide cleavage, transport kinetics, and glycosylation. It is conceivable, that the alternate signal sequences may alter these aspects of LIF maturation in the ER and may therefore affect protein targeting.

LIF is a heavily glycosylated protein. Murine glycosylated LIF is 38-67 kDa, which can be de-glycosylated to approximately 20 kDa protein. Unglycosylated LIF produced in *E.coli* is biologically active, indicating that glycosylation is not a requirement for receptor activation. There are six possible asparagine-linked glycosylation sites (Asn 9, 34, 63, 73, 96, 116), which are conserved amongst human, mouse, rat, sheep and pig. Aikawa *et al.*(1998) demonstrated that of the glycosyl moieties at each of the N-glycosylation sites (when knocked out individually), none were essential to the function of the protein, but the reduced ability to promote the proliferation of DA-1a cells observed for some mutants suggests a biochemical role.

Immunoprecipitation of the conditioned medium of hLIF-D and hLIF-M transfected cells treated with tunicamycin (an inhibitor of N-linked glycosylation), revealed two apparently identical proteins of approximately 20 and 22kDa. These were deduced to be unmodified and the O-glycosylated forms of the mature hLIF protein. Extracellular proteins encoded by hLIF-M share an identical core polypeptide with the secreted proteins encoded by hLIF-D and differ only in their degree of N-linked glycosylation. Proteins produced by hLIF-M transfected cells contained large amounts of N-linked glycosylation producing a protein approximately 45kDa. Secreted hLIF-D proteins demonstrated size heterogeneity, producing modified proteins ranging from ~20-45kDa (Voyle *et al.* 1999).

1.8.2 N-glycans as sorting determinants.

The existence of specific signals and mechanisms that target proteins to one domain or another are becoming more evident. The polarity of LIF secretion, and the mechanism of trafficking have yet to be defined, but potentially lie within signal patches encoded within the structure of the mature protein. Proteins destined for apical/basolateral domains are sorted into distinct post-golgi carriers in the TGN via various types of signals. These can be encoded in proteinaceous, carbohydrate or lipid moieties and may operate hierarchically.

It has been hypothesised that N-glycans act as apical targeting signals. The role of N-glycans in apical targeting has been studied through the use of drugs affecting the process of N-glycosylation. Treatment of cells with tunicamycin (an inhibitor of N-linked glycosylation) was shown to lead to a random secretion of the normally apically secreted glycoprotein erythropoietin. Unfortunately, studies using tunicamycin are often difficult to interpret, as tunicamycin can result in the intracellular accumulation of incorrectly folded proteins (Huet *et al.* 2003).

Mutations of the N-glycosylation sites of glycoproteins have demonstrated the potential for glycans as apical sorting determinants. Furthermore, they have shown that not all N-glycans in a given glycoprotein have the same ability to promote apical targeting. For the apically targeted glycoprotein, erythropoietin deletion of all of its three N-glycans caused loss of polarised apical secretion. But single or double site removal had effects running from none to complete disruption of apical delivery. The role of N-glycans in apical targeting was also shown through the modification of the

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unglycosylated protein rat growth hormone (GH) by the addition two N-glycosylation sites, which resulted in the almost exclusive 62-92% secretion of GH into the apical medium (Huet *et al.* 2003).

In the carbohydrate sorting signal hypothesis, N-glycans interact with (*trans* Golgi network) TGN lectin sorters to mediate incorporation of the transported protein into apical carrier vesicles. Two lectin-like proteins have been described in the TGN and post–Golgi compartments: VIP-36 a protein with homology to legume lectins, such as conacavalin A and the thyroglobulin receptor, which binds N-acetlyglucosamine residues. They could play a role in the apical targeting of glycoproteins (Rodriguez-Boulan and Gonzalez 1999).

However, a growing body of evidence has begun to cast doubt on the necessity of glycosylation as an apical targeting determinant. A number of apically targeted soluble secretory proteins show no effect of N-glycan removal, including the soluble ectodomain of P75 neurotrophin receptor and VEGF (Rodriguez-Boulan and Gonzalez 1999). Su *et al.*(1999) demonstrated that when MDCK cells were transfected with mutants of GH having none, 1, or 2 sites for N-glycosylation, N-glycosylation promoted apical sorting. However, in ECV304 cells, GH was secreted predominately to the basolateral medium independent of the number N-glycans. This indicates that not all cell lines recognise N-glycans as a signal for apical sorting and may rely upon other signal patches present within a given protein.

In light of the conflicting evidence concerning the role of N-glycans in the apical targeting of glycoproteins, it is conceivable that they could indirectly facilitate

apical sorting of some proteins. N-glycans could stabilise a proteinaceous apical sorting signal required for exiting the TGN or allow the protein to assume a transport permissive conformation (Rodriguez-Boulan and Gonzalez 1999).

In this way, if the signal sequence for LIF determines the glycosylation of LIF, that glycosylation could influence the presentation of proteinaceous signal patches on LIF to the protein targeting machinery. Mechanisms of protein targeting are complex. The importance of each mechanism will inevitably vary according to the cell type and thus for LIF it will need to be assessed in multiple cell types.

1.9   Aims and objectives of study

The aim of this study is to investigate the polarity of LIF secretion. Whether LIF secretion is polarised is an important question. The answer will enlighten our understanding of the trafficking of LIF within cells and inform how dysregulation of LIF trafficking may contribute to disease.

As the polarity of LIF secretion has not yet been described, the endogenous secretion of LIF will initially be studied in Caco-2 cells, a polarised intestinal epithelial cell line. The effects of paracrine factors on the polarity of LIF secretion will also be examined.

Two isoforms of murine LIF that differ in their signal peptides have been described, which exhibit alternate localisation of the mature protein. The effects of the alternate signal peptides on the polarity of LIF secretion will be investigated in polarised epithelial cells. Since no cell lines are known to express the individual LIF transcripts, stable cell lines that express these LIF transcripts will be created. As the cell lines used for expression may already express LIF, LIF will be epitope tagged with FLAG. Epitope tagging is useful as it allows the separation of exogenous from endogenous proteins. A LIF-FLAG ELISA will also be developed in order to detect the tagged proteins.

Prior to the creation of cell lines, a number of LIF expression plasmids will be produced and tested. This developmental work will identify and address any potential problems with the expression plasmids prior to the stable expression of the plasmids

in epithelial cell lines. The work will also allow the selection of appropriate ELISAs for detecting LIF.

Chapter 2: Creation and validation of LIF expression plasmids

2.1   Introduction

Cloning of the cDNA encoding a particular protein, is the first of many steps needed to produce a recombinant protein for analysis. Large amounts of a desired protein can be produced in living cells using expression vectors. These are generally plasmids that have been designed to produce a large amount of stable mRNA that can be translated into protein in transformed bacterial, or transfected yeast, insect or mammalian cells.

Bacterial cells offer simplicity, short generation times, and large yields of product with low cost. However, expression in prokaryotic systems has a number of disadvantages, the most notable being that bacterial cells lack the enzymes that are present in eukaryotic cells that add posttranslational modifications to proteins. These modifications are often required for the proper folding and functioning of proteins. Therefore, transient expression in mammalian cells is often used for evaluating the activity of a newly cloned gene or engineered protein.

A common strategy used to purify recombinant proteins or differentiate them from related cellular proteins is epitope tagging. A fusion protein is produced that contains the entire protein being analysed, plus a tag (epitope). The size of the tag can range from only a few amino acids to a complete protein, which can be attached to either the N- or C-terminus of the desired protein. A major advantage of epitope tagging is that well characterised and highly specific antibodies can be used to study the protein of interest. Epitope tagging facilitates the identification of the fusion protein, as well as to provide a one-step purification procedure for the fusion protein,

by passing the cell extracts or supernatants through columns of the appropriate affinity matrix. Specific cleavage sites engineered between the affinity tag and the protein of choice can enable the removal of tags or release of fusion proteins from the appropriate affinity matrix. Several factors, such as the possibility of an adverse effect of the epitope tag on protein function, requires thorough consideration when selecting the appropriate tag and positioning it within the protein.

To understand which signal peptide has given rise to the mature LIF protein that a cell secretes, cell lines are required that only make a single signal peptide isoform. However, such cells do not naturally exist. The single isoforms could also be expressed exogenously, but it would be necessary to distinguish them from endogenously secreted LIF. In this thesis, epitope-tagging with FLAG™ was used to separate exogenous from endogenous protein expression. FLAG™ tag is a hydrophilic and highly immunogenic tag, which was specifically designed for antibody-mediated identification using Anti-FLAG (M1, M2 or M5) antibody. The FLAG peptide sequence is limited to eight amino acids (AspTryLysAspAspAspAspLys). Such hydrophilic sequences have been shown to express strong antigenicity and are likely to adopt a highly exposed conformation in three-dimensional folding. Due to its small size the FLAG-tag can be encoded by a synthetic oligonucleotide simplifying its addition to the protein of interest (Einhauer and Jungbauer 2001).

The rationale for the tagging of LIF with FLAG, is that it would allow identification of exogenous LIF secreted from cells, which may already be producing LIF. Human LIF-D and LIF-M proteins tagged with FLAG have been described and

expressed in COS cells (Voyle *et al.* 1999). Therefore, FLAG appeared to be a good choice of epitope with which LIF could be tagged. Tagged LIF proteins could be identified separately from any endogenous LIF, by using an enzyme linked immunosorbant assay (ELISA) that incorporated an Anti-FLAG antibody. For example, an anti-FLAG antibody could be used as the capture antibody and an anti-LIF antibody could be used as the detection antibody.

Some of the LIF constructs were expressed as a glutathione-S-transferase (GST) fusion protein to allow simple purification of LIF proteins produced in bacteria. GST is an enzyme that binds glutathione, a tri-peptide. When the coding sequence of the LIF gene is fused to GST, then the fusion protein can be purified in one step by putting the bacterial extract onto a column matrix to which glutathione is immobilised. The GST-LIF protein should be the only protein bound. A protease cleavage (3C rhinovirus protease or thrombin) site can be introduced between the GST coding region and the coding region for LIF to allow the release of LIF protein from the column leaving GST bound.

To purify LIF proteins produced in mammalian cells LIF was expressed as a fusion protein with the Fc region of human IgG1 (hinge-CH2-CH3). The Fc region of IgG1 is associated with multiple effector functions that are associated with different regions, for example, complement fixation, placental transfer and binding to staphylococcal protein A. Protein A is a component of the staphylococcal cell wall that binds to the Fc region of most IgG subclasses, and provides a method with which to purify Fc fusion proteins as well as antibodies. As with the GST fusion, a protease cleavage (3C rhinovirus protease or thrombin) site can be introduced between the

coding region for LIF and the Fc region of human IgG1, to allow the release of LIF protein from the column leaving the Fc bound.

Expressed proteins were analysed by a Ba/F3 cell proliferation assay. Bioassays are an important tool, which may allow an indirect measurement to test the ability of expressed proteins to activate the LIF receptor, in comparison to a known standard. BA/F3 cells are a pro-B-cell line that can be stably transfected with cDNAs encoding cytokine receptors to provide a bioassay for a particular cytokine. They are useful because they express very few endogenous receptors that could interfere with the assay. In this manner, BA/F3 cells have been stably transfected with either both components of the mLIF receptor complex mLIFR and mgp130 (BA/F3-mLIFR-mgp130); (kind gift John K Heath), or both components of the hLIF receptor complex hLIFR and hgp130 (BA/F3-hLIFR-hgp130); (Robinson *et al.* 1994) to generate LIFR ligand dependent cell lines. In this study, BA/F3-mLIFR-mgp130 cells were used to evaluate both hLIF and mLIF constructs, as the murine LIF receptor demonstrates a high affinity for both mouse and human LIF.

In Ba/F3 cells, the Stat3 pathway plays a key role in the G1 to S phase cell cycle transition and prevention of apoptosis, whereas the ERK1/2 and AKT pathway is implicated in the S to G2/M transition and is essential for mitogenesis. In Ba/F3 cells, expressing either IL-11Rα-gp130 or LIFRα-IL-6Rα-gp130, IL-6 type cytokines (LIF, IL-6 and IL-11) were unable to activate ERK1/2 or AKT at low concentrations (10ng/ml) in an acute assay (15 minutes), but did activate Stat3 in serum free medium. High concentrations of cytokine (100ng/ml) were able to activate these pathways but the activation was always low in comparison to activation achieved in the presence of

serum. This suggests that additional factors present in serum are needed to complement signal transduction by low doses of IL-6 type cytokines. The activation of ERK1/2 and AKT appears to be initiated by activation of the calcium independent mannose 6-phosphate receptor (CIMPR) by insulin like growth factor II (IGF-II), which is present in Foetal calf serum (FCS). It has been proposed, that the specific transduction pathways, activated by IL-6 type cytokines and IGF-II, complement each other to sustain proliferation and survival of BA/F3 cells (Duplomb *et al.* 2003). When modified LIF proteins promote the proliferation of BA/F3 mLIFR/mgp130 cells, it can be concluded, that at least the activation of Stat3 is acting normally.

2.2 Materials and Methods

2.2.1 Molecular biology

2.2.1.1 Bacterial strains.

Subcloning efficiency DH5α cells (Gibco) were used to carry out transformation experiments. Cells were used in accordance with the manufacturer's guidelines (appendix 1). 100μl aliquots of the transformed cells were plated on Luria Broth (LB) agar (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% bacteriological agar) containing 100μg/ml ampicillin and incubated at 37°C overnight.

2.2.1.2 Purification of plasmid DNA.

Plasmid DNA was isolated from transformed DH5α cells, using QIAprep spin miniprep and Endofree plasmid maxiprep kits (Qiagen), and were used according to the manufacturer's guidelines (appendix 2,3).

2.2.1.3 Agarose gel electrophoresis.

Gels were made using 1% agarose (Gibco BRL) in 1 X TAE (40mM Tris-acetate, 1mM EDTA) for purification of DNA fragments or 1 X TBE (45mM Tris-borate, 1mM EDTA) for visualisation of DNA and ethidium bromide (0.005%). Prior to loading, samples were prepared in 1 X loading buffer (0.09% bromophenol blue, 0.09% xylene cyanol, 60% glycerol, 60mM EDTA). In order to estimate fragment size, 1KB DNA ladders (MBI fermentas) were run on gels in addition to the samples. Electrophoresis was carried out at 100mA. Gels were visualised under UV light using a transilluminator. For documentation, gels were analysed using a Genelink

(Syngene) camera and software (exposures were varied between gels to optimise the detection of the DNA fragments).

2.2.1.4 Purification of DNA from agarose gels.

DNA bands of the desired size were removed from agarose gels using sterile scalpels. DNA was recovered using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions (appendix 4).

2.2.1.5 Restriction digests.

Restriction enzymes were used in conjunction with appropriate buffers as recommended by the manufacturer (Promega). Typical reactions consisted of DNA at a concentration of 50ng/μl.

2.2.1.6 Ligation reactions.

Ligations were performed using standard protocols. Ligations were carried out in reaction volumes of 10μl including 1unit T4 DNA ligase (Promega) and 1 X ligation buffer (Promega), approximately 30fmol of appropriate vector and an equal ratio of insert DNA. DNA fragments were added together in sterile water and warmed to 45°C for five minutes. The mixture was then chilled to 0°C on ice before adding the other reagents. Reactions were incubated at 16°C for sixteen hours (Techgene thermocycler).

2.2.1.7 Alkaline phosphatase (dephosphorylation of cDNA ends).

Reactions were carried out in 50μl volumes containing DNA (up to 10 pmol of 5'-ends), 1X Calf intestinal alkaline phosphatase (CIAP) reaction buffer and CIAP (up to 0.05 units) (Promega). Reactions were incubated for 30 minutes at 37°C, after which time a further aliquot of CIAP (up to 0.05 Units) was added, and incubated for a further 30 minutes. Following the incubation, 300μl of stop solution (10mM Tris-HCL pH 7.5, 1mM EDTA pH 7.5, 200mM NaCl, 0.5% SDS) was added.

2.2.1.8 DNA sequencing.

Sequencing of all constructs created by PCR was carried out at either Alta-Biosciences or the Functional Genomics Laboratory, Birmingham University, using appropriate sequencing primers (appendix 6).

2.2.1.9 Polymerase Chain Reaction (PCR).

Reactions were performed in 50μl volumes, containing 1 X PFU buffer (Stratagene), 2.5 units PFU DNA polymerase (Stratagene), 100μM dNTPs (Promega), 20μM of appropriate forward and reverse primers (MWG Biotech); (appendix 7), and 5ng DNA template. PCR reactions were carried out in a Techgene thermocycler. A PCR cycle of 25 cycles was carried out at the following temperatures. For a hot start, one cycle at 95°C for 3min was carried out; PFU DNA polymerase was then added, followed by 25 cycles of 94°C for 30 seconds, 45°C for 45 seconds followed by 72°C

for one minute. The final cycle consisted of 94°C for 30 seconds, 45°C for 45 seconds followed by a final extension of 72°C for 10 minutes

2.2.2   Production of mLIF constructs.

2.2.2.1 Construction of pGex-3C-mLIF-FLAG (EJH-1).

Murine LIF cDNA, excluding the signal sequence, was isolated from the plasmid pGex 2T mLIF (Mereau *et al.* 1993) by polymerase chain reaction (PCR) using oligonucleotide primers (appendix 7) directed against the 5' and 3' end of the mLIF coding region. To create the mLIF-FLAG insert, primers were designed to incorporate a Bam H1 restriction site in the forward primer. The Flag epitope (DYKDDDDK) was inserted in frame prior to the stop codon, and was followed by the restriction site Eco R1, in the reverse primer. PCR products were purified using the QIAquick PCR purification kit (appendix 5); (Qiagen); overhangs were created by Bam H1, Eco R1 digestion (section 2.2.1.5). The mLIF-FLAG insert was then ligated (section 2.2.1.6) into the Plasmid pGex-3C (kind gift of JK Heath), which had been linearised by the restriction endonucleases Bam H1 and Eco R1 (section 2.2.1.5), to create the plasmid pGex-3C-mLIF-FLAG (EJH-1). Inserts produced using PCR were sequenced (section 2.2.1.8) to ensure that no mutations were present in the insert (For vector maps, see appendix 8).

2.2.2.2 Construction of pcDNA3-mLIF-M-FLAG (EJH-2) and pcDNA3-mLIF-D FLAG (EJH-3).

Murine LIF cDNA encoding the signal sequences for the diffusible and matrix associated form of LIF were isolated from the plasmids pXMT2 mLIF-D, and

pXMT2 mLIF-M respectively (Rathjen *et al.* 1990). It was possible to excise the desired N-terminal half of the mLIF cDNA by virtue of a Sma 1 restriction endonuclease site in the centre of the LIF coding sequence. Both mLIF-D and mLIF-M fragments were excised using the endonuclease Xho 1 (5') and the Sma 1 (3') izoschizmer Xma 1. The 3' end of epitope tagged mLIF cDNA was isolated from the plasmid pGex-3C-mLIF-FLAG (EJH-1) (section 2.2.2.1) and by excising it with the restriction endonucleases Xma 1 and Eco R1. Fragments were isolated by gel electrophoresis and extracted using QIAquick gel extraction kit (Qiagen). Three way ligations were set up using the 5' mLIF-M/mLIF-D fragments, the 3'mLIF-FLAG fragment and pcDNA3.1(-) (Invitrogen) linearised with the restriction endonucleases Xho 1 and Eco R1 to create the plasmids pcDNA3-mLIF-D-FLAG (EJH-3) and pcDNA3-mLIF-M-FLAG (EJH-2).

2.2.2.3 Construction of pcDNA3.1-mLIF-M (EJH4) and pcDNA3.1-mLIF-D (EJH5).

Murine LIF cDNA encoding the LIF-D signal peptide was isolated from the plasmid pXMT2 mLIF-D (Rathjen *et al.* 1990) using the enzyme EcoR1. The fragment was ligated into the plasmid pcDNA3.1 (-) that had been linerised with the restriction endonuclease EcoR1. The linearised plasmid was treated with Calf intestinal alkaline phosphatase to prevent recircularisation of the plasmid during ligation (section 2.2.1.7). The fragment was then ligated into the plasmid, pcDNA3.1 (-), that had been linearised with the enzyme EcoR1, to create the plasmid pcDNA3-mLIF-D (EJH-5). Murine LIF cDNA encoding the LIF-M signal peptide was taken from the plasmid pXMT2 mLIF-M (Rathjen *et al.* 1990) using the endonuclease

EcoR1 and Xho 1. The fragment was then ligated in to the plasmid, pcDNA3.1(-), that had been linearised with the enzymes EcoR1 and Xho 1, to create the plasmid pcDNA3.1-mLIF-M (EJH4). These plasmids were used as intermediaries, to prepare pBI-L-mLIF-M (EJH17) and pBI-L mLIF-D (EJH18) plasmids (section 4.2.4)

2.2.2.4 Construction of pcDNA3.1-mLIF-D-Fc (EJH6).

In order to create C-terminal fusion proteins with the Fc region of human IgG it was necessary to produce the entire coding sequence for the mature mLIF protein excluding the stop codon (TAG). Murine LIF cDNA including mLIF-D signal peptide followed by the coding sequence for the mature mLIF protein excluding the stop codon (TAG) was created by PCR. The 5' oligonucleotide primer was directed at the 5' end of the signal peptide and the 3' oligonucleotide was directed to the 3' end of the LIF coding sequence. The 5' oligonucleotide incorporated a BamH1 site for cloning and the 3' oligonucleotide incorporated an EcoR1 site. cDNA encoding a 3C Rhinovirus protease cleavage site and the Fc portion of IgG was isolated from the plasmid pSecTag2BHSC-Fc (kind gift of JK Heath). Three way ligations were performed that included the plasmid pcDNA3.1(-) linearised with the restriction enzymes BamH1 and EcoR1, the modified to create the plasmid pcDNA3.1-mLIF-D-Fc (EJH6).

2.2.3 Construction of hLIF expression plasmids.

2.2.3.1 Construction of pcDNA3.1-hLIF-D (EJH7) and pcDNA3.1-hLIF-D-FLAG (EJH8).

Although a plasmid that already expressed human hLIF-D was available in the lab, it was not known how the human LIF coding sequence was cloned in to this plasmid. To ensure which restrictions sites flanked the coding sequences of human LIF, cDNA was isolated from the plasmid pcDNA3.1-hLIF (kind gift of Ann Vernallis) by PCR. The 5′ oligonucleotide was directed at the 5' signal peptide and included a BamH1 site and the 3′ oligonucleotide was directed to the 3' end of the LIF coding sequence and included EcoR1 restriction sites. Two way ligations were set up that included the hLIF cDNA and pcDNA3.1 (+) that had been linearised using the restriction enzymes BamH1 and EcoR1, to create the plasmid pcDNA3.1-hLIF-D (EJH7). pcDNA3.1-hLIF-D-FLAG (EJH8) was created in the same manner, with the exception that the oligonucleotide that was directed to the 3'end of the LIF coding sequence, contained the FLAG peptide sequence and a stop codon.

2.2.3.2 Construction of pcDNA3.1-hLIF-D-FLAG-no stop codon (EJH9) and pcDNA3.1-hLIF-M-no stop codon (EJH10).

In order to create additional hLIF constructs it was necessary to create intermediate constructs, which could then be manipulated. Human LIF cDNA was isolated from the plasmid pcDNA3.1-hLIF (kind gift of Ann Vernallis) by PCR. Human LIF cDNA including BamH1 (5') and EcoR1 (3') restriction sites, the hLIF-D or hLIF-M signal peptides, and either hLIF excluding a stop codon or hLIF FLAG (3') excluding a stop codon (TAG), were created using oligonucleotide primers directed at the 5′ and 3′ ends of the hLIF coding region. This process was used to create the plasmids; hLIF-D-FLAG-no stop codon (EJH9) and hLIF-M-no stop codon

(EJH10). The no stop codon constructs were used in the creation of the Fc constructs described subsequently.

2.2.3.3 Construction of pcDNA3.1-hLIF-M (EJH11) and pcDNA3.1-hLIF-M-FLAG (EJH12) and pcDNA3.1-hLIF-D-with no stop codon (EJH13).

By virtue of an internal Sma1 site within the hLIF cDNA it was possible to create additional hLIF plasmids by shuttling alternative 5′ (BamH1-Sma1) fragments containing either the hLIF-D or hLIF-M signal peptides or 3′ (Sma1-EcoR1) fragments containing the 3' end of the LIF coding sequence (with or without the FLAG-tag) between previously created plasmids. This reduces the number of PCR reactions necessary to create the required plasmids and saves on sequencing.

To create the plasmid pcDNA3.1-hLIF-M (EJH11), the 5'(BamH1-Sma1) portion of hLIF-M-no stop codon (EJH10) containing the hLIF-M signal peptide, was ligated together with the 3′ (Sma1-EcoR1) portion of pcDNA3.1-hLIF-D (EJH7) containing the 3' end of the LIF coding sequence, and BamH1 and EcoR1 linearised plasmid pcDNA3.1 (+) in a three way ligation.

pcDNA3.1-hLIF-M-FLAG (EJH12) was created using the 5'(BamH1-Sma1) portion of hLIF-M-no stop codon (EJH10) containing the hLIF-M signal peptide, the 3′ (Sma1-EcoR1) portion of pcDNA3.1-hLIF-D-FLAG (EJH8) containing the FLAG tagged 3' end of the LIF coding sequence and the BamH1 and EcoR1 linearised plasmid pcDNA3.1 (+) in a three way ligation.

The plasmid pcDNA3.1-hLIF-D-with no stop codon (EJH13) was created using the 5'(BamH1-Sma1) portion of pcDNA3.1-hLIF-D-FLAG (EJH8) containing the hLIF-D signal peptide, the 3' (Sma1-EcoR1) portion of hLIF-M-no stop codon (EJH10) containing the hLIF coding sequence lacking a stop codon, and the BamH1 and EcoR1 linearised plasmid pcDNA3.1 (+) in a three way ligation. This plasmid was an intermediate plasmid, which was used to create the Fc constructs described subsequently.

2.2.3.4 Construction of pcDNA3.1-hLIF-D-FLAG-Fc (EJH14) and pcDNA3.1-hLIF-D (EJH15).

Fc tagged versions of pcDNA3.1-hLIF-D-FLAG and pcDNA3.1-hLIF-D, were created by firstly digesting the plasmid Psectag2B-HSC-Fc (Kind gift JK Heath) with the restriction endonucleases EcoR1 and Xho1 to produce a fragment containing the Fc portion of immunoglobulin G and an upstream 3C Rhinovirus protease cleavage site. This fragment was then ligated into the plasmid pcDNA3.1-hLIF-D-FLAG-no stop codon and pcDNA3.1-hLIF-D-no stop codon, that had been linearised using the restriction endonucleases EcoR1 and Xho1, to create the plasmids pcDNA3.1-hLID-D-FLAG-Fc (EJH14) and pcDNA3.1-hLIF-D-Fc (EJH15).

2.2.4 Bacterial LIF expression.

2.2.4.1 Culture growth and induction of bacterial mLIF, hLIF and mLIF-FLAG.

Shaking Cultures (50ml) of LB 50µg/ml Ampicillin (Sigma) were inoculated with a single colony of *E.coli* strain JM109 containing the pGex-2T-mLIF, pGex-2T-

hLIF or DH5α containing the expression plasmid pGex-3C-mLIF-FLAG (EJH-1) (section 2.2.2.1) and grown overnight at 37°C. The Following morning cultures were diluted 1:10 with LB (50μg/ml Ampicillin) and grown with agitation (37°C) until optical density at 600nm (O.D$_{600}$) reached 0.7 (for approximately two to three hours). The temperature was then reduced to 25°C, and left for twenty minutes to allow the temperature to equilibrate. To induce protein production, 0.001M isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma) was added and the culture was left to grow until the O.D$_{600}$ had climbed to 1.2 (approximately four hours). Cultures were then centrifuged at 6,370 X g at 4°C (Beckman) for ten minutes. The pellet was then resuspended in 5ml MTPBS (150mM NaCl, 16mM Na$_2$HPo$_4$, 4mM NaH$_2$Po$_4$), plus Complete^{TM} mini protease inhibitor tablets (Roche) (1 tablet/10ml MTPBS) and frozen at -70°C until required.

2.2.4.2 Protein purification of bacterial mLIF and hLIF.

Proteins were purified in a similar manner as outlined by (Hudson *et al.* 1996). Bacteria (section 2.2.4.2) were lysed by sonication (Jencons ultrasonic processor) at 60-80% power in pulses until the lysate became a dark brown. Triton X-100 (Roche) was added to a final concentration of 1%. Lysates were then centrifuged at 27,200 X g for 20 minutes at 4°C (Beckman). A 0.8 X 4cm disposable polypropylene column (Biorad) was set up, containing a volume of 400μl glutathione sepharose (Amersham Pharmacia Biotech). The column was then washed; once with MTPBS alone, and once with MTPBS plus 1% Triton X-100. Supernatant was then loaded, and allowed to pass through the column. Further washes were then carried out to remove any unbound protein; 1X 2.5ml MTPBS plus 1% Triton, 1X 2.5ml

MTPBS alone, 2X 3ml wash buffer (50mM Tris pH 8.5, 150mM NaCl), 2X 3ml digestion/elution buffer (50mM Tris-Cl pH 8.5, 150mM NaCl, 2.5mM $CaCl_2$). The column was then closed off and 500μl digestion/elution buffer containing 35 units of thrombin (Sigma) was added and left for four hours at room temperature with mixing every hour. After four hours the digestion was eluted, and the column washed with two further aliquots of digestion/elution buffer and each fraction saved. To remove any aggregates and possible microbial contamination, fractions were spun for 30 minutes in a refrigerated microfuge at full speed (Sanyo Hawk 15/05) at 4°C. Protein concentrations were determined using the Coomassie plus protein assay reagent kit (Pierce), (section 2.2.6.1) against a Bovine Serum Albumin (BSA) standard (Pierce). The purified proteins were stored at -70°C until required.

2.2.4.3 pGex3C bacterial mLIF-FLAG protein purification.

Isolation of bacterial mLIF-FLAG was carried out in the same manner as outlined above, with the exception that cleavage of the fusion protein was achieved using 10μg 3C Rhinovirus protease (Staunton et al. 1998) incubated at 4°C overnight. Buffers for washing and eluting proteins from the column were also changed to optimise conditions; wash buffer for 3C (50mM Tris pH 8.0, 150mM NaCl, 10mM EDTA), digestion/elution buffer for 3C (50mM Tris-Cl pH 8.0, 150mM NaCl, 10mM EDTA and 1mM DTT added fresh).

2.2.5 Mammalian LIF expression.

2.2.5.1 Culture of 293T cells.

293T cells are a human epithelial kidney cell line, transformed with the SV40 large T-antigen and have been selected for transient expression studies because of their high transfectability. Cells were cultured for the transient transfection (section 2.2.5.2) with expression vectors. 293T cells were maintained in DMEM with L-glutamine, high glucose (Gibco), 10% FCS (Labtech), 50 units/ml penicillin and 50μg/ml streptomycin (Gibco). When cultures reached 90% confluency, cells were split by tapping the culture vessel until all cells had become loose. Cells were then transferred to a sterile universal container and centrifuged at 194 X g for five minutes to pellet the cells. Cells were then resuspended in fresh culture medium and re-plated at a ratio of 1:10.

2.2.5.2 Transient transfection of 293T cells.

The day before transfection, cells were plated at 5 X 10^4 cells/cm^2 in a 25cm^2 flask, so that on the following day they were at 50-70% confluence. The DNA/CaPO$_4$ precipitate was prepared by aliquoting 26μl 2M CaCl$_2$, 10μg DNA was then added. The solution was made up to 250μl with sterile H$_2$O. The DNA/ CaPO$_4$ mixture was added drop wise to an equal volume of 2 X Hanks Buffered Saline (HBS) adjusted to pH 7.12 with NaOH (prepared from a 10 X HBS stock; 8.18% w/v NaCl, 5.94% w/v Hepes, 0.2% w/v Na2HPO4), to form a fine white precipitate. The precipitate was incubated at room temperature for five minutes, and then added drop wise to flasks containing cells, and placed in a humidified 37°C, 5% CO$_2$, 95% air mixture incubator for twenty four hours. To check the efficiency of transfection, additional cells were transfected at the same time with a plasmid containing the reporter β-

galactosidase. Transfected cells were then visualised after twenty-four hours using β-gal staining (section 2.2.5.3).

2.2.5.3 β-Galactosidase staining.

Prior to fixing cells that had been transfected with the plasmid pcDNA3-β-galactosidase, cultures were aspirated and washed two times with PBS. Cells were fixed by adding 2-3ml of cell fixative (2% v/v formaldehyde, 0.2% v/v glutaraldehyde in 1 X PBS). Cells were washed once using 1X PBS. To stain the cells, 5ml β-galactosidase stain (5mM $K_3Fe[CN]_6$, 5mM $K_4Fe[CN]6$, 2mM $MgCl_2$ in 1X PBS and 1mg/ml 5-bromo-4-chloro-3-indolyl-B-galactoside in DMF) was added to the cells. Cells were then incubated for twenty-four hours at 37°C and then washed with PBS. Monolayers were then visualised microscopically to assess the efficiency of transfection by estimating the percentage of blue cells.

2.2.5.4 Harvesting of proteins from transiently transfected 293T cells.

After sixteen hours (section 2.2.5.2) incubation with $DNA/CaPO_4$ precipitate, the medium was aspirated from the dishes and then washed once with complete medium (section 2.2.5.1) and again with PBS (Sigma). UltraCho cell medium (Bio-Whittiker), which is serum free, was then gently added to the flasks, which were then returned to the incubator. The cells were left for three days to express protein, after which time conditioned medium was removed. Conditioned medium was firstly centrifuged at 194 X g for 10 minutes to remove cellular debris, and then sterile filtered before it was stored at 4°C.

2.2.5.5 Purification of LIF-Fc fusion proteins.

293T cell conditioned media (section 2.2.5.4) were collected from five 162cm^2 flasks and centrifuged firstly at 194 X g for five minutes, transferred to fresh 50ml tubes, and then centrifuged at 1750 X g for 15 minutes to remove cellular debris. To protect the protein from degradation, 100mM Tris pH 8.0, 5mM EDTA and 1mM phenylmethylsulphonyl fluoride (PMSF) were incorporated into the conditioned medium. A vacuum filtration system was employed to further clarify the conditioned medium using a 47mm extra thick glass fiber filter (Gelman sciences, Michigan) and 0.45μm Supor$^®$ sterilization membrane (Pall corp, Michigan) to remove particulates. A disposable 0.8 X 4cm polyprop column (Biorad) was set up, containing a volume of 400μl protein A sepharose (Amersham Pharmacia Biotech). The column was washed three times using wash buffer for 3C protease (section 2.2.4.3). Conditioned medium was loaded and allowed to pass through the column, and then recirculated to allow efficient binding of Fc-tagged proteins. The column was then washed a further 2 times using wash buffer for 3C, and then using digestion/elution buffer for 3C (section 2.2.4.3) to remove any unbound protein. The column was then closed off and 500μl digestion/elution buffer containing 2μg of 3C rhinovirus protease was added and the column was incubated for sixteen hours at 4°C to allow digestion. After 16 hours the free proteins were eluted, and the column was washed with two further aliquots of digestion/elution buffer and each fraction saved. To remove any aggregates and possible microbial contamination, fractions were spun for 30 minutes in a refrigerated microfuge at full speed (Sanyo Hawk 15/05) at 4°C. Protein concentrations were determined using the Coomassie plus protein assay reagent kit (Pierce); (section 2.2.6.1) against a Bovine Serum Albumin (BSA) standard (Pierce).

2.2.5.6 Protein A sepharose immunoprecipitations.

A bead volume of 10µl of protein A sepharose was resuspended in 1ml 100mM Tris pH 8.0 and pelleted at 16,249 X g for 10 seconds. The pellet was then resuspended in a further 1ml 100mM Tris pH 8.0 and washed a further two times in the same manner. 500µl of clarified conditioned medium (containing 100mM Tris pH 8.0, 5mM EDTA and 1mM PMSF) was added to 10µl protein-A sepharose. The reaction was incubated for 90min at 4°C on a shaking platform. The sepharose was then pelleted at 16,249 X g for 10 seconds and washed three times to remove any unbound protein. An equal volume (approximately 10µl) of 2 X sample buffer (deionised H_2O, 5% glycerol, 12.5mM Tris-HCl pH 6.8, 0.4% SDS, 0.002% bromophenol blue and 1% β-mercaptoethanol added fresh) was then added, and incubated at 100°C for three minutes. The sample was then centrifuged at 16,249 X g for 10 seconds and the supernatant examined by SDS PAGE (section 2.2.6.2).

2.2.5.7 FLAG-fusion protein immuopreciptiations.

Flag fusion proteins were immunoprecipitated using Anti-Flag® M2 affinity gel (Sigma). The Anti-flag M2 affinity gel was thoroughly resuspended, and a 40µl aliquot was immediately transferred to a microcentrifuge tube. The resin was then centrifuged at 9700 X g to pellet the resin and allowed to settle for 2 minutes before the supernatant was removed. The 20µl packed gel volume was then washed twice with 500µl TBS (50mM Tris-HCl, 150mM NaCl, pH 7.4). In order to remove any of the unbound Anti-flag antibody the resin was washed with 500µl 0.1M glycine-HCl,

pH 3.5, followed by three washes with 500μl TBS. To the washed resin 500μl of conditioned medium or purified protein in TBS was added and gently agitated for 2 hours at room temperature. The resin was then centrifuged for five seconds at 9,740 X g and the supernatant was removed. The resin was then washed three times with 500μl TBS. In order to elute the flag fusion protein an equal volume of 2 X sample buffer (excluding 2-mercaptoethanol) was added to each sample, which was then incubated at 100°C for three minutes. Following the incubation, samples were centrifuged at 9,740 X g for five seconds to pellet any undissolved agarose and examined using SDS PAGE (section 2.2.6.2).

2.2.6   Protein analysis

2.2.6.1 Protein assays

Protein concentrations were estimated using the Coomassie® Plus protein assay reagent kit (Pierce), according to the manufacturer's guidelines. Briefly, 100μl of samples and BSA standards (0-25μg/ml) diluted in sodium azide (150mM NaCl, 2% w/v azide) were added to microplates (Corning), together with Coomassie reagent (100μl) and the A_{570} was measured (MRX microplate reader, Dynex Technologies).

2.2.6.2 SDS PAGE.

Protein gels were elecrophoresed using the Mini Protean® 3 Cell (Biorad). Gels were cast according to the manufacturer's guidelines. Samples were prepared in sample buffer (section 2.2.5.6) and loaded onto the gel, together with molecular weight markers. Electrophoresis was carried out at 200V (max), 60mA (max) for

approximately forty-five minutes or until the bromophenol blue reached the bottom of the resolving gel. Plates were then carefully separated, and the gel placed in Coomassie Brilliant blue stain (0.1% Coomassie, 50% methanol, 7% acetic acid), for approximately one hour on a slowly rocking platform. Gels were then destained (50% methanol, 7% acetic acid) and photographed.

2.2.6.3 Western blot transfer.

Proteins for western blot transfer were first separated by SDS PAGE (section 2.2.6.2); gels were then equilibrated in transfer buffer (25mM Tris, 192mM glycine, 10% methanol) before being transferred to pre-wetted (in methanol) immobilon-P transfer membrane (Millipore) trimmed to the exact dimensions of the gel. The gel, and immoblilon-P were then sandwiched between four pieces of Whatman filter paper and two pieces of sponge, pre-soaked in transfer buffer. Transfers were performed using the mini trans-blot electrophoretic transfer cell (Biorad) at 30V, 90mA overnight.

2.2.6.4 Western blot analysis.

Immobilon-P membranes (section 2.2.6.3) were blocked in TBS (8g NaCl, 0.2g KCl, 3g Tris-base in one litre H_2O) 5% powdered milk for two hours at room temperature, then rinsed in TBS 0.01% Tween (Sigma). They were then placed in TBS 0.01% Tween, 3% powdered milk containing the appropriate primary antibody and incubated overnight at 4°C on a shaking platform. Mouse anti-FLAG M2 monoclonal antibodies (Sigma, catalogue #F3165) were used as primary antibody

(1:5000) to detect the FLAG epitope. The following day membranes were washed extensively 6X for five minutes in 100ml TBS 0.01% Tween, to remove unbound antibody, and then placed in TBS 0.01% Tween 3% powdered milk, containing the appropriate secondary antibody for one hour at room temperature, with agitation. To detect the anti-FLAG M2 antibody, the secondary antibody, anti-mouse immunoglobulin peroxidase (1:10000 dilution); (Amersham Pharmacia) was used. After extensive washes in TBS 0.01% Tween, and then TBS alone, membranes were placed in Supersignal west-pico chemiluminescent substrate (Pierce) for five minutes. Membranes were then immediately sandwiched between acetates (Lloyd Parton, Manchester) and exposed to photographic film (Amersham Pharmacia Biotech) in a developing cassette. To visualize the bound antibody, exposed films were developed using developer (Sigma), and following extensive washes, fixed using fixer (Sigma) in a darkroom.

2.2.7 Biological assays.

2.2.7.1 BAF cell proliferation assay.

BA/F3 mLIR-mgp130 cells are a pro-B-cell line that have been stably transfected with cDNA, encoding both components of the mLIF receptor: mLIFR and mgp130 (BA/F3-mLIFR-mgp130) (kind gift JK Heath). They were maintained in RPMI 1640 (Gibco), supplemented with 10% FCS (Labtech), 50U/ml penicillin, 50µg/ml streptomycin (Gibco), and recombinant human LIF (20ng/ml) (section 2.2.4.2).

Activity of purified LIF proteins and conditioned medium was assessed using the BAF proliferation assay. Assays were carried out in ninety-six well (low evaporation) flat-bottomed plates (Costar). RPMI 1640, 10% FCS was used to prepare two-fold dilutions (triplicates) of purified LIF or conditioned medium, across the plate. BA/F3-mLIFR-mgp130 cells were washed 4X with RPMI 1640, 10% FCS before being diluted to a density of 5×10^4/ml. Cells (50µl) were added to 50µl of purified LIF protein or conditioned medium (total volume of 100µl), and incubated for seventy-two hours at 37°C in a humidified 5% CO_2, 95% air mixture incubator. Proliferation was measured using 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay. In the MTT assay, the tetrazolium salt MTT is reduced by metabolically active cells in part by dehydogenase enzymes to form a coloured, water insoluble formazan salt. The resulting intracellular purple formazan can be solubilized, and quantified by spectrophotometric means (Mosmann 1983).

2.2.7.2 MTT reduction assay

After a seventy-two hour growth period 20µl of filter sterilised (0.2µm sized pore) MTT (2.5mg/ml in PBS), was added to each well of BA/F3-mLIFR-mgp130 cells and incubated for four hours. After such time, formazan crystals that had formed in viable cells were solubilised by adding 100µl 0.01% SDS 0.01 N HCL to each well, and returned to the incubator overnight. The optical density of each triplicate sample was measured the following day using a microplate spectrophotometer (MRX microplate reader, Dynex Technologies) at 570 nm and the background was subtracted. Background was determined by measuring the OD_{570} of well that received medium alone (RPMI 1640) 10% FCS, with no additional cytokines) plus cells.

2.2.7.3 Data analysis.

The EC50 of LIF protein in BA/F3-mLIFR-mgp130 cell assays was determined using Graphpad Prism non-linear regression curve fit (variable slope).

2.3 Results.

2.3.1 Purification and analysis of bacterial mLIF, mLIF-flag and hLIF proteins.

In order to determine whether the D- or the M- form of LIF has given rise to the mature LIF protein secreted by a cell, cell lines are required that only make one of the signal peptide isoforms. Alternatively, the single isoforms could be expressed exogenously, however it would be necessary to distinguish them from endogenously secreted LIF. As there are no known cell lines that exclusively make D or M LIF isoforms, systems for exogenous expression were developed. The first approach to distinguish exogenous from endogenous LIF was to epitope-tag LIF with the FLAG epitope tag. An ELISA set up using an anti-FLAG antibody as the capture antibody and an anti-LIF antibody as the detection antibody would be able to distinguish exogenously produced LIF from untagged endogenously produced LIF. Since epitope tagging can alter protein function, the activity of the LIF-FLAG protein was carefully compared with the wildtype LIF. It is important to note that the wild-type counterparts were also used, in the development of the ELISAs to detect secreted LIF, this will be discussed in the next chapter. To test the effect of the FLAG, murine and human LIF-D and LIF-M were epitope tagged with FLAG and tested to see if they fared comparably to their wild type counterparts.

In the first instance, the tagged LIF protein was produced in bacteria and following affinity purification it was analysed by SDS PAGE. The protein was produced in bacteria in preliminary experiments, as this is a cheap and efficient way of producing high yields of recombinant protein for analysis prior to eukaryotic

expression. The mLIF and hLIF proteins were produced in JM109 cells expressing the plasmid pGex-2T-mLIF (Mereau *et al.* 1993) and pGex-2T-hLIF (Robinson *et al.* 1994) respectively. The mLIF-FLAG was produced in DH5α expressing the plasmid pGex-3C-mLIF-FLAG (EJH-1) (section 2.2.2.1). Following a suitable period of growth, the bacteria were lysed and the proteins were purified on a glutathione-sepharose column. After a number of washes, purified proteins expressed from pGex-3C-mLIF-FLAG were cleaved from the column using the 3C rhinovirus protease (section 2.2.4.3). Alternatively, proteins expressed from pGex-2T-hLIF and pGex-2T-mLIF were cleaved from the column using thrombin (section 2.2.4.2). The total yield of protein collected from the eluate of the column as determined by Coomassie protein assay, was 6.7mg/l for mLIF-FLAG, 3.5mg/l for mLIF and 3mg/l for hLIF. The reason why mLIF-FLAG was expressed at such a high level is unknown.

Bacterial mLIF-FLAG, isolated by 3C protease cleavage, and bacterial mLIF, isolated by thrombin cleavage, were examined for purity by visualising the proteins using SDS PAGE (12% acrylamide and stained using Coomassie) (Fig. 2.1A). Double bands can be seen around the expected size for mLIF-FLAG and mLIF (~21kDa), which is often seen in recombinant LIF proteins (Ann Vernallis, personal communication). No contaminants were observed in either preparation when 10μg of protein was loaded. Immunoblotting carried out using the anti-FLAG M2 antibody was able to detect bands in lanes containing LIF-FLAG (Fig. 2.1B). Comparison to molecular weight markers revealed detected bands of a molecular weight of approximately 21kDa. FLAG protein was not detected in lanes containing untagged mLIF protein; therefore antibody binding is specific to LIF proteins containing the

A
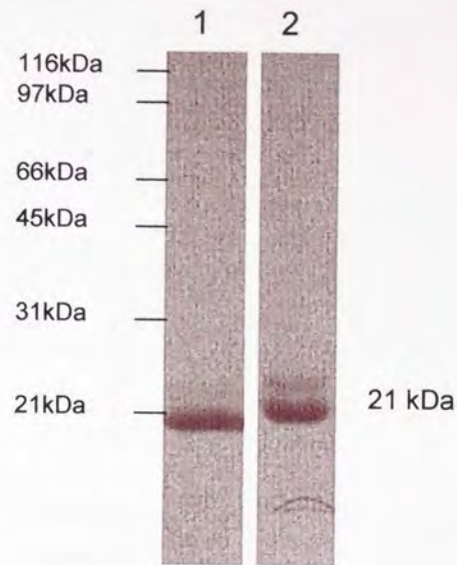
B
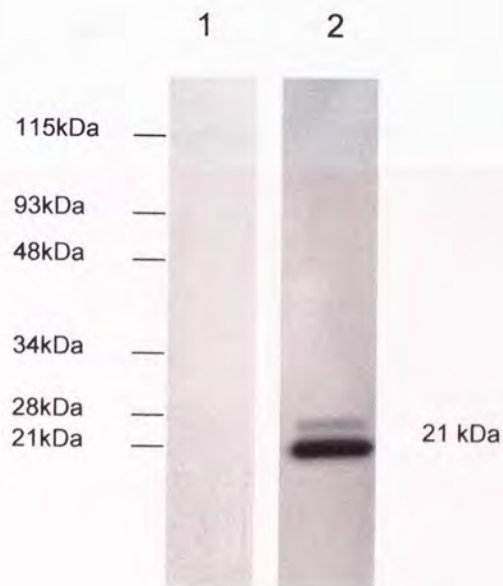
Figure 2.1 Purification and analysis of bacterial mLIF and mLIF-FLAG proteins. Purified proteins were analysed by SDS-PAGE and western blot. (A) SDS-PAGE (12% acrylamide gel stained with Coomassie). Lane 1) 10µg bacterial mLIF. Lane 2) 10µg bacterial mLIF-FLAG. Following interpolation with protein standards bacterial mLIF-FLAG can be estimated at ~21kDa. (B) Anti-FLAG immunoblot transferred from 12% acrylamide SDS PAGE. Lane 1) 8ng bacterial mLIF. Lane 2) 8ng bacterial mLIF-FLAG. Following interpolation with pre-stained proteins standards ran alongside samples, the band detected by anti-FLAG antibodies can be estimated at approximately 21kDa, which would be expected for bacterial LIF proteins, containing the FLAG epitope.
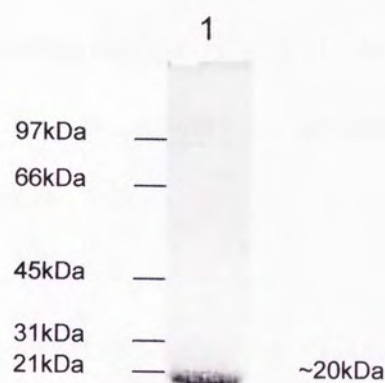
Figure 2.2 Purification and analysis of bacterial hLIF protein. Protein was purified using glutathione sepharose and cleaved using thrombin. Purified proteins were analysed by SDS-PAGE (12% acrylamide gel stained with Coomassie). Lane 1) 10µg bacterial hLIF. Following interpolation with protein standards bacterial hLIF can be estimated at ~20kDa.

FLAG epitope. Immunoblotting using the Anti-FLAG M2 antibody was able to detect as little as 4ng of the LIF-FLAG protein (data not shown). Despite detecting only the small number of bands shown around 21kDa (fig. 2.1B) when 8ng of protein was loaded, higher protein concentrations (64ng) revealed the presence of an extra band at 46kDa (data not shown). These possibly represent uncleaved GST-LIF-FLAG. Purified bacterial hLIF was also analysed by SDS PAGE (12% acrylamide and stained using Coomassie) and no contaminants were observed when 10ug of protein was analysed (loaded on the gel) (Fig. 2.2). As mLIF-FLAG was successfully expressed and purified, it was possible to determine its biological activity in comparison to wild type mLIF.

2.3.2 Biological activity of mLIF and mLIF-FLAG proteins produced in *E.coli*.

Since addition of the FLAG may alter the conformation of the LIF protein purified, mLIF-FLAG was assayed for its ability to activate LIF-R and gp130 in a bioassay. Proliferation assays were carried out using BA/F3-mLIFR-mgp130, a LIFR-ligand-dependent cell line that proliferates in response to both human and murine LIF. Bacterial mLIF-FLAG and mLIF proteins were titrated in a 2-fold dilution series across a 96-well plate starting at 2ng/ml and were assayed for biological activity (section 2.2.7.1). BA/F3-mLIFR-mgp130 cells were able to proliferate in response to mLIF-FLAG and mLIF protein (Fig. 2.3). The BA/F3-mLIFR-mgp130 cells demonstrated a sigmoidal growth curve in response to both mLIF-FLAG and mLIF with a 1.5 fold higher EC50 for the mLIF-FLAG (mLIF-FLAG EC50: 3.12×10^{-12}M; mLIF EC50: 1.96×10^{-12}M as calculated by Graphpad Prism). Although the maximal
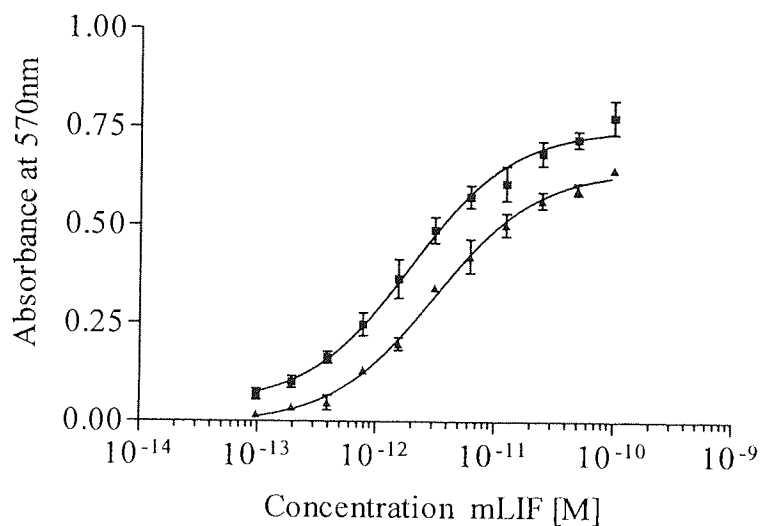
Figure 2.3 BA/F3-mLIFR-mgp130 cells proliferated in response to both mLIF and mLIF-FLAG proteins produced in *E.coli*. Sigmoidal dose response curve (standard slope) in the presence of purified bacterial mLIF and mLIF-FLAG proteins. (▲) Bacterial mLIF-FLAG (2ng/ml starting dilution). (■) Bacterial mLIF (2ng/ml starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

response of the curves is not equal between mLIF and mLIF-FLAG, it is probably not significant given the variability frequently observed in the BA/F3 mLIFR-mgp130 proliferation assay. Importantly, LIF-FLAG protein retained its biological activity, indicating that protein conformation had not been significantly affected by the addition of the FLAG epitope and that the FLAG epitope was unlikely to greatly inhibit receptor binding, or receptor activation. As production of mLIF-FLAG in bacteria was successful it was decided that tagging of murine and human LIF-D and LIF-M proteins on the C-terminus with FLAG would be possible.

2.3.3 Biological activity of conditioned medium from 293T cells transiently transfected with mLIF cDNA encoding tagged and untagged proteins.

The next stage in the development of the LIF-FLAG proteins was to create the LIF-D and LIF-M isoforms and their tagged counterparts in eukaryotic expression vectors. Once this was achieved, the plasmids could be transiently expressed in mammalian 293T cells, and the conditioned medium tested for biological activity.

The LIF expression vectors were constructed as described (section 2.2.2). The LIF constructs pcDNA3-mLIF-D (EJH5), pcDNA3-mLIF-M (EJH4) and their epitope-tagged counterparts (EJH2, EJH3) were expressed transiently in 293T cells. After three days, the conditioned media were assayed for biological activity in a BA/F3-mLIFR-mgp130 assay at a starting dilution of 1:500 (Fig. 2.4). No LIF activity was evident in the conditioned medium of pcDNA3 (-); (control vector) transfected cells at the dilutions tested, verifying that the activity observed was due to
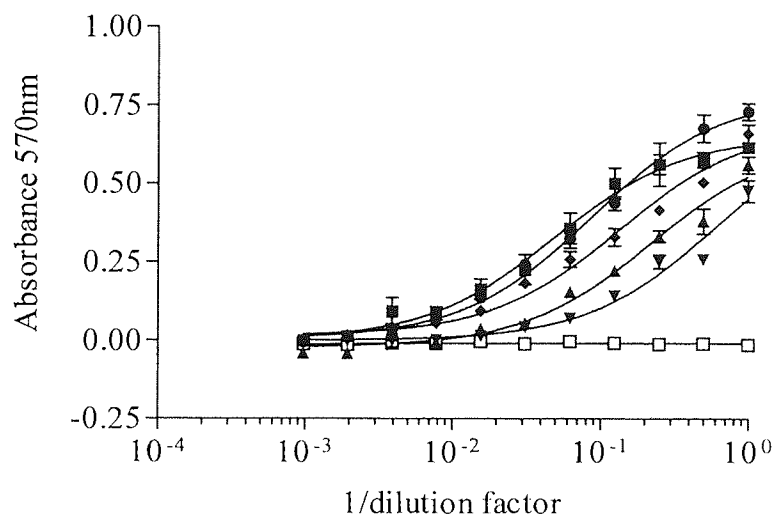
Figure 2.4 BA/F3-mLIFR-mgp130 cells proliferated in response to conditioned medium from 293T cells transiently transfected with mLIF cDNA. Sigmoidal dose response curve (standard slope) in the presence of conditioned medium from 293T cells transiently transfected with the plasmids, (□) pcDNA3 (vector control), (▲) pcDNA3-mLIF-D-FLAG, (▼) pcDNA3-mLIF-M-FLAG, (●) pcDNA3-mLIF-M, (♦) pcDNA3-mLIF-D, in comparison to (■) bacterial hLIF (2ng/ml starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

the transfected LIF cDNAs. Bacterial hLIF (section 2.2.4.2) demonstrated an EC50 of 4.89×10^{-12} M (as calculated by Graphpad Prism). Conditioned media from mLIF-D (EJH5) and mLIF-M (EJH4) were able to maintain BA/F3 cells at dilutions up to 1 in 256,000. In comparison, media from mLIF-M-FLAG (EJH2) and mLIF-D-FLAG (EJH3) transfected cells retained biological activity in this assay to dilutions of 1 in 64,000. Therefore each of the mLIF transcripts (mLIF-D and mLIF-M) directed the production of comparable levels of extracellular LIF activity in 293T cells. The 4-fold difference in maximal dilution between tagged and untagged LIF may reflect genuine differences in activity or expression, or may simply reflect differences in transfection efficiency. As such differences were not observed between the bacterial mLIF and mLIF-FLAG (Fig 2.3) and eukaryotic hLIF and hLIF-FLAG (Fig. 2.5), the four fold difference here is probably not significant. However, in order to calculate meaningful EC50 values for the expressed LIF proteins, it is necessary to produce purified preparations from each construct.

2.3.4 Biological activity of conditioned medium from 293T cells transiently transfected with hLIF cDNA encoding tagged and untagged proteins.

As for the mLIF constructs, it was necessary to create eukaryotic hLIF expression vectors and analyse their expression in 293T cells. hLIF-FLAG expression vectors were constructed as described (section 2.2.3). The LIF constructs pcDNA3-hLIF-D (EJH5), pcDNA3-hLIF-M (EJH4) and their epitope-tagged counterparts (EJH8, EJH12) were expressed transiently in 293T cells and the conditioned medium was assayed for biological activity (Fig. 2.5) in a BA/F3-mLIFR-
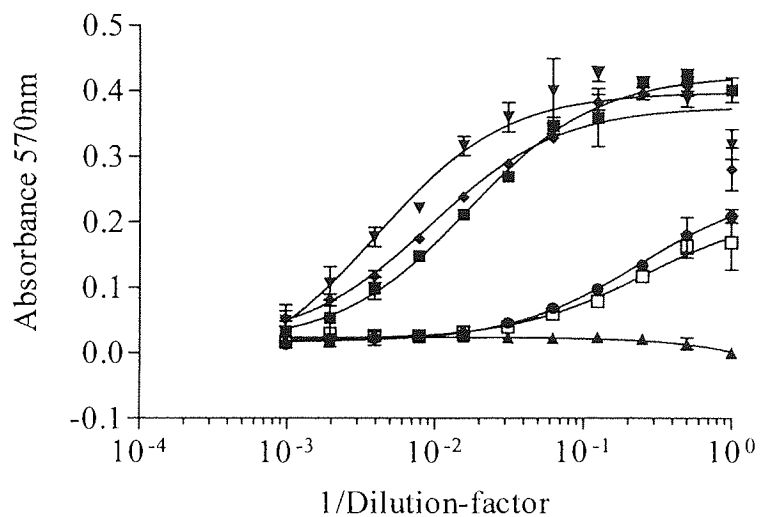
Figure 2.5 BA/F3-mLIFR-mgp130 cells proliferated in response to conditioned medium from 293T cells transiently transfected with hLIF cDNA. Sigmoidal dose response curve (standard slope) in the presence of conditioned medium from 293T cells transfected with the plasmids, (▲) pcDNA3 (vector control) (□) pcDNA3-hLIF-M-FLAG, (♦) pcDNA3-hLIF-D-FLAG, (●) pcDNA3-hLIF-M, (▼) pcDNA3-hLIF-D, in comparison to (■) Bacterial hLIF (2ng/ml starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

mgp130 assay at a starting dilution of 1:500. No LIF activity was evident in the conditioned medium of pcDNA3 (control vector) transfected cells at the dilutions tested, verifying that the activity observed was due to the transfected LIF cDNAs. Bacterial hLIF (section 2.2.4.2) demonstrated an EC50 of 1.7×10^{-12} M (as calculated by Graphpad Prism). Conditioned medium from pcDNA3-hLIF-D (EJH7) and pcDNA3-hLIF-D-FLAG (EJH8) transfected cells were able to maintain BAF cells at dilutions up to 1 in 512,000. In comparison, medium from pcDNA3-hLIF-M-FLAG and pcDNA3-hLIF-M transfected cells retained biological activity in this assay to dilutions of 1 in 64,000. Here the FLAG tag does not appear to make much difference, however, expression of LIF-M results in an eight-fold lower activity than expression of LIF-D. Thus, hLIF-M transcripts directed the production of lower levels of extracellular LIF activity in 293T cells than the D transcripts. This is not an unexpected result in light of experiments published (Voyle *et al.* 1999) (see chapter 2 discussion).

As both sets of hLIF and mLIF constructs expressed active LIF proteins in a eukaryotic expression system, the next step was to create purified eukaryotic LIF proteins, for a more direct assessment of biological activity.

2.3.5 Purification and analysis of eukaryotic hLIF, hLIF-FLAG and mLIF.

The initial strategy was to express LIF-FLAG proteins in stable cell lines to study secretion and detect them by a LIF-FLAG ELISA. The ELISA would be calibrated with purified eukaryotic hLIF-D-FLAG. In order to produce purified eukaryotic hLIF-D, hLIF-D-FLAG, mLIF-D-FLAG and mLIF-D, LIF cDNAs were

expressed as fusion proteins with the Fc region of human IgG1 (hinge-CH2-CH3) in 293T cells (appendix 25). A 3C site was included immediately prior to the Fc region to allow cleavage by human rhinovirus 3C protease, following purification on protein-A-sepharose. Due to the discovery of mutations in the mLIF-FLAG cDNA following sequencing, it was decided to purify the hLIF-FLAG and assay its activity before returning to the mLIF-FLAG construct.

The total yield of protein collected from the protein A column as determined by Coomassie protein assay, was 2.5mg/l for mLIF, 0.5mg/l for hLIF and 2.4mg/l for hLIF-FLAG. Eukaryotic hLIF and mLIF isolated by 3C protease cleavage was analysed for purity by SDS PAGE (10% acrylamide and stained using Coomassie) (Fig. 2.6A). Bands can be seen as a smear around the expected size for eukaryotic hLIF (~45kDa) and mLIF (~45kDa). The high molecular weight compared to bacterial hLIF and mLIF indicate that the proteins are glycosylated in 293T cells. No contaminants were observed when 2μg of protein was loaded. Immunoblotting was carried out using the anti-FLAG M2 antibody to detect the FLAG epitope in hLIF-FLAG. Comparison to molecular weight markers, revealed the tagged proteins to have a molecular weight of approximately ~45kDa (Fig. 2.6B.). The FLAG-epitope was not detected in lanes containing untagged hLIF protein; therefore antibody binding is specific to LIF proteins containing the FLAG epitope. No contaminants were observed when 10ng hLIF Protein was analysed in the immunoblot, indicating little if any contamination with uncleaved hLIF-D-FLAG-Fc. As eukaryotic hLIF-FLAG and hLIF were successfully expressed and purified using the Fc constructs it was possible to determine their biological activity.
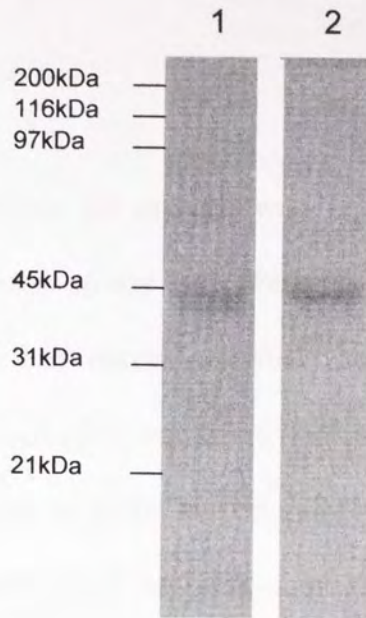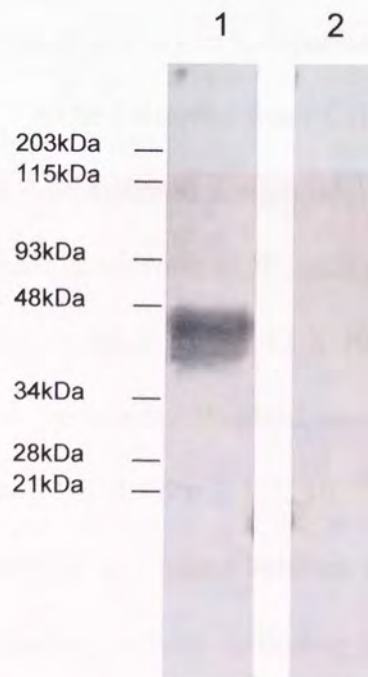
Figure 2.6 Purified eukaryotic hLIF and mLIF proteins. Proteins were purified using protein A sepharose analysed by SDS-PAGE and stained with Coomassie. A) Lane 1) eukaryotic mLIF 2µg, Lane 2) eukaryotic hLIF 2µg. (B) Anti-FLAG immunoblot transferred from 10% acrylamide SDS PAGE. Lane 1) 10ng eukaryotic hLIF-FLAG. Lane 2) 10ng eukaryotic hLIF. Following interpolation with pre-stained protein standards ran alongside samples, the band detected by anti-FLAG antibodies as a smear can be estimated at ~45kDa.

2.3.6 Biological activity of purified eukaryotic LIF proteins produced in 293T cells.

As the purified eukaryotic LIF proteins were to be used to calibrate the LIF-FLAG ELISAs, it was necessary to test their ability to bind and activate LIFR and gp130 to ensure that they had maintained their correct conformation. More importantly, a deranged conformation would interfere in a secretion assay in which protein trafficking may depend upon the correct conformation of LIF. Proliferation assays were carried out using BA/F3-mLIFR-mgp130. Isolated eukaryotic LIF proteins were titrated in a 2-fold dilution series across a 96-well plate starting at 2ng/ml and assayed for biological activity in comparison to the Bender Medsystems ELISA kit eukaryotic hLIF Standard derived from CHO cells (section 3.2.7). The BA/F3-mLIFR-mgp130 cells demonstrated a sigmoidal growth curve in response to both eukaryotic hLIF-FLAG and eukaryotic hLIF, with a 1.95 fold higher EC50 with eukaryotic hLIF than hLIF-FLAG (hLIF EC50: 12 X 10^{-12}M; hLIF-FLAG EC50: 6.2 X 10^{-12}M). In comparison to the Bender standard, eukaryotic hLIF demonstrated a 5.2-fold higher EC50 (Bender hLIF EC50: 2.3 X 10^{-12}M) (Fig. 2.7). The maximal response of the curves was roughly equivalent between all three proteins. The hLIF-FLAG protein retained its biological activity, indicating that protein conformation had not been significantly altered by the addition of the FLAG epitope. As tagging of eukaryotic hLIF does not affect its ability to bind and activate the LIF receptor, it may be possible to use this purified product as a standard in an hLIF-FLAG ELISA and use hLIF-FLAG in a secretion assay.
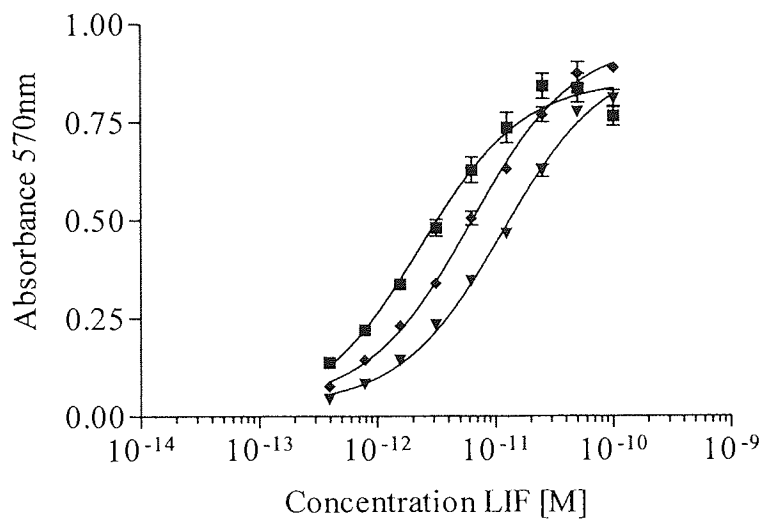
Figure 2.7 BA/F3 mLIFR-mg130 cells proliferated in response to both purified eukaryotic hLIF and hLIF-FLAG proteins produced in 293T cells. Sigmoidal dose response curve (standard slope) in the presence of purified eukaryotic hLIF and hLIF-FLAG proteins. (♦) eukaryotic hLIF-FLAG (2ng/ml starting dilution). (▼) eukaryotic hLIF (2ng/ml starting dilution). (■) Bender hLIF (2ng/ml starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

The biological activity of purified eukaryotic mLIF was also compared to purified eukaryotic hLIF, bacterial mLIF and the Bender Medsystems standard (Fig. 2.8). Purified eukaryotic mLIF exhibited a 3-fold higher EC50 than hLIF (mLIF EC50: 6.1×10^{-11}M; hLIF EC50: 1.9×10^{-12}M), a 2.7 fold higher EC50 than bacterial mLIF (EC50: 2.3×10^{-12}M) and a 21-fold higher EC50 than the Bender Medsystem standard (EC50: 3×10^{-13}). The eukaryotic mLIF fared worse (2.7-fold higher EC50) than the bacterial mLIF, suggesting that it is not as active. Note that the difference in this assay between Bender Medsystem hLIF and eukaryotic hLIF (6.5 fold) is somewhat greater than in the preceding assay (5.2 fold); (Fig. 2.7) as a result of both a lower EC50 for the Bender Medsystem standard and a higher EC50 for eukaryotic hLIF, demonstrating some inter-assay variation.

Interestingly, conditioned media from 293T cells expressing the complete fusion proteins hLIF-D-Fc and hLIF-D-FLAG-Fc also demonstrated biological activity and was able to maintain BAF cells at dilutions up to 1 in 512,000 (Fig. 2.9). This indicates that the addition of the Fc region of IgG does not adversely affect the binding of LIF to its receptor. However, until a purified version of the protein is produced a true comparison to the wild type protein cannot be made.

2.3.7 Immunoprecipitation of LIF-FLAG proteins using Anti-FLAG M2 sepharose.

The LIF-FLAG proteins in the conditioned medium from cells expressing the alternate LIF isoforms would be detected using an anti-LIF-FLAG ELISA, using the
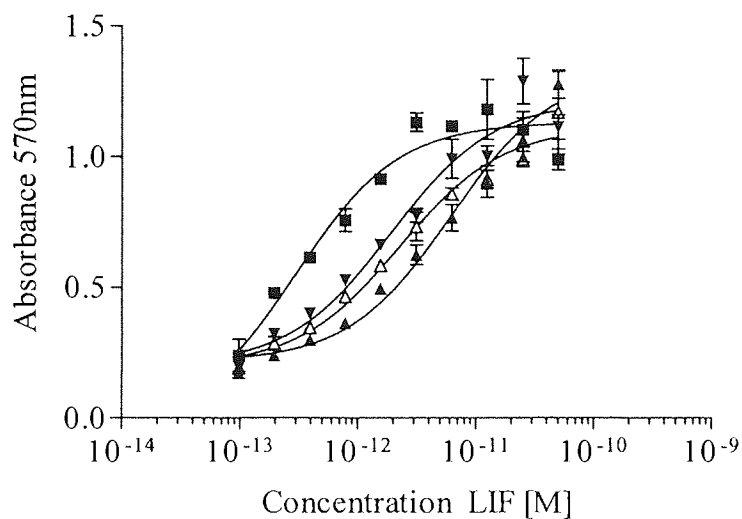
Figure 2.8 Purified eukaryotic hLIF and mLIF proteins demonstrated substantial biological activity. Sigmoidal dose response curve (standard slope) in response to purified LIF proteins. (■) Bender hLIF (1ng/ml starting dilution), (▲) eukaryotic mLIF (1ng/ml starting dilution), (Δ) bacterial mLIF (1ng/ml starting dilution), (▼) eukaryotic hLIF (1ng/ml starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.
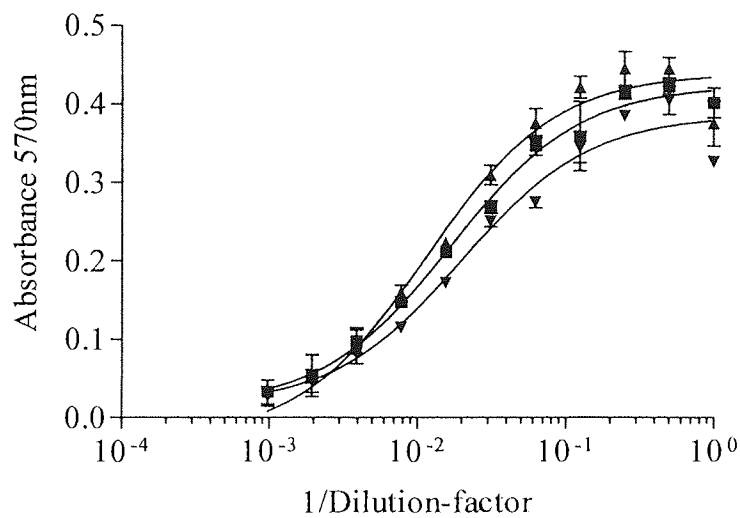
Figure 2.9 Uncleaved hLIF-Fc and hLIF-FLAG-Fc proteins demonstrated biological activity. Sigmoidal dose response curve (standard slope) in response to hLIF-Fc proteins. (■) Bacterial hLIF (2ng/ml starting dilution), (▲) eukaryotic hLIF-D-Fc (1:500 starting dilution), (▼) eukaryotic hLIF-FLAG-Fc (1:500 starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

anti-FLAG M2 antibody (Sigma), to specifically capture tagged LIF. Voyle *et al.*(1999) had not used an anti-FLAG antibody to detect their LIF-FLAG proteins, therefore, it was necessary to test the ability of this antibody to detect native LIF-FLAG proteins. Anti-FLAG M2 sepharose was used to immunoprecipitate murine and human LIF-FLAG proteins. The purified eukaryotic hLIF-FLAG protein and the purified bacterial mLIF-FLAG protein were compared, to investigate any differences in detection caused by the glycosylation state of the protein. Purified eukaryotic hLIF was used as a control, to ensure that immunoprecipitation was specific to the FLAG epitope. Eukaryotic hLIF-D-FLAG-Fc (uncleaved) conditioned medium was used to investigate the effect of the uncleaved Fc on the ability of the antibody to detect the FLAG. The control protein FLAG-bacterial alkaline phosphatase (BAP) was obtained commercially (Sigma) and is known to interact with the anti-FLAG M2 antibody.

To the washed anti-FLAG M2 sepharose (20μl), 500μl of samples containing either eukaryotic hLIF-FLAG (8μg/ml) bacterial mLIF-FLAG (8μg/ml), FLAG-BAP (4μg/ml) and hLIF-D-FLAG-Fc (500μl conditioned medium), were added. Immunoprecipitated proteins were analysed by SDS PAGE (10% acrylamide gel and stained using Coomassie) (Fig. 2.10). Neither eukaryotic hLIF-FLAG, nor bacterial mLIF-FLAG were immunoprecipitated. In contrast, the control protein (FLAG-BAP), indicated by the bands at 45-55kDa was successfully immunoprecipitated. Interestingly, the hLIF-D-FLAG-Fc was also successfully immunoprecipitated, shown by the band at approximately 63kDa. It is possible that the conformation of hLIF-D-FLAG-Fc is different to that of the cleaved proteins, thus allowing access of the anti-FLAG M2 antibody to the FLAG tag and subsequent immunoprecipitation. The immunoprecipitation was also attempted in the presence of either reducing agents (β-

200kDa —
116kDa —
97kDa —
66kDa —
                    ~63kDa
45kDa —

31kDa —

   1   2   3   4
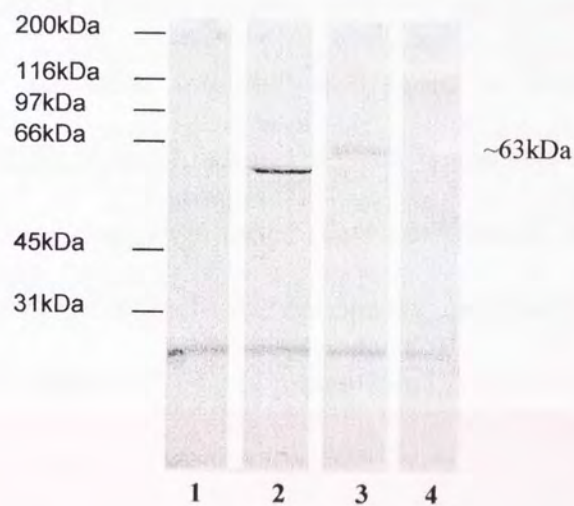
Figure 2.10 Purified LIF-FLAG proteins were not detected by SDS-PAGE following immunoprecipitation using Anti-FLAG M2 sepharose. Proteins were immunoprecipitated using anti-FLAG M2 sepharose and following resuspension in sample buffer analysed by SDS-PAGE (10% acrylamide gel stained with Coomassie). Lane 1) Eukaryotic hLIF-FLAG, Lane 2) FLAG-BAP (control), Lane 3) eukaryotic hLIF-D-FLAG-Fc, Lane 4) bacterial mLIF-FLAG. Following interpolation with protein standards ran alongside samples the band in lane 2 can be estimated at approximately 45kDa and the band in lane 3 can be estimated at approximately 63kDa.

mercaptoethanol up to 30mM), or RIPA buffer (10mM Tris HCL pH 8.0,100mM NaCl, 1mM EDTA, 1% NP40, 0.1% SDS and 0.5% sodium deoxycholate), to try to partially denature the protein in an attempt to increase the exposure of the FLAG. However, both methods were insufficient to enhance detection of the FLAG epitope (data not shown).

An anti-hLIF-FLAG ELISA (coating with M2 and detecting with the Bender Medsystem anti-hLIF detection antibody) also failed to detect hLIF-FLAG. In contrast, the Bender Medsystem ELISA (section 3.2.7) was able to detect hLIF-FLAG within the same concentration range tested (data not shown). As a consequence of this result it was concluded that the FLAG epitope tag, as placed at the C-terminus did not allow detection of native LIF-FLAG proteins and the LIF-FLAG was unsuitable for studies of LIF secretion.

2.4   Discussion.

The alternate human and murine transcripts encode biologically active proteins when produced in bacteria and 293T cells. Although there were no apparent differences between the murine LIF isoforms, different levels of extracellular LIF bioactivity resulted from expression of the hLIF-D and hLIF-M transcripts. In addition, epitope tagging both murine and human LIF with FLAG did not significantly alter its conformation or biological activity. However, the anti-FLAG M2 antibody was unable to detect LIF-FLAG proteins in both an immunoprecipitation assay and in a hLIF-FLAG ELISA. Therefore, the intention to use the FLAG epitope to distinguish exogenous LIF could not proceed with the existing constructs.

Similarities in the biological activity of conditioned medium from 293T cells transfected with murine LIF-D or LIF-M, suggests that there is no significant difference between the levels of protein secreted between the two isoforms. A comparison of the expression levels of each isoform has not been described previously, indicating that this is a novel finding. In contrast, there are apparent differences in conditioned medium from human LIF-D and LIF-M transfected cells. Conditioned medium from human LIF-D transfected cells was able to maintain BA/F3 cells up to 1 in 512,000. Whilst conditioned medium from human LIF-M transfected cells was able to maintain BA/F3 cells up to 1 in 64,000 dilution, demonstrating an eight-fold lower level of activity in comparison to hLIF-D. Similar differences in the biological activity of hLIF transcripts were observed by (Voyle *et al.* 1999). They demonstrated LIF bioactivity present in conditioned medium from transfected COS-cells, using a biological assay based upon maintenance of

undifferentiated murine ES cell colonies. Conditioned medium from hLIF-D transfected cells maintained ES cell colonies at dilutions up to 1 in 20,000, in contrast, medium from hLIF-M transfected cells retained bioactivity up to dilutions of 1 in 2000. They also claimed an identical ten-fold lower bioactivity in the conditioned medium from hLIF-M transfected 293T cells (Voyle *et al.* 1999).

These results demonstrate that hLIF-M transcripts, like the mLIF-M transcripts encode secreted proteins. The translation of secreted proteins from the hLIF-M transcript suggests translation of these secreted proteins must initiate at a non-AUG codon. The relative inefficiency of initiation from non-AUG start codons may explain the differences observed in the conditioned medium between human and murine LIF-D and LIF-M transfected cells. Mehdi *et al.*(1990) demonstrated that the natural ACG start codon of the C' protein of Sendai virus was 10% as efficient as AUG in COS cell transient transfections. Changing the start codon to a CUG or GUG codon increased the efficiency to 30% and 20% respectively. The presence of an in frame GUG in the hLIF-M first exon may allow translation of the hLIF-M transcript in the absence of an in-frame AUG, but less efficiently than from the AUG.

Alternatively initiated forms of proteins are sometimes functionally distinct and can be differentially localised. Many proto-oncogenes use non-AUG start codons to produce overlapping proteins with different N-termini. In proteins such as bFGF, alteration of the N-terminal amino acids alters the localisation of the protein. The AUG initiated forms enter the secretory pathway, whereas the CUG initiated forms migrate to the nucleus (Hann 1994). For hLIF-M, preferential entry into the nucleus is unlikely, since Voyle *et al.* (1999) only observed truncated proteins intracellularly.

Nonetheless, the example of bFGF illustrates that LIF is not alone in the association between alternate N-termini and alternate localisation.

Eukaryotic hLIF and mLIF proteins were successfully purified as Fc fusion proteins that were subsequently cleaved to release free LIF. The purified proteins also demonstrated comparable biological activity in a Ba/F3 mLIFR-mgp130 assay. Jostock et al.(1999) created an IL-6-Fc fusion protein, and by introducing a protease cleavage site between IL-6 and the Fc region they were able to successfully purify eukaryotic IL-6, suggesting that this may be a general way to purify IL-6 type cytokines. Although mammalian cells do not reach the expression levels seen in bacterial cells this system has several advantages. Mammalian cells are able to carry out post-translational modifications such as glycosylation, which may contribute to protein activity and stability of the protein. In addition, expression in bacteria carries the risk of contaminating microbial toxins, which may interfere in biological assays. When compared to their monomeric counterparts, the hLIF-D-Fc fusion proteins were efficiently expressed in 293T cells and demonstrated comparable biological activity. Jostock et al. (1999) demonstrated that IL-6-Fc also exhibited agonistic activity in Ba/F3 IL-6R-mgp130 proliferation assay, and demonstrated comparable biological activity when used in equimolar concentration to IL-6. They suggested that as biological activity was comparable, only one of the two-cytokine molecules included in the dimeric Fc protein is involved in the assembly of a signalling receptor complex. The hLIF-Fc proteins could be used as important research tools in binding experiments, to study LIFR expression on cells, or in LIF/monoclonal antibody immunoprecipitations to study LIF-interacting proteins. The advantage of the Fc fusion is that it can be easily detected with protein A or antibodies to human Fc.

Quantifying the purified eukaryotic protein with the Bender Medsystem ELISA will be presented in section 3.3.4, however it will be discussed here since it illuminates the BA/F3 mLIFR-mgp130 assay results. Using the ELISA (section 3.2.7), it was possible to test the purified eukaryotic hLIF produced in section 2.3.5 and compare it with the values obtained from the Bender standard. The eukaryotic hLIF purified from 293T cells, was approximately 4 fold lower (n=3) than the expected amount of protein determined by Coomassie protein assay. In BA/F3 mLIFR-mgp130 cell assay, the purified eukaryotic hLIF demonstrated a 5-6 fold lower EC50 than the Bender Medsystem standard. Thus, the reduced activity in the ELISA correlates well with the reduced activity in the BA/F3 mLIFR-mgp130 assay, suggesting the genuine differences in specific activity may be small.

The difference between the two proteins could be due to the way in which proteins are purified, or the method used to quantify the amount of LIF present. The Bender Medsystem eukaryotic LIF was produced from CHO cells transfected with the pC10-6R plasmid encoding the cDNA of human LIF. The supernatant was quantified using the biological assay based on the LIF induced proliferation of the myeloid leukemic cell line DA1a. One unit, is defined as the quantity of LIF, able to trigger half maximum proliferation of this cell line and corresponds to 25pg of the glycosylated protein (Taupin *et al.* 1997). The purified hLIF standard produced here was quantified by the amount of protein eluted from the column (section 2.2.6.1) and not by its specific activity on the same DA1a cell line. This may explain the differences between the biological activities, when apparently similar amounts of protein were compared.

The inability to detect FLAG proteins using the hLIF-FLAG ELISA and immunoprecipitation would suggest that the FLAG-tag is unavailable for antibody binding and may be masked by the LIF protein. Subsequent experiments carried out within the lab have utilised an hLIF-3XFLAG protein. 293T cells were transiently transfected with the plasmid phLIF-3XFLAG and the conditioned medium collected. When tested with the hLIF-FLAG ELISA hLIF-3XFLAG was detected up to 1 in 1000 dilution of 293T conditioned medium, however even a 1 in 10 dilution of the hLIF-FLAG was not detected (Ann Vernallis personal communication). Hernan et al. (2000) compared 3X FLAG-BAP with the original FLAG-BAP protein in a western blot using the M2 antibody. Although the authors were able to detect FLAG-BAP at concentrations as low as 5ng, they were able to detect 3X FLAG-BAP at concentrations as low as 500pg. In addition, when tested in an ELISA and a dot blot, they were able to demonstrate a ten-fold increase in detection with the 3X FLAG-BAP protein. The greater difference in the detection of the hLIF-3XFLAG versus the hLIF-FLAG (at least 100-fold), compared to the 3XFLAG-BAP vs. FLAG-BAP (ten-fold), indicate that the sensitivity has been increased more than one would expect from simply repeating an exposed epitope 3X. This supports the view that the single FLAG epitope was inaccessible in the original hLIF-FLAG. Thus, the hLIF-3XFLAG may prove suitable for secretion studies, if purified hLIF-3XFLAG demonstrate comparable biological activity to untagged hLIF.

Chapter 3: Endogenous LIF expression.

3.1 Introduction.

Epithelial cells can exist as polarised monolayers and are capable of secreting a variety of cytokines and other proteins in a polarised manner. The direction in which cytokines are secreted could determine the targets of the cytokine. Cytokines secreted in an apical direction, would enter the luminal environment and could target homologous cell types. Alternatively basal secretion could target cells underlying the epithelium such as fibroblasts. The configuration of the human colon suggests a highly organised structure that creates specific microenvironments. Rockman *et al.* (2001) used isolated and cultured colonic pericryptal fibroblasts and epithelial crypt cells from the human colon and compared their cytokine and receptor expression. Cultures of pericryptal fibroblasts were analysed for the expression of IL-6 and LIF by RT-PCR. The fibroblasts were found to express transcripts for both IL-6 and LIF. To confirm the RT-PCR the conditioned medium was analysed for the presence of LIF and IL-6. The IL-6 protein was detected, but the LIF protein could not be detected in the conditioned medium. A low level of production of LIF by pericryptal fibroblasts cannot be ruled out. However, since LIF has been suggested to be a molecule that is incorporated into the extracellular matrix of fibroblast cell lines such as STO and 10T1/2 (Rathjen *et al.* 1990) this may also be true for the ECM of pericryptal fibroblasts. An examination of the cytokine receptors demonstrated the expression of LIFR and gp130, but not IL-6 Rα. Conversely overlying colonic epithelial cells were found not to secrete IL-6 but did express the IL-6Rα and gp130. In addition the epithelial cells secreted LIF but did not express the LIFRα. This suggests that LIF and IL-6 may be going in opposite directions with LIF coming from

epithelial cells and IL-6 coming from fibroblasts and they may act as Paracrine/autocrine factors, creating a mechanism for communication between the epithelial cells and pericryptal fibroblasts.

In addition to communication with underlying fibroblasts, epithelial cells may also interact with cell types in the apical luminal environment such as neutrophils. Adenosine is generated during active inflammation in the intestinal lumen by the interaction of neutrophils with the intestinal epithelia. Epithelial ectonucleotidases convert neutrophil-derived ATP into adenosine, which then acts through the A2b receptor on the epithelium. Sitaraman *et al.*(2001) demonstrated that stimulation of T84 (intestinal epithelial cell line) monolayers with either apical or basolateral adenosine induces a polarised apical increase in IL-6 secretion. IL-6 released in the luminal compartment, may achieve a sufficient concentration to activate neutrophils. This would allow an epithelial-dependent intracellular calcium flux and subsequent degranulation of neutrophils, thus enhancing their microbiocidal activity.

Adenosine is not unique in stimulating polarised cytokine secretion. Cytokines of the IL-6 family are secreted in a polarised manner in response to a range of paracrine factors. Mascarenhas *et al.*(1996) using the rat IEC-6 (intestinal epithelial) cell line grown on microporous membrane inserts determined that these cells were capable of secreting IL-6 preferentially to the basolateral surface when stimulated basolaterally with IL-1β. In contrast stimulation with TNFα resulted in an equal level of IL-6 secretion to the apical and basolateral surface regardless of whether the cells were stimulated on the apical or basolateral surface. This suggests that IEC are capable of secreting IL-6 in different patterns and therefore of

communicating with different localised populations of cells, depending on the stimulation received.

When cultured *in vitro*, Caco-2 cells take on the feature of differentiated small intestinal enterocytes, forming polarised monolayers with tight junctions and brush border microvilli at their apical surface. Moon *et al.*(2000) tested if IL-β induced C3 (complement component) and IL-6 production is differentially regulated at the apical and basolateral membranes of the enterocyte. They found that the release of C3 was greatest in the basal chamber regardless of whether the cells were stimulated at the apical or basal membrane. In contrast, the production of IL-6 was highest at the cell membrane that was stimulated with IL-1β, with greatest levels when stimulated basolaterally. The mechanism behind this observation is not known. Caco-2 cells express binding sites for IL-6 at both poles and for IL-1β at the basolateral pole and to a lesser extent at the apical pole (Molmenti *et al.* 1993). As the level of C3 production was identical after apical and basolateral stimulation the results must reflect differences in intracellular pathways responsible for C3 and IL-6 secretion distal to the IL-1β receptor. IL-6 mRNA levels were increased in IL-1β stimulated Caco-2 cells and this effect was seen after 30 minutes and persisted for at least four hours, suggesting that IL-6 recovered in the culture medium after stimulation most likely represents newly synthesised IL-6 (Moon *et al.* 2000).

Epithelial cells exhibit variability with regards to the polarised distribution of proteins and the intracellular sorting of these molecules. It has been reported that Caco-2 cells use both a direct route and an indirect (transcytotic) route to the cell surface. Most cells secrete a small amount of their newly synthesised lysosomal

enzymes. Wick *et al.*(1999) showed that in Caco-2 cells, the majority of secreted phosphorylated lysosomal enzymes accumulated in the apical medium. This was shown to occur in part via an indirect pathway. The calcium independent mannose 6-phosphate receptor (CIMPR), which is enriched on the basolateral surface of Caco-2 cells, is able to recapture secreted lysosomal enzymes at the basolateral surface and deliver them to an apical endosomal compartment from which some escape apically. As LIF is also capable of binding CIMPR (Blanchard *et al.* 1998), its distribution may also be influenced by indirect trafficking. Caco-2 cells are also able to deliver proteins via a direct apical route. Caco-2 cells expressing exogenous gp80 glycoprotein complex were shown to efficiently release the majority (80%) of the secretory protein directly to the apical surface (Appel and Koch-Brandt 1994).

As Caco-2 cells have demonstrated the ability to secrete IL-6 in a polarised fashion in response to paracrine factors, whether or not Caco-2 cells may also secrete LIF in a polarised manner is an important question. The answer will inform our understanding of the role of LIF in the gut and contribute to our understanding of LIF trafficking.

3.2   Materials and methods.

3.2.1   Caco-2 cell culture.

Caco-2 (intestinal adenocarcinoma) cells were maintained in DMEM, high glucose, with L-glutamine (Gibco), 20% FCS (Labtech), 1% MEM (Gibco), defined as medium X, in a humidified 37°C, 5% CO_2, 95% air mixture incubator. Cells were allowed to grow to 60-80% confluence before trypsinising in 0.25% trypsin, 0.2% EDTA (Gibco) and seeding into new 75cm^2 flasks at a one in ten dilution.

3.2.2   Harvesting conditioned medium from Caco-2 cells

Caco-2 cells, cultures were grown in 75cm^2 flasks and allowed to reach confluency. Cells were then left for two weeks with feeding every two-three days to allow them to fully differentiate. After two weeks medium was aspirated from the flasks, which were then washed once with complete medium X (section 3.2.1). Fresh medium (20ml) was then gently added to the flasks, which were then returned to the incubator. The cells were left for 24-48 hours, after which time conditioned medium was removed. Conditioned medium was firstly centrifuged 194 X g for 10 minutes to remove cellular debris, and then sterile filtered before it was stored at 4°C.

3.2.3   Culture of Parental BA/F3 cells.

Parental (BA/F3 cells not transfected with LIFR-gp130) BA/F3 cells were maintained in RPMI 1640 (Gibco), supplemented with 10% FCS (Labtech), 50U/ml penicillin, 50μg/ml streptomycin (Gibco), and bacterial IL-3 (100pg/ml); (R&D systems). Parental BA/F3 bioassays were carried out in the same manner as BA/F3-

mLIFR-mgp130 assays (section 2.2.7.1) with the exception that the control used was bacterial IL-3 (R&D).

3.2.4 R&D hLIF Enzyme-Linked Immunoabsorbant Assay (ELISA).

An ELISA to detect LIF in medium conditioned by Caco-2 cells (section 3.2.2) was set up using commercially available antibodies and standard operating procedure as recommended by R&D systems. 100µl of monoclonal mouse anti-human LIF antibody (3µg/ml) (R&D systems, catalogue #MAB250), diluted in PBS (Sigma), was transferred to each well of a maxi-sorp immuno-plate (NUNC). Immediately after coating, the plate was sealed and incubated overnight at room temperature. The next day wells were washed 3X (0.05% Tween 20 in PBS, pH 7.4) and aspirated using an autowasher (Labsystems, Well wash 4 Mk2). Plates were blocked by adding 300µl of PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 to each well. Plates were incubated for 1 hour. Plates were then aspirated/washed as previously. 100µl aliquots of standards (1500-23.4pg/ml) and samples were added per well and mixed by gently tapping the plate for 1 minute. Plates were then covered and incubated for two-hours at room temperature. The aspiration/wash was repeated as before. 100µl of the biotinylated anti-human LIF antibody (200ng/ml); (R&D systems, catalogue #BAF250) was added to each well. The plate was then covered and incubated for two hours at room temperature. The aspiration/wash was repeated as before. 100ul of streptavidin horseradish peroxidase (1:500 dilution) (Amersham Pharmacia Biotech) was added to each well. The plate was covered and incubated for 20 minutes at room temperature. The aspiration/wash was repeated as before. 100µl of 1,2-phenylenediamine dihydrochloride (OPD); (DAKO) substrate solution was

used according to the manufacturers guidelines (1 tablet/12ml H_2O, plus 5μl 30% H_2O_2). After a suitable colour had developed the reaction was stopped with 50μl/well 0.5M H_2SO_4. The optical density of each well was measured at 490nm (MRX microplate reader, Dynex Technologies).

3.2.5   Neutralization of LIF activity using anti-bacterial hLIF antibodies.

An assay to neutralize LIF-like activity in a BA/F3 mLIR-mgp130 cell assay was set up (section 2.2.7.1) with 100ng/ml anti-hLIF antibody (R&D catalogue # MAB250) per well.

3.2.6   Neutralization of LIF activity using anti-eukaryotic hLIF antibodies.

An assay to neutralize LIF-like activity of Caco-2 cell supernatants in a BA/F3 mLIR-mgp130 cell assay was set up (section 2.2.7.1) using 10ug/ml anti-glycosylated hLIF antibody (Chemicon catalogue # AB1886) per well.

3.2.7   Bender Medsystems hLIF ELISA.

An ELISA to detect glycosylated LIF was used in accordance with the manufacturer's instructions. To coat the plate with capture antibody 100μl of anti-human LIF antibody (2.5μg/ml), diluted in PBS, was transferred to each well of a maxi-sorp immuno-plate (NUNC). Immediately after coating, the plate was sealed and incubated overnight at 4°C. The next day wells were washed 3X (0.05% Tween 20 in PBS) and aspirated using an autowasher (Labsystems, Well wash 4 Mk2). Plates were blocked by adding 300μl assay buffer (PBS containing 0.5% BSA, 0.05%

Tween 20) to each well, and incubated for two hours at room temperature. Plates were then aspirated/washed as previously. 100μl aliquots of standards (200-3.13pg/ml) and samples were then added per well. Plates were then covered and incubated for two hours at room temperature. The aspiration/wash was repeated as before. Next 100μl of biotin-conjugate was added per well. Plates were then covered and incubated for one hour at room temperature. The aspiration/wash was repeated as before. 100ul of streptavidin- horseradish peroxidase was added to each well. The plate was covered and incubated for thirty minutes at room temperature. The aspiration/wash was repeated as before. 100μl of tetramethylbenzidine (TMB) solution was added to each well. After a suitable colour had developed the reaction was stopped with 50μl/well of 0.5M H_2SO_4. The optical density of each well was measured at 450nm (Anthos labtech instruments).

3.2.8 Bender Medsystems hIL-6 ELISA.

An ELISA kit from Bender Medsystems was used to detect hIL-6 and was used in accordance with the manufacturer's instructions. To coat the plate with capture antibody 100μl of anti-human IL-6 antibody (2.5μg/ml), diluted in PBS, was transferred to each well of a maxi-sorp immuno-plate (NUNC). Immediately after coating, the plate was sealed and incubated overnight at 4°C. The following day, wells were washed 3X (0.05% Tween 20 in PBS) and aspirated using an autowasher (Labsystems, Well wash 4 Mk2). Plates were blocked by adding 300μl assay buffer (PBS containing 0.5% BSA, 0.05% Tween 20) to each well, and incubated for two hours at room temperature. Plates were then aspirated/washed as previously. 50μl of assay buffer and 50μl aliquots of standards (100-1.6pg/ml) and unknowns, and 50μl

of biotin-conjugate (diluted 1 in 1000 with assay buffer) were added per well and mixed by gently tapping the plate for 1 minute. Plates were then covered and incubated for two hours at room temperature on a microplate shaker set at 200rpm. The aspiration/wash was repeated as before. 100ul of streptavidin horseradish peroxidase was added to each well. The plate was covered and incubated for one hour at room temperature. The aspiration/wash was repeated as before. 100μl of TMB solution was added to each well. After a suitable colour had developed the reaction was stopped with 50μl/well of 0.5M H_2SO_4. The optical density of each well was measured at 450nm (Anthos labtech instruments).

3.2.9 Caco-2 growth on transwell inserts.

Caco-2 cells were grown on 4.7 cm^2, 0.4μm pore size polycarbonate membrane transwell inserts (Costar) in medium X (section 3.2.1) containing 100U/ml penicillin, and 100μg/ml streptomycin (Gibco), herein named PS medium. Cells were seeded into transwell inserts at a density of 4 X 10^5 cells/well. Apical chambers contained 1ml PS medium, whereas basolateral chambers contained 2ml of PS medium. Medium in each chamber was replaced every two-three days for fourteen days, after which time monolayer integrity was routinely demonstrated by measuring transepithelial resistance.

3.2.10 Measurement of epithelial cell monolayer integrity.

Transepithelial electrical resistance (TEER) of cells grown on transwell inserts was measured in PS medium. The TEER of each monolayer was measured with an

epithelial voltohmeter using 'chopstick electrodes' (EVOM World Precision Instruments, USA). The 'chopstick electrode' was positioned with its longest electrode in the basal chamber and the shorter electrode in the apical chamber. The mean TEER was calculated from three different positions. The intrinsic resistance of the insert (permeable support alone) was subtracted from the total resistance (cell monolayer and permeable support) to calculate the resistance of the monolayer. The resistance was corrected for surface area of the permeable support ($4.7cm^2$) and TEER expressed as Ohms cm^2 ($\Omega.cm^2$). A value of $250\Omega.cm^2$ (Caco-2 cells) and $160 \Omega.cm^2$ (MDCK cells), calculated from three different positions, was considered to represent sufficient development of monolayer integrity.

3.2.11 Measurement of polarised endogenous cytokine expression in Caco-2 cells.

Once Caco-2 cell monolayer integrity was established (section 3.2.10), monolayers were stimulated either apically, basolaterally or were dual stimulated with 1ng/ml IL-1β (R&D systems) for twenty-four hours in a humidified 37°C, 5% CO_2, 95% air mixture incubator. Apical chambers contained 1ml of PS medium whereas basolateral chambers contained 1.5ml of PS medium. Cell supernatants were analysed for the production of IL-6 (section 3.2.8) and LIF (section 3.2.7).

3.2.12 Data analysis.

Results for polarity of secretion are presented as ± standard error of the mean (SEM). Analysis of variance followed by the Tukey's test was used for statistical comparison between means and was calculated by Graphpad Prism software.

3.3   Results.

3.3.1   Biological activity of Caco-2 cell conditioned medium.

A study using the intestinal epithelial cell line, Caco-2, was set up in order to characterise the polarity of endogenous LIF secretion. The Caco-2 cell line was used because it is a well-characterised human polarised epithelial cell line, which is able to secrete cytokines and other proteins in a polarised manner. Preliminary experiments to determine if Caco-2 cells secrete LIF were carried out on confluent cultures of Caco-2 cells grown in 75cm flasks over a 48-hour period (section 3.2.2). LIF-like activity was assessed using the BA/F3-mLIFR-mgp130 bioassay using conditioned medium titrated at a two-fold dilution series across a ninety-six well plate (section 2.2.7.1). Biological assays are important as they provide a method for comparing the effects of a known standard and unknown preparations. Over a forty-eight hour period, the conditioned medium accumulated LIF-like activity, which was able to maintain the proliferation of BA/F3-mLIFR-mgp130 up to 1 in 16 dilution without reaching the maximal response exhibited by bacterial hLIF (Fig 3.1). In comparison, the hLIF standard (starting at 2ng/ml) was able to maintain the proliferation of BA/F3-mLIFR-mgp130 cells up to a 1 in 512 dilution. If all of the biological activity were due to LIF, and if the specific activities of the LIF standard and the LIF in the Caco-2 conditioned medium was similar, then the expected concentration may be estimated as approximately 62-125pg/ml.

BA/F3 cells also proliferate in response to other (non- IL-6 family) cytokines such as IL-3. To determine whether the source of BA/F3-mLIFR-mgp130 cell proliferation was LIFR/gp130 dependent, BA/F3 parental cells (lacking

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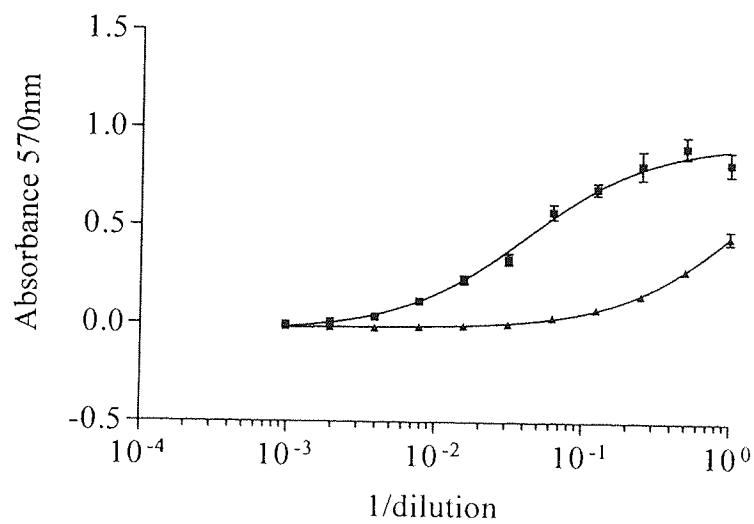
Figure 3.1. mLIFR-mgp130 cells proliferate in response to 48 hour Caco-2 conditioned medium. Sigmoidal dose response curve (standard slope) in the presence of (■) Bacterial hLIF (starting dilution 2ng/ml) or (▲) 48 hour Caco-2 conditioned medium from confluent $75cm^2$ tissue culture flask. Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

mLIFR/mgp130) were used in the same manner as outlined above. BA/F3 parental cells did not proliferate in response to Caco-2 conditioned medium, whereas in comparison the cells proliferated in the presence of IL-3 (Fig 3.2). It can therefore be concluded that Caco-2 cells must be secreting a LIFR and/or a gp130-dependent ligand.

3.3.2 Biological activity of bacterial (but not eukaryotic) LIF is inhibited by anti-hLIF antibodies raised against bacterial hLIF proteins.

Results shown in the last section determined that Caco-2 cells must secrete a LIFR and/or gp130-dependent ligand. However, the result is not a specific indication that LIF is responsible for the proliferative responses observed; another member of the IL-6 family may be responsible for the activity. To further determine if the proliferation of BA/F3-mLIFR-mgp130 cells was due to LIF itself, or other members of the IL-6 family that utilise LIFR and/or gp130, neutralising antibodies against bacterial hLIF (R&D systems) were used. The concentration of antibody required to neutralise hLIF activity is dependent, amongst other things, on the amount of cytokine present. To overcome this, the concentration of hLIF was titrated out in the presence of a constant amount of antibody allowing for a number of ratios of antibody to LIF to be tested. The effect of the antibody, if successful, would be to shift the growth curve to the right to higher concentrations of LIF, thus indicating antagonism of LIF activity in the medium. Initial experiments compared the effect of an R&D antibody (MAB250), raised against bacterial LIF, on eukaryotic hLIF produced in 293T cells and bacterial hLIF (starting from 2ng/ml). The manufacturer claims the neutralisation dose$_{50}$ of the antibody to be approximately 60-200ng/ml in the presence of 1.5ng/ml
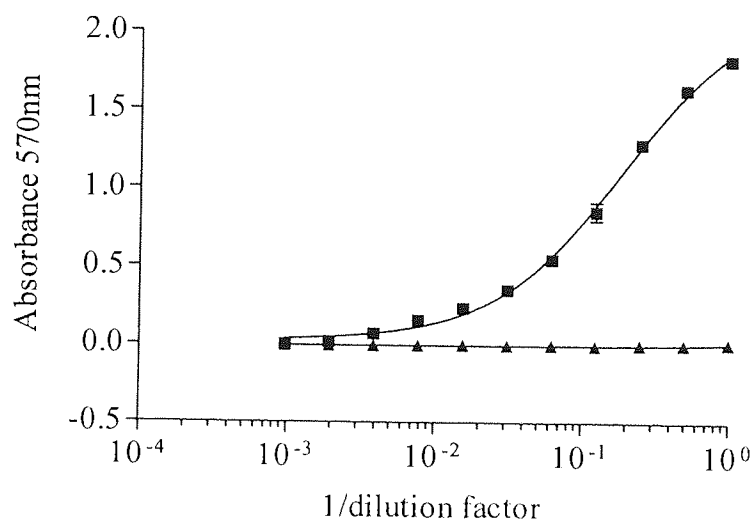
Figure 3.2. BA/F3 parental cells do not respond to 48-hour Caco-2 conditioned medium. Sigmoidal dose response curve (standard slope), in the presence of (■) IL-3 (200pg/ml) and (▲) 48 hour Caco-2 conditioned medium from confluent 75cm^2 tissue culture flask. Results are expressed as the A$_{570}$ of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

hLIF. The resultant growth curves revealed that although MAB250 (100ng/ml) was able to induce a 3.7 fold shift in EC50 (from 4.5 x 10^{-12}M to 1.67 x10^{-11}M) for bacterial hLIF, the monoclonal antibody was unable to induce a shift in EC50 for eukaryotic hLIF proteins (Fig. 3.3). The R&D antibody was only effective in antagonising the activity of bacterial hLIF causing a shift of the curve to the right. At saturating concentrations of LIF, the antibody did not reduce the maximal response of bacterial hLIF, indicating that the action of the antibody was not due to toxicity, since it was overcome by higher concentrations of hLIF. Since the R&D anti-hLIF antibody (MAB250), raised against bacterial hLIF was unable to neutralise eukaryotic hLIF activity in a bioassay, it was unsuitable for testing Caco-2 conditioned medium. This highlights the importance of considering glycosylation in determining the ability of antibodies to detect LIF.

3.3.3 Biological activity of Caco-2 cell conditioned medium and eukaryotic hLIF was inhibited by antibodies raised against eukaryotic hLIF.

Antibodies raised against bacterial LIF proved insufficient to inhibit the activity of eukaryotic hLIF, so alternate antibodies were sought in order to neutralise the LIF-like activity in Caco-2 cell conditioned medium. An antibody raised against eukaryotic human LIF (Chemicon) was used to determine its effects on the proliferation of BA/F3-mLIFR-mgp130 cells, using a known concentration of eukaryotic hLIF (section 2.2.5.5). This antibody was able to induce a five-fold shift in the EC50 of eukaryotic hLIF (from EC50 9.6 X 10^{-13} M to EC50 4.8 X 10^{-12} M), without affecting the maximal response (Fig 3.4). It was also able to inhibit the proliferative responses of BA/F3-mLIFR-mgp130 cells to Caco-2 cell conditioned
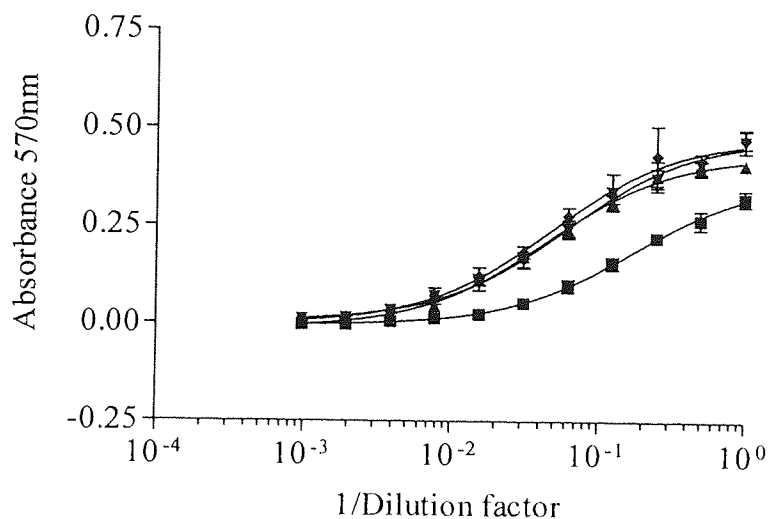
Figure 3.3. Proliferation of BA/F3-mLIFR-mgp130 cells was inhibited by the addition of 100ng/ml anti-hLIF antibodies to bacterial hLIF proteins. Sigmoidal dose response curve (standard slope) in response to ■ Bacterial hLIF (2ng/ml starting dilution) plus 100ng/ml anti-hLIF antibody, ▲ Bacterial hLIF (2ng/ml starting dilution), ▼ eukaryotic hLIF (1:2,000 starting dilution) plus 100ng/ml anti-hLIF antibody, ◆ eukaryotic hLIF (1:2,000 starting dilution). Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.
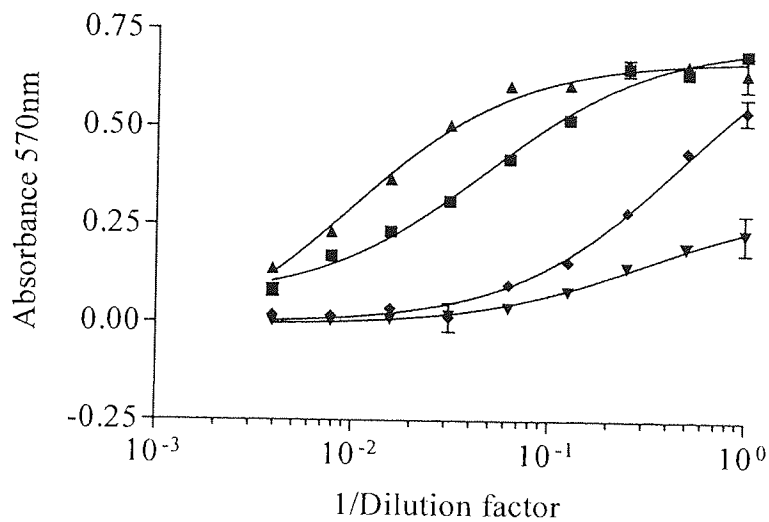
Figure 3.4. Proliferation of BA/F3-mLIFR-mgp130 cells was inhibited by the addition of 10μg/ml anti-glycosylated hLIF antibodies to eukaryotic hLIF and Caco-2 cell conditioned medium. Sigmoidal dose response curve (standard slope) in response to (■) Eukaryotic hLIF (2ng/ml) plus 10μg/ml anti-glycosylated hLIF antibody, (▲) Eukaryotic hLIF (2ng/ml), (▼) Caco-2 cell conditioned medium plus 10μg/ml anti-glycosylated hLIF antibody, (♦) Caco-2 cell conditioned medium. Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

medium, inhibiting the maximum proliferative response by three-fold. The significant neutralisation of LIF-like activity from Caco-2 cell medium, using anti-LIF antibodies, would suggest that a large proportion of this activity is actually LIF, and not other LIFR and/or gp130-dependent ligands.

3.3.4 The R&D hLIF ELISA underestimated the concentration of eukaryotic LIF.

Enzyme linked immunoassays (ELISA) provide a sensitive method of specifically quantifying a given substrate in solution. An hLIF ELISA utilising R&D antibodies raised against bacterial LIF was used to determine if eukaryotic hLIF protein could be detected. Initially the ELISA was tested for its ability to detect bacterial hLIF (Fig 3.5). Although the R&D ELISA claimed to detect hLIF within a range of 23.4–1500pg/ml, in our laboratory it was only able to detect as low as 80pg/ml bacterial hLIF. The lack of sensitivity in practice may be a genuine result or may represent differences in the preparation of R&D's preparation of LIF and our own. The R&D ELISA was used to test the Caco-2 cell conditioned medium, but was insufficiently sensitive to detect the protein responsible for LIF-like activity in the BA/F3-mLIFR-mgp130 bioassays (data not shown).

As the R&D ELISA proved inadequate to detect eukaryotic LIF in Caco-2 medium, and the capture antibody was unable to neutralise eukaryotic hLIF, an alternative ELISA was purchased. This ELISA from Bender Medsystems, used antibodies raised against eukaryotic hLIF protein, claimed a detection range of 3–200pg/ml and was able to detect at least 10pg/ml Bender Medsystem hLIF standard in
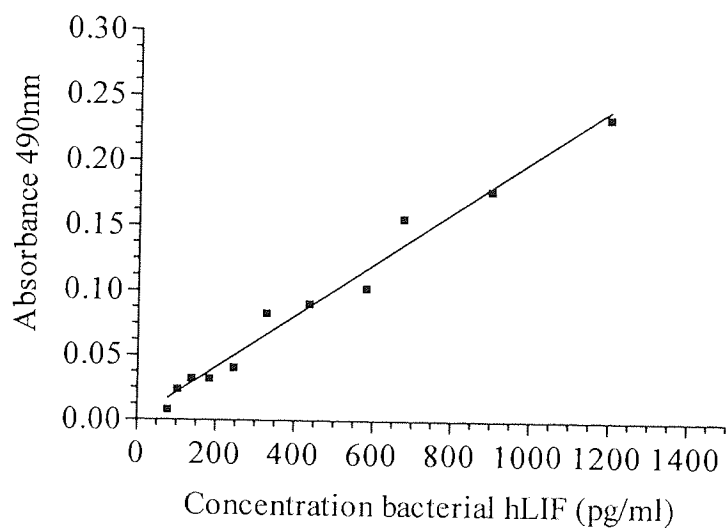
Figure 3.5. R&D hLIF ELISA detected approximately 80pg/ml bacterial hLIF.
Linear regression showing detection of bacterial hLIF using R&D hLIF ELISA ($R^2=$ 0.978 as calculated by Graphpad prism). Data points represent the mean of duplicate samples from a single experiment.

our laboratory (Fig 3.6). This ELISA was originally developed by Taupin *et al.*(1997) and later commercialised by Bender Medsystems.

To further test the ability of the R&D ELISA to detect eukaryotic hLIF, a known concentration of eukaryotic hLIF standard from the Bender Medsystems ELISA kit, (section 3.2.7) was titrated and compared to the estimated LIF values of the R&D hLIF ELISA (Fig 3.7). Values from the Bender standard were found to be approximately three-fold lower than that expected over a range of concentrations. In addition, the R&D ELISA was not sensitive enough to detect levels less than 180 pg/ml of eukaryotic hLIF protein. This data supports the suggestion that the monoclonal antibody (MAB250) used in the previous section might be unable to interact efficiently with eukaryotic LIF.

The Caco-2 cell conditioned medium was tested with the Bender Medsystem and this ELISA was able to detect approximately 32pg/ml hLIF from 20ml of 48-hour conditioned medium ($\sim$43pg/5cm^2). The amount detected by the ELISA is not far off the estimate from the BA/F3 mLIFR-mgp130 cell assay (62-125pg/ml). These results demonstrate for the first time, the production of LIF from unstimulated Caco-2 cells and underlines the importance of selecting an appropriate ELISA. This ELISA was also tested to determine its ability to detect bacterial hLIF (Fig 3.8), but it was unable to sensitively detect bacterial LIF over a range of concentrations. In comparing the R&D and Bender ELISA, they have different sensitivities toward bacterial and eukaryotic LIF. The R&D ELISA is better at detecting bacterial hLIF and the Bender Medsystem ELISA is better at detecting eukaryotic hLIF. This is perhaps unsurprising, since they were raised against either bacterial (R&D), or eukaryotic
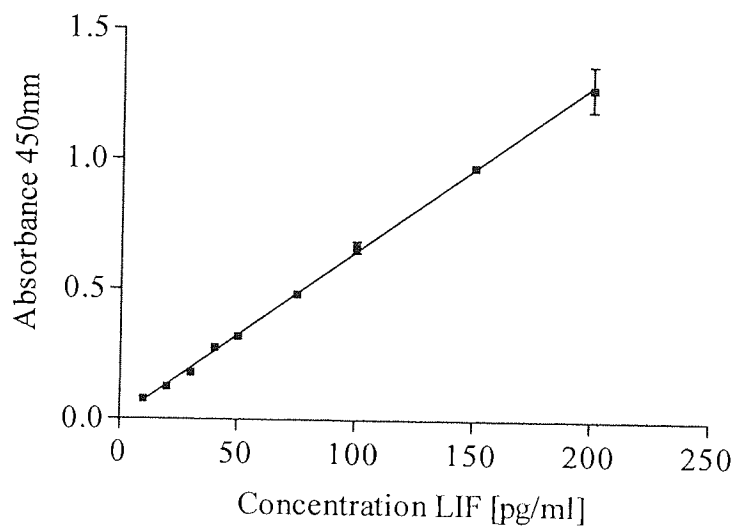
Figure 3.6 Bender Medsystem ELISA was able detected approximately 10pg/ml eukaryotic hLIF. Linear regression showing detection of eukaryotic hLIF using Bender Medsystem hLIF ELISA ($R^2 = 0.999$ as calculated by Graphpad prism). Data points represent the mean of duplicate samples from a single experiment.
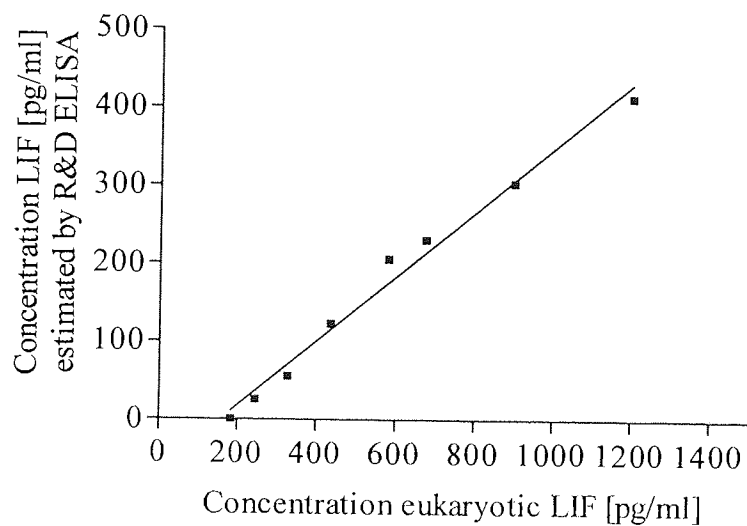
Figure 3.7. R&D hLIF ELISA underestimated the concentration of eukaryotic hLIF. Linear regression showing detection of eukaryotic hLIF (from Bender kit) using R&D hLIF ELISA using bacterial hLIF as the standard (R^2 = 0.986 as calculated by Graphpad prism). Data points represent the mean of duplicate samples from a single experiment.
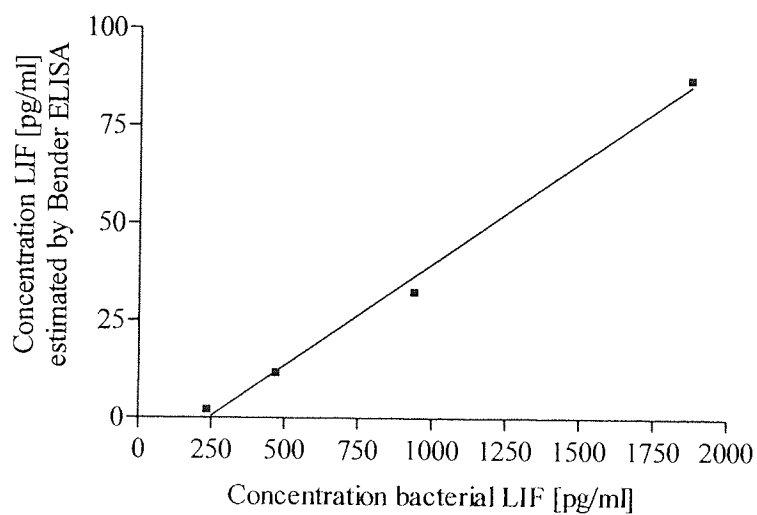
Figure 3.8. Bender hLIF ELISA underestimated the concentration of bacterial hLIF. Linear regression showing detection of bacterial hLIF using Bender hLIF ELISA ($R^2 = 0.99$ as calculated by Graphpad prism). Data points represent the mean of duplicate samples from a single experiment.

(Bender Medsystem) hLIF, and as such favoured the protein to which they were raised. This result is in agreement with the neutralisation assay, suggesting that Caco-2 cells secrete LIF when unstimulated. The conditioned medium tested, was obtained from cells that were cultured on tissue culture plastic. As such, it was unknown if the production of LIF was different at the apical and basolateral membranes. This would require the culture of cells on Transwell permeable supports, which allow independent access to both sides of a monolayer.

3.3.5 IL-1β stimulated the secretion of LIF from Caco-2 cells.

Characterising endogenous LIF levels is important since it defines target levels for exogenous expression, such that exogenous expression can be set to within a natural LIF secretion range. Since LIF is an inducible cytokine, experiments were conducted to determine whether IL-1β could stimulate LIF production by Caco-2 cells. To investigate the effects of IL-1β on the secretion of LIF, Caco-2 cells, grown in $75cm^2$ flasks, were stimulated with IL-1β (1ng/ml) for twenty-four hours. LIF-like activity was assessed using BA/F3-mLIFR-mgp130 cells. Medium was titrated as a 2-fold dilution series across a 96 well plate, and compared to LIF-like accumulation from unstimulated Caco-2 cells. Medium from IL-1β treated Caco-2 cells exhibited a 2.6 fold increase in maximal A_{570}, indicating that IL-1β was able to stimulate an increase in LIF secretion (Fig 3.9).

Medium from stimulated cells was subsequently tested using the Bender Medsystem ELISA and was shown to contain >200pg/ml hLIF. Together with the data from the bioassay, this suggests that Caco-2 cells can be induced to secrete LIF

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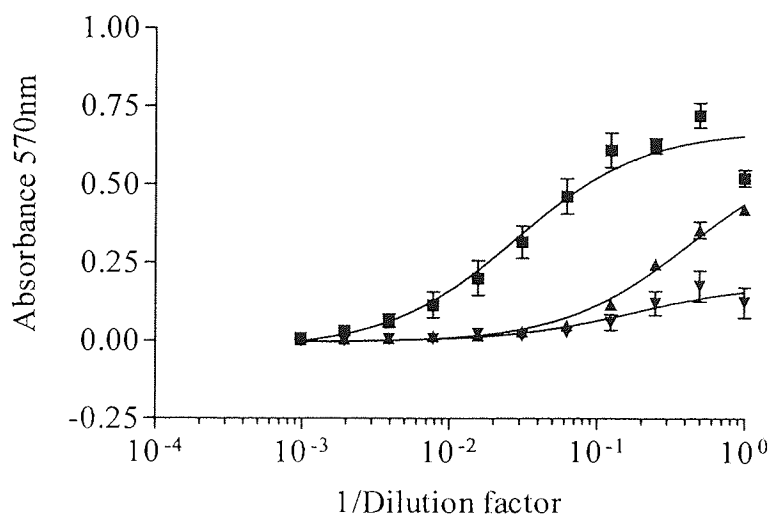
Figure 3.9. **BA/F3-mLIFR-mgp130 cells showed greater proliferation in response to IL-1β stimulated Caco-2 cell conditioned medium when compared to conditioned medium from unstimulated cells.** Sigmoidal dose response curve (standard slope) in response to (■) bacterial hLIF (2ng/ml starting dilution), (▲) 24-hour IL-1β stimulated Caco-2 conditioned medium, (▼) 48 hour Caco-2 conditioned medium from cells grown in 75cm^2 flasks. Results are expressed as the A_{570} of cells assayed for proliferation by MTT assay. Data points represent the SEM of triplicate samples from a single experiment.

that is biologically active. As Caco-2 cells were shown to respond to IL-1β, it will be possible to investigate the LIF secretion in Transwell cultures at both membranes in both unstimulated and stimulated cells.

3.3.6 The polarity of LIF secretion in Caco-2 cells when stimulated with IL-1β.

Since the Caco-2 cells studied in the previous section were grown on tissue culture plastic, it was not known from these results whether the production of LIF was greater at the apical or basolateral membrane. It was also unknown if LIF production was differentially regulated by IL-1β at the apical and basolateral membranes. To determine the polarity of LIF secretion, sealed monolayers of Caco-2 cells were grown on transwell filters. The formation of a sealed monolayer after 14 days was confirmed by measurements of the transepithelial electrical resistance (TEER), which, in unstimulated cells reached approximately 1,800 $\Omega.cm^2$. In addition, as some cytokines are capable of disrupting monolayer integrity, the effect of IL-1β was tested. Stimulation with IL-1β was found not to have any significant effect on monolayer permeability following a twenty-four-hour incubation (Fig 3.10).

The contents of the conditioned media of the apical or basolateral chamber were analysed by the Bender Medsystem ELISA. As the volumes in the apical and basolateral chambers differ, amounts rather than concentrations were compared when analysing the proportion secreted into each chamber. Unstimulated Caco-2 cells produced 19 ± 3pg LIF in the apical chamber and 27 ± 5pg in the basolateral chamber

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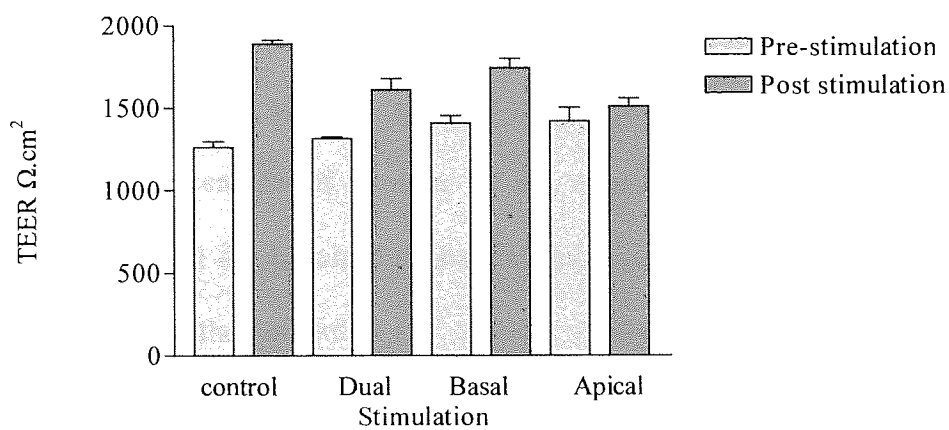
Figure 3.10. Stimulation with IL-1β (1ng/ml) did not significantly affect the transepithelial electrical resistance. TEER measured before and following stimulation with IL-1β (1ng/ml). Values are expressed as SEM (n=3) of three determinations/well for triplicate wells.

(Fig 3.11A). The total amount of LIF secreted $46pg/4.7cm^2$ in transwells, was comparable to the amount of LIF present in the conditioned medium of cells grown in $75cm^2$ flasks ($\sim43pg/5cm^2$). When analysed as a percentage of protein in the apical or basolateral chamber, the proportion of LIF when unstimulated was $59 \pm 1\%$ in the basolateral chamber (Fig 3.11B). Apical treatment with IL-1β resulted in increased LIF production to $81 \pm 20pg$ in the apical chamber and $104 \pm 31pg$ in the basal chamber with no significant change in the polarity of secretion. In contrast, basolateral treatment of cells with IL-1β showed a shift in the amount of LIF secreted to $39 \pm 8pg$ in the apical chamber and $159 \pm 39pg$ in the basolateral chamber. When analysed as a percentage of protein in the apical or basolateral chamber, the proportion of LIF in the basolateral chamber increased to $80 \pm 1\%$ from $59 \pm 1\%$. Further increases in the amount of LIF secreted were observed with dual stimulation, up to $111 \pm 22pg$ in the apical chamber and $214 \pm 39pg$ in the basolateral chamber. When analysed as a percentage of protein in the apical or basolateral chamber, the proportion of LIF in the basolateral chamber increased to $67 \pm 2\%$ from $59 \pm 1\%$. Figure 3.11 demonstrates that LIF was predominantly secreted in a basolateral direction irrespective of the side of stimulation. However, the amount of LIF secreted in the basolateral chamber differed significantly according to the side stimulated. Dual and basolateral stimulation lead to the largest amount secreted basolaterally. In addition they also have the largest effect on the polarity of secretion, leading to an increase in the proportion-secreted basolaterally. It should be noted that although the variation in the amount of LIF secreted is high between experiments, there is much less variation in the proportion of LIF secreted.

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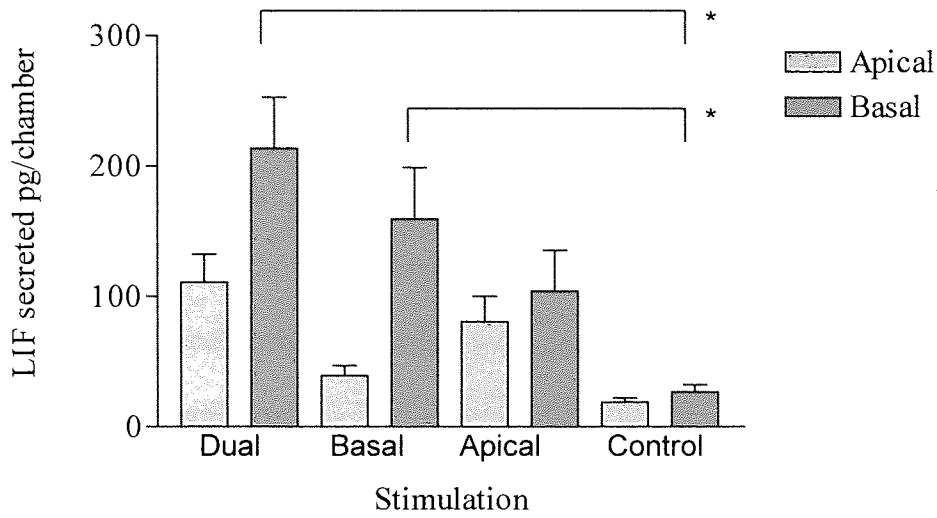
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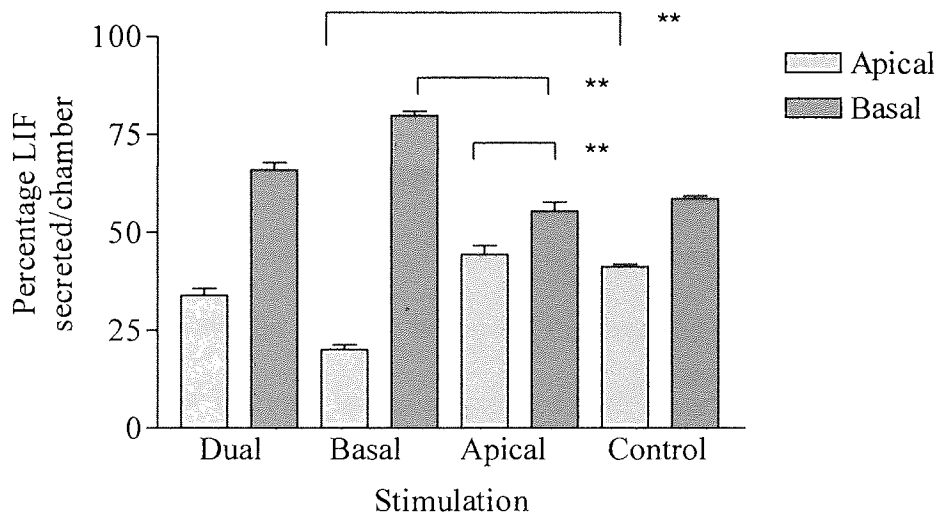
Figure 3.11 The polarity of LIF secretion in Caco-2 cells when stimulated with IL-1β. Polarised Caco-2 cells grown on Transwell filters were stimulated with IL-1β (1ng/ml) either in the apical chamber, basolateral chamber, both chambers simultaneously or none (control). Cell supernatants were collected from the apical and basolateral chambers and were analysed for LIF by ELISA. (A) LIF secreted (pg)/chamber in response to 1ng/ml IL-1β. (B) The same data as in (A) displayed as the percentage of LIF secreted/chamber in response to 1ng/ml IL-1β. Values are expressed as SEM (n=3). Brackets indicate compared samples. *P<0.05; **P<0.01; ***P<0.001. For a complete ANOVA analysis see appendix 26, 27.

3.3.7 The polarity of IL-6 secretion in Caco-2 cells when stimulated with IL-1β.

The differential regulation of IL-6 has previously been described by Moon *et al.*(2000). We therefore decided to repeat their experiments, in order to confirm their results and compare them to those observed with LIF. The possibility that the two cytokines were differentially localised in response to IL-1β, may indicate specific characteristics for each cytokine during inflammation. To test the polarity of IL-6 secretion, samples collected for LIF secretion were tested again but this time for the presence of IL-6. The synthesis of IL-6 was undetectable in untreated cells using the Bender IL-6 ELISA (Range 1.6-100pg/ml). Apical treatment with IL-1β resulted in 71 ± 11pg in the apical chamber and 55 ± 11pg in the basolateral chamber (Fig 3.12A). When analysed as a percentage of protein in each chamber, the polarity of IL-6 when apically treated with IL-1β was 57 ± 3% in the apical chamber and 43 ± 3% in the basolateral chamber (Fig 3.12B). In contrast, basolateral treatment of cells with IL-1β showed a similar increase in the amount secreted to 60 ± 11pg in the apical chamber and a larger increase in the amount of IL-6 secreted to 122 ± 29pg in the basolateral chamber. When analysed as a percentage of protein, the proportion of IL-6 found in the basolateral chamber was 67 ± 1%. Similar results were observed with stimulation of both sides of the monolayer in terms of the percentage of protein in the apical or basolateral media when compared to basolateral stimulation. However, significant increases to 176 ± 34pg in the basolateral chamber were observed when compared to that seen with apical or basolateral stimulation alone. In contrast to LIF production, unstimulated Caco-2 cells do not produce IL-6, therefore a comparison in the polarity of secretion with unstimulated cells is not possible. Interestingly, the

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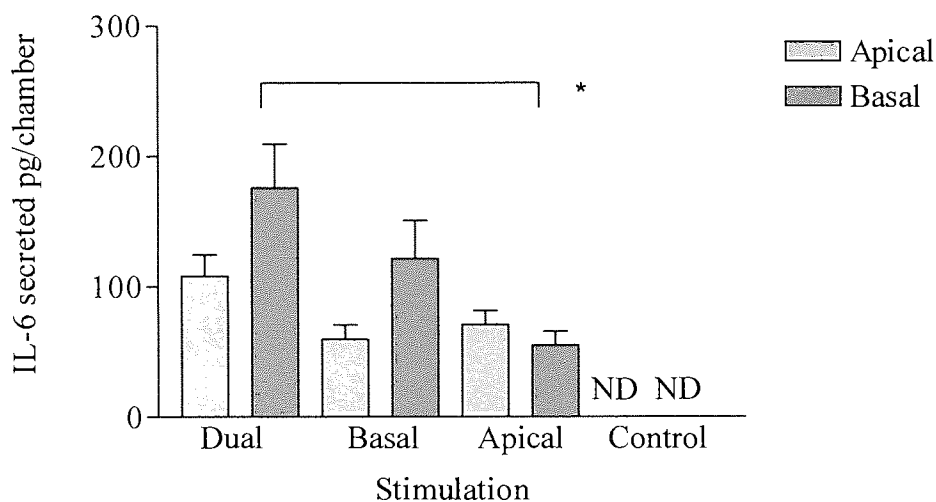
B
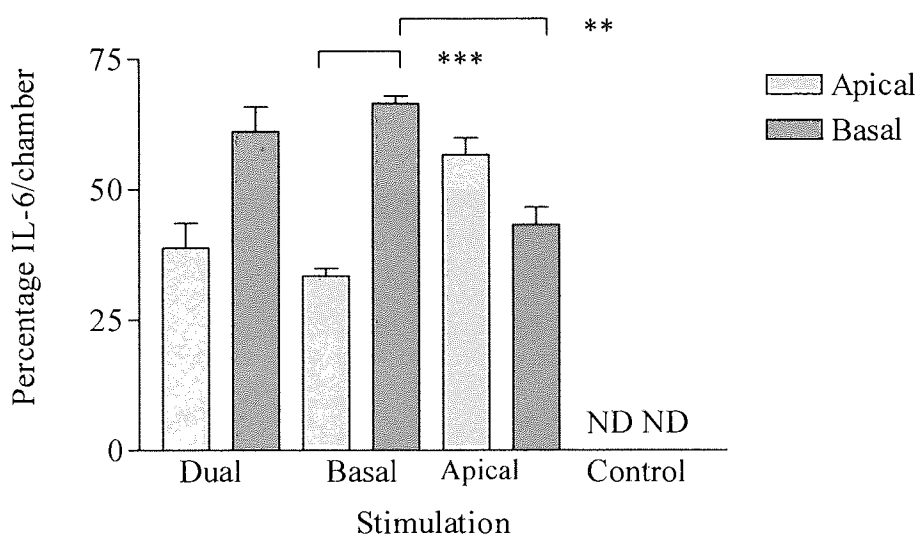
Figure 3.12 The polarity of IL-6 secretion in Caco-2 cells when stimulated with IL-1β. Polarised Caco-2 cells grown on Transwell filters were incubated with IL-1β (1ng/ml) either to the apical or basolateral sides or both simultaneously. Cells incubated without IL-1β produced no detectable amounts of IL-6. Cell supernatants were collected from the apical and basolateral chambers and were analysed for IL-6 by Bender ELISA. (A) IL-6 secreted (pg)/chamber in response to 1ng/ml IL-1β. (B) The same data as in (A) displayed as the percentage of IL-6 secreted/chamber in response to 1ng/ml IL-1β. Values are expressed as SEM (n=3). Brackets indicate compared samples. *P<0.05; **P<0.01; ***P<0.001. For a complete ANOVA analysis see appendix 28, 29.

production of IL-6 was differentially regulated by IL-1β at the apical and basolateral membranes, being secreted predominantly through the membrane that was stimulated with IL-1β. Although the percentage of LIF secreted apically is higher in apically stimulated cells than in basolaterally-stimulated cells, the apical membrane never becomes the predominant membrane for secretion of LIF as it does for IL-6.

3.4   Discussion.

Unstimulated Caco-2 cells produce detectable amounts of biologically active LIF. However, the detection of LIF in ELISAs is dependent upon the ELISA used, as well as the glycosylation state of the protein being analysed. When stimulated with IL-1β, Caco-2 cells produced significantly more LIF as well as IL-6. LIF was secreted predominantly at the basolateral surface, independent of the side stimulated with IL-1β. In contrast, the polarity of IL-6 secretion was dependent upon the side of the cell stimulated with IL-1β. For both proteins the amount of protein secreted was highest at the basolateral membrane when stimulated basolaterally.

The expression of pro-inflammatory cytokines and their receptors by normal (unstimulated) intestinal epithelial cells may point towards a role for these cytokines in the normal growth and differentiation processes e.g. crypt to villus maturation. Alterations in either cytokine or receptor expression may have a role in immune and inflammatory events by promoting or suppressing other molecules

Guimbaud et al.(1998) reported that patients with ulcerative colitis have a high production of LIF as detected from colonic perfusion. In addition, they used HT29 and Caco-2 cells (both are intestinal epithelial cells) as a model of the production and effects of LIF. They demonstrated that although HT29 cells make LIF, Caco-2 cells do not. They also showed that HT29 but not Caco-2 cells proliferate in a dose dependent fashion in response to LIF. HT29 cells are an intestinal cell line that does not generate a tight monolayer or display segregation of apical and basolateral membrane proteins. The results in section 3.3.4 indicate the failure to detect LIF by

Guimbaud *et al.*(1998) in Caco-2 cell conditioned medium may have been a result of the ELISA they used. Using the Bender ELISA we detected approximately 32pg/ml of LIF from Caco-2 cells conditioned medium. Guimbaud *et al.*(1998) used the R&D hLIF ELISA, which was shown here to greatly underestimate the quantities of eukaryotic LIF and which did not detect LIF in the conditioned medium from Caco-2 cells. Experiments using anti-LIF antibodies (R&D) that were raised against bacterial LIF revealed that they were also unable to neutralise the bioactivity of eukaryotic LIF.

We used bacterial LIF as a standard in the R&D ELISA (as recommended by the manufacturer) and BA/F3-mLIFR-mgp130 assays, however proteins produced in *E.coli* have severe drawbacks as denaturated and aggregated proteins may make up a significant proportion of the total protein concentration (Taupin *et al.* 1997). In addition using bacterial proteins as a standard could impair the real sensitivity and accuracy of the assay especially since glycosylation accounts for a significant part of the LIF protein (Taupin *et al.* 1997). We were able to demonstrate that an ELISA from Bender Medsystems was able to detect low levels of eukaryotic LIF protein, whilst antibodies from Chemicon raised against eukaryotic LIF, were able to neutralise the biological activity of eukaryotic LIF. Whereas those obtained from R&D that were raised against the bacterial protein were unable to correctly estimate the level of LIF produced or neutralise its activity in a bioassay. An important consideration when obtaining antibodies is the ability of the antibody to detect glycosylated proteins, especially in proteins such as LIF, where glycosylation makes up a significant portion of the mature protein.

Using the Bender Medsystem ELISA it was possible to accurately quantify the eukaryotic hLIF produced in section 2.3.5 and compare it with the values obtained from the Bender standard. The eukaryotic hLIF purified from 293T cells was on average 4-fold lower than the expected amount of protein determined by Coomassie protein assay. This protein also exhibited a 5.2-fold lower EC50, when compared to the Bender Medsystem standard in the BA/F3 mLIFR/mgp130 cell assay. Suggesting that the Bender Medsystem ELISA reflects the concentration of active proteins. Differences in the amount of protein detected by the ELISA may result from differences in the way the protein concentration was determined, as well as the specific activity of the purified protein.

Having established that Caco-2 cells produced LIF, it was possible to determine the polarity of LIF secretion and compare that to IL-6. When cultured on transwells Caco-2 cells form polarised monolayers with tight junctions and exhibit substantial TEERs, indicating the formation of a sealed monolayer. To induce LIF secretion in Caco-2 cells, IL-1β was used, as it is a pro-inflammatory cytokine that is capable of up-regulating LIF production in several cell types (Rathjen *et al.* 1990). However, it has been demonstrated that there is a correlation between exaggerated nitric oxide (NO) production induced by endotoxin, or endogenous inflammatory mediators such as IL-1β and the loss of mucosal barrier function. Xu *et al.*(2002) tested whether enterocytes could be induced to express iNOS and produce NO after endotoxin or cytokine challenge and tested the relationship between NO production and increased monolayer permeability. Using a cocktail of IL-1β, TNFα and IFNγ increases in Caco-2 monolayer permeability, NO production and iNOS expression were observed. This effect was blocked using NOS inhibitors, supporting the concept

that increased NO production is associated with loss of barrier function. As LIF was to be induced with IL-1β we tested the effects of IL-1β on monolayer integrity, but observed no differences following a 24-hour incubation.

To validate the TEER results in preliminary experiments we tested the permeability of Caco-2 monolayers to LIF. When large amounts of LIF were placed in the basolateral chamber, small amounts of LIF were detected in the apical chamber. However, when added to the apical chamber less than 1% of the LIF crossed the monolayer. The passage of LIF across the monolayer was specific for the basolateral to apical direction (data not shown). The restriction of passage from basolateral to apical chamber would indicate that monolayer integrity was not compromised. Disruption of the monolayer would have resulted in LIF traversing through the filter and cell layer to the opposite chamber, independent of the chamber to which it was added. Vreugdenhil *et al.*(2000) demonstrated the specific basolateral-apical passage of lipopolysaccharide binding protein across Caco-2 monolayers. Therefore mechanisms may exist in this cell line to transport proteins including LIF from the basolateral to apical side of the cell.

In Caco-2 cells, the calcium independent mannose 6-phosphate receptor (CIMPR) is enriched 3-fold on the basolateral surface, and is thought to recapture secreted lysosomal enzymes exclusively at this surface. The enzymes are subsequently delivered to the apical surface via an apical endosomal compartment; a site where the endocytic pathways from the apical and basolateral surfaces meet (Wick *et al.* 1999). Blanchard *et al.*(1998) demonstrated that CIMPR is capable of binding and endocytosing LIF via its carbohydrate moieties. The capture and sorting

of LIF to the apical surface may be mediated by CIMPR. Indeed, LIF may be carried in addition to lysosomal enzymes or IGFII. Investigators have shown that mannose 6-phophate containing lysosomal enzymes can compete with IGFII for binding to CIMPR suggesting overlap between the binding sites. LIF and IGFII do not compete for binding, and may suggest endocytosis of more than one ligand by an individual CIMPR (Blanchard *et al.* 1999).

In unstimulated Caco-2 cells the majority of LIF is secreted basally. Stimulation at the apical surface increases the amount of LIF secreted, but does not significantly alter the polarity of LIF secretion from that seen in unstimulated cells. In comparison IL-6 secretion is predominantly directed towards the apical membrane when stimulated at this pole. Thus both cytokines appear to be differentially secreted when cells are stimulated apically with IL-1β. Conversely, basal stimulation with IL-1β results in the release of both these cytokines towards the basal membrane, with increases in the amount of protein secreted. Similarly, dual stimulation results in the predominantly basal release of both cytokines, but with increased levels of protein secreted, probably as a result of the additive effects of increased receptor stimulation at both poles of the cell. The predominant release of IL-6 at the membrane that was stimulated is similar to results reported by Moon *et al.*(2000). As Caco-2 cells express IL-6 receptor at both apical and basolateral surfaces, IL-6 may act as a paracrine/autocrine factor, targeting nearby intestinal cells that are physically close and more distant cells in the lumen of the intestine. As IL-6 is a potent inducer of acute phase proteins (APP), the signal could then induce other cells to produce APP.

Vreugdenhil *et al.* (2000) demonstrated the polarised release of a number of acute phase response proteins. Lipopolysaccharide binding protein was secreted exclusively from the apical side of unstimulated Caco-2 cells. When stimulated apically with IL-1β, IL-6, TNFα and dexamethasone, the secretion of LBP into the apical medium was significantly enhanced whilst none was detected basolaterally. In contrast, basolateral stimulation resulted in secretion from both surfaces with the majority of LBP secreted basolaterally and a further increase in the amount secreted. In contrast to LBP, serum amyloid A (SAA) another positive APP, was detectable at both surfaces in response to cytokines at either side of the monolayer, but predominantly in the compartment corresponding to the side of stimulation. The negative acute phase proteins apoA1 and apoB are normally secreted from both sides of the cell but their secretion predominates at the basolateral surface. However, the total amount secreted decreases in response to cytokine stimulation with the greatest reduction observed in the basolateral compartment when stimulated basolaterally. These observations emphasise that the secretory response of intestinal cells is predominantly directed toward the pole at which inflammatory processes take place. A recurring theme indicates the basolateral secretion is greatest when inflammatory process take place at the subepithelium.

The production of LIF from intestinal epithelia both constitutively and in response to IL-1β raises important possibilities as to the influence of LIF. Results shown here add to the suggestion that enterocyte production and the release of at least some acute phase proteins is differentially regulated at the apical and basal membrane in response to paracrine factors. The detection of LIF in unstimulated cells is consistent with the detection of LIF from human colonic epithelial cells (Rockman *et*

al. 2001) and the presence of LIF mRNA obtained from murine intestinal tissue (Robertson *et al.* 1993). The basal production of LIF under normal conditions may have a number of paracrine roles. The persistence of high levels of LIF transcripts in the murine intestine is noteworthy in this regard, since it is a continuously renewing tissue and is characterised by the retention of active stem cell populations (Robertson *et al.* 1993), suggesting a role for basally secreted LIF. Instead of stem cells, Rockman *et al.*(2001) suggested an interaction of LIF produced by colonic epithelial cells with pericryptal fibroblasts, whilst Kalabis *et al.*(2003) went further to demonstrate that in 3-dimensional organotypic culture, LIF was the most significant factor for proliferation of isolated fetal colonic epithelial cells. The proliferation of LIF-stimulated epithelial cells was dependent upon the presence of fibroblasts in the matrix, further indicating a role for LIF in the cross talk between epithelial and mesenchymal compartments (Kalabis *et al.* 2003).

In addition to the potential targets of LIF, interesting questions have emerged as to how the site of stimulation is able to influence the pattern of protein secretion and this shall be discussed under future work in section 5.1. In conclusion, the results discussed in this chapter provide evidence for the vectorial secretion of LIF and IL-6 by intestinal epithelial cells. The potential interactions with other cells and mechanisms of vectorial transport demands further investigation.

Chapter 4: Exogenous LIF expression.

4.1   Introduction.

In order to understand which signal peptide has given rise to the mature LIF protein that a cell secretes, cell lines are required that only make a single signal peptide isoform endogenously. However there are no known cell lines that exclusively make D or M LIF isoforms. Alternatively the single isoforms could be expressed exogenously but it would be necessary to distinguish them from endogenously secreted LIF. In addition, in order to allow investigations into the effects of the level of LIF expression on the polarity of secretion, LIF would be best expressed under the control of an inducible promoter. This system would not only provide a system for switching on gene expression, but would also allow fine control over the level of expression.

In this thesis Caco-2 cells and Mandin-Darby canine kidney (MDCK) cells were used for the creation of inducible cell lines in order to express the alternate LIF isoforms. For a full discussion of Caco-cells see chapter 3. The MDCK cell line is a well studied polarised epithelial cell line that provides a useful model for studying the polarised sorting of proteins and the signals involved in these processes. An antigenic determinant that is expressed only on the epithelial cells of the thick ascending limb of the loop of Henle and the distal convoluted tubule is present on MDCK cells and indicates the possible origin of the cells (Herzlinger *et al.* 1982).

When determining the polarity of exogenous proteins it is crucial to consider the level of protein expression. Whilst investigating the mechanism of apical sorting

in the secretory pathway Marmorstein *et al.*(2000) expressed varying amounts of vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGFβ1). They found that at low expression levels apical sorting was efficient but was saturated at high expression levels, with excess protein detected increasingly in the basolateral medium. Consequently, matching the level of exogenous protein expression to physiological levels may be prudent.

One way of controlling the level of protein expression is to use regulated expression systems. Two such systems are the BD^Tm Tet-off and BD^Tm Tet-on gene expression systems, which allow regulated high level gene expression. In the Tet-off system gene expression is turned on when tetracycline (Tc) or doxycycline is removed from the culture medium. In contrast expression is turned on in the Tet-on system by the addition of doxycycline (Fig 4.1).

In *E.coli* the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon. TetR blocks the transcription of genes by binding to the tet operator sequence (Tet-O) in the absence of Tc or doxycycline. In order to set up a functional Tet system, the first step is to create a stable line expressing TetR. Once this is achieved the second step is to create a double stable cell line with a response plasmid containing a tetracycline response element (TRE) that contains the Tet-O and the gene of interest. The first component of the Tet system is the regulatory protein based on TetR. In the Tet-off system this 37kDa protein is a fusion of amino acids 1-207 of TetR and the C-terminal 127 amino acids of the herpes simplex virus VP16 activation domain. Addition of the VP16 domain converts the TetR from a
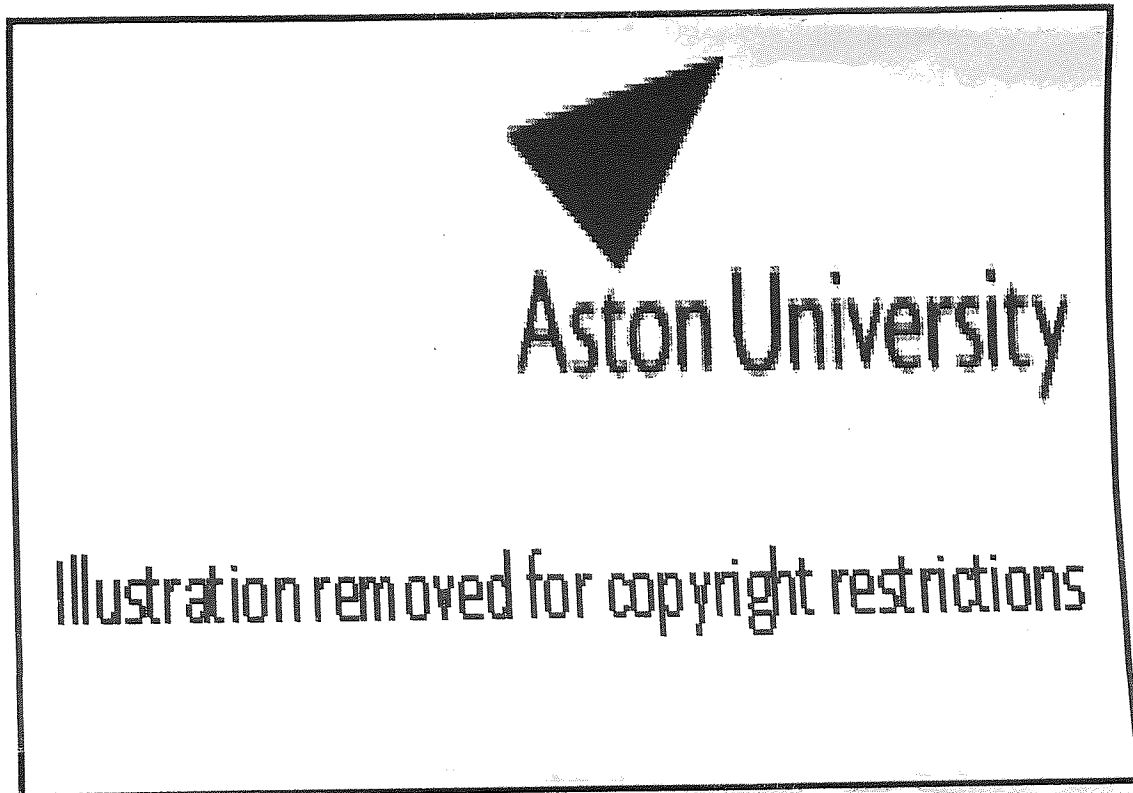
Figure 4.1. Schematic of gene regulation in the Tet-Off and Tet-On systems. A) Tet-On: The tetracycline response element (TRE) is located upstream of the minimal early promoter which is silent in the absence of activation. The "reverse" Tet repressor rtTA binds the TRE and activates transcription in the presence of doxycycline. B) Tet-Off: tTA binds the TRE and activates transcription in the absence of doxycycline. Reproduced from BD Biosciences protocol # PT3001-1.

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transcriptional repressor to a transcriptional activator (tTA). The Tet-on system is similar to the Tet-off system but the regulatory protein is based on a "reverse" Tet repressor (rtTA). As a result of these changes rtTA binds the TRE in the presence of doxycycline.

The Tet-On system is only responsive to doxycycline and not tetracycline. In contrast, the Tet-Off system is equally responsive to both doxycycline and tetracycline. It is recommended that doxycycline is used for both systems, because significantly lower concentrations of doxycycline are required for complete activation or inactivation (0.01-1µg/ml doxycycline vs. 1-2µg/ml tetracycline). In addition, doxycycline has a longer half-life (Twenty-four hours) than tetracycline (twelve-hours).

The second critical component is the response plasmid, which expresses the gene of interest under control of the TRE. The bi-directional (pBI) Tet vectors are specifically designed response vectors that allow co-regulated expression of two genes under the influence of a single TRE (Fig 4.2). This vector is ideal to allow the selection of clones based on the expression of the co-regulated marker gene luciferase or GFP, which provides a convenient test for stable expression of the plasmid. In theory, the presence of a co-regulated reporter may also enable an estimation of the level of expression that underlies the production of the protein of interest. To summarise, the ultimate goal in setting up functional Tet-systems is creating a double stable Tet-cell line, which contains the regulatory plasmid (introduced first) and the response plasmid (introduced second). The gene of interest is only expressed upon binding of tTA or rtTA protein to the TRE. (Gossen and Bujard 1992).

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Illustration removed for copyright restrictions
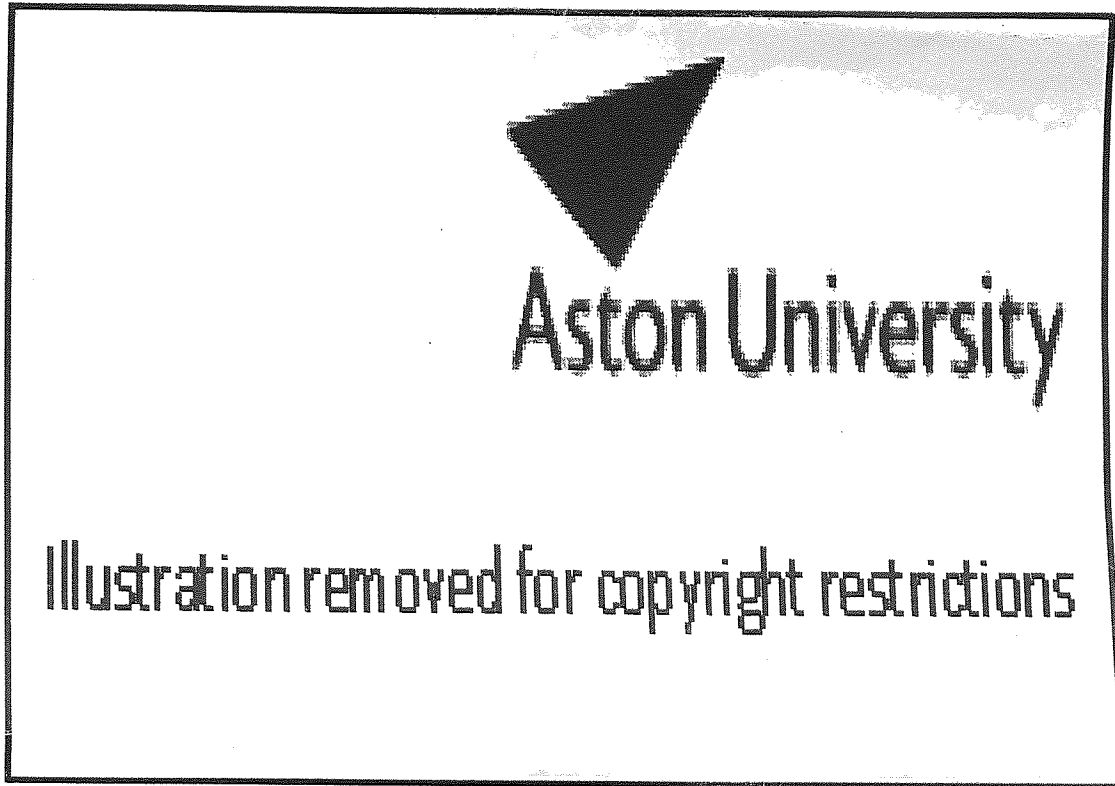
Figure 4.2   Schematic of gene regulation the pBI-L reporter plasmid (BD Biosciences) under control of rtTA. The pBI-L expression cassette allows a gene of interest (LIF) and a reporter (luciferase) to be expressed simultaneously under the control of a single tetracycline response element (TRE) in the presence of doxycycline in the Tet-On system and in the absence of doxycycline in the Tet-Off system. Reproduced from BD Biosciences protocol # PT3001-1.

The time needed to establish a double stable Tet cell line can be greatly reduced by purchasing a premade Tet cell line, which already expresses the appropriate regulatory protein. During this study (Madin-Darby canine Kidney) MDCK Tet-off cell lines were used for the production of double stable Tet-Off cell lines. The MDCK Tet-off cell line was created by stable transformation with the plasmid pUHD15-1 and pSVpuro (BD Biosciences).

Examining exogenous LIF production in MDCK cells will address whether the alternate signal peptides have a major influence in the polarised secretion of the mature LIF protein. It may also inform our understanding of the role of LIF in the kidney. Morel *et al.*(2000) demonstrated LIF secretion from normal isolated human renal tubular epithelial cells. In addition they demonstrated a dramatic increase of LIF in the urine (but not blood) of kidney transplant recipients undergoing acute rejection episodes and urinary tract infections. Studies of receptor localisation in MDCK cells revealed that LIFR is localised to both apical and basolateral plasma membranes, whilst gp130 is restricted mainly to the basolateral membrane. Stat3 activation was observed with stimulation by human LIF at both membranes, with greater responses observed when added basolaterally. A small fraction of activated Stat3 was detected in unstimulated cells, and this is possibly due to an autocrine factor produced by the MDCK cells. This observation was supported when conditioned medium from MDCK cells was applied to HepG2 cells, causing a prominent Stat3 activation (Buk *et al.* 2004). These results clearly demonstrate that MDCK cells express LIFR and gp130 and may also produce ligands capable of activating Stat3 such as LIF and/or other LIFR ligands. The production of LIF by normal tissue as

well as by inflamed tissue suggests that it may function as an important regulator of

kidney epithelial function.

4.2   Materials and methods.

4.2.1   Stable transfection of Caco-2 cells.

The day before transfection, cells were plated at 2 X 10^5 cells/cm^2 in a 75cm^2 flask, so that on the following day they were at 50-70% confluence. The DNA/CaPO^4 precipitate was prepared as described in section 2.2.5.2. Cells were then rinsed with 15% DMSO in 1X HBS for one minute, after which time the cells were rinsed and placed in medium (section 3.2.1). After 72 hours, the cells were passaged and plated into six well plates at 2 X 10^5 cells per well. After 48 hours genistein (G418) 400μg/ml was added. Cells were observed every two days. After five days massive cell death occurred, plates were then continually checked for the appearance of large healthy colonies. Colonies were selected using cloning discs (section 4.2.2).

4.2.2   Selection of colonies using cloning discs.

Colonies were selected at a diameter of approximately <5mm. Tissue culture medium was aspirated from the tissue culture plate, which was then washed twice in PBS. Cloning discs (Sigma) were then dipped in trypsin solution (section 3.2.1) and placed on the selected colonies and the plates returned to a humidified 37°C, 5% CO_2, 95% air mixture incubator for approximately seven minutes. Following the incubation cloning discs from the plate were removed and placed into a 24 well plate containing the appropriate selective agents (section 4.2.1). When cells approached confluence, the cells were trypsinised and transferred to larger culture vessels for expansion and characterisation.

4.2.3 Screening stable Tet-on cell lines (Caco-2 cells).

In order to identify G418 resistant clones that could meet the criteria suitable for stable Tet-on cell lines, transient transfection assays with pTRE2hyg-Luc (BD Biosciences) were used. Once clones had been picked and expanded they were screened once they had reached 50-80% confluency in a 6 well plate. The cells were trypsinsed and split about 1/3 into a single well of a six well plate. The cells in this "stock" plate were propagated depending upon the results of the screening assay. The remaining 2/3 of the cells were transfected with 1-2µg of pTRE2hyg-Luc, using the appropriate transfection method (section 4.2.1). The cells were then split into two wells of a six well plate. Into one of the two wells 1-2µg/ml doxycycline was added and the transfected cells were incubated for 48 hour in a humidified 37°C, 5% CO_2, 95% air mixture incubator. Cells were then assayed for induction using a luciferase assay by comparing cells with and without doxycycline (section 4.2.9). The fold-induction of luciferase was calculated using the following equation; Tet-on fold induction = +Dox RLU/-Dox RLU. The background (lysis buffer plus luciferase assay buffer) was subtracted from the total luciferase activity. Clones were selected with the highest fold induction (preferably clones that exhibited a >20 fold induction) for propagation and further testing (protocol PT3001-1 BD Biosciences Clontech).

4.2.4 Production of pBI-L-mLIF-M (EJH17) and pBI-L-mLIF-D (EJH18).

Murine LIF cDNA encoding both the LIF-D signal peptide and LIF-M signal peptide was isolated from the plasmids pcDNA3.1-mLIF-D (EJH4) and pcDNA3.1-mLIF-M (EJH5) using the restriction Nhe1 and HindIII. The resultant fragments were subsequently gel purified, and ligated in to the plasmid pBI-L (BD Biosciences)

linearised with the restriction endonucleases NheI and HindIII. For cloning procedures see the methods section chapter 2.

4.2.5   Stable transfection of MDCK TET-off cells.

Mandin-Darby canine kidney (MDCK) Tet-off cells are a premade cell line, which stably expresses the tTR regulatory protein. They were maintained in DMEM, high glucose (Gibco), supplemented with 10% FCS (Labtech), 50U/ml penicillin, 50μg/ml streptomycin (Gibco) and 1μg/ml puromycin (sigma). The later is to maintain the tTR DNA. Double stable cell cultures also contained 1μg/ml doxycycline. Cells were allowed to grow to 60-80% confluence before trypsinising in 0.25% trypsin, 0.2% EDTA (Gibco) and seeding into new 75cm^2 flasks at a one in ten dilution.

The day before transfection, cells were plated at 5 X 10^4 cells/cm^2 in a 75cm^2 flask so that on the following day they were at 50-70% confluence. Following transfection of the cells using the calcium phosphate method (section 2.2.5.2), cells were passaged and plated into 10 six well plates (unless otherwise stated) at 4 X 10^4 cells/well and placed back into a humidified 37°C, 5% CO$_2$, 95% air mixture incubator for twenty four hours (any remaining transfected cells were discarded). After 48 hours hygromycin 100ng/ml was added, together with, doxycyclin 1μg/ml (replaced every two days). Cells were observed every 2 days. After five days massive cell death occurred, plates were then continually checked for the appearance of large healthy colonies. Colonies were selected using cloning cylinders (section 4.2.6).

4.2.6 Selection of colonies using cloning cylinders.

Colonies were selected at a diameter of approximately < 8mm X 8mm. Cloning cylinders (Sigma) were firstly placed over the selected colonies using silicon grease on one end to seal the colony from the rest of the plate. Tissue culture medium was aspirated from the cylinder with a Gilson tip p1000 and the cells were washed twice with 100µl PBS. Trypsin solution (section 4.2.5) was then added to the cylinder and the plates returned to a humidified 37°C, 5% CO_2, and 95% air mixture incubator for approximately ten minutes. Following the incubation cells were removed by repeated pipeting using a Gilson tip P1000. Cells were then removed and placed into a 24 well plate containing the appropriate selective medium (section 4.2.5). When cells approached confluence, the cells were trypsinised and transferred to larger culture vessels for expansion and characterisation.

4.2.7 Titrating G418 and Hygromycin (Kill curves).

Cells were plated at a fixed density at 2 x 10^5 into each well of a six well culture dish containing 2ml of the appropriate medium plus varying concentrations (0, 50, 100, 200, 400 and 800µg/ml) of G418 or hygromycin. The cells were then incubated for 10-14 days in a humidified 37°C, 5% CO_2, 95% air mixture incubator, replacing the selective medium every four days. The cells were then examined every two days to identify the lowest concentration that began to give massive cell death in 5 days and that killed all cells within two weeks. Once the optimal drug concentration had been determined, it was necessary to determine the optimal plating density. Cells were plated at several different densities (9 X 10^5, 2 X 10^5, 9 X 10^4, 4 X 10^4, 2 X 10^4

and 9×10^3 cell/well) in each well of a six-well plate containing the appropriate selective medium. The cells were then incubated for 5-14 days in a humidified 37°C, 5% CO_2, 95% air mixture incubator, replacing the selective medium every four days. The cells were then examined every two days to identify the plating density that allowed the cells to reach 80% confluency before massive cell death at day 5 occurred and that killed all cells within two weeks.

4.2.8   Screening double stable MDCK Tet-off cell lines.

In order to identify clones exhibiting doxycycline-regulated gene expression they were screened once they had reached 50-80% in a 6 well plate. The cells were trypsinsed and split about 1/3 into a single well of a six well plate. The cells in this "stock" plate were propagated depending upon the results of the screening assay. The remaining 2/3 of the cells were split into two wells of a six well plate. Into one of the two wells 1-2µg/ml doxycycline was added and the cells were incubated for 48 hour in a humidified 37°C, 5% CO_2, 95% air mixture incubator. Cells were then assayed for induction using a luciferase assay (section 4.2.9). The fold-induction of luciferase was calculated using the following equation; Tet-off fold induction = -Dox RLU/+Dox RLU. The background (lysis buffer plus luciferase assay buffer) was subtracted from the total luciferase activity. Clones were selected with the highest fold induction for propagation and further testing (protocol PT3001-1 BD Biosciences Clontech). In addition to measuring the expression of luciferase, cells were also assayed for the presence of LIF in cell supernatants by ELISA (section 3.2.7 and 4.2.13).

4.2.9 Luciferase assay.

A luciferase assay to measure induction of luciferase in pBI-L stably transfected cells (sections 4.21 and 4.25) was obtained from Promega (E1500) and used according to the manufacturers guidelines. Growth medium was removed from the cells to be assayed and cells were rinsed with PBS. To lyse cells, 375µl of 1X lysis buffer was added per 9.6cm^2 tissue culture well. The culture was rocked several times to ensure complete coverage of the cells. Lysed cells were then scraped from the dish and transferred to a microcentrifuge tube and placed on ice. The microcentrifuge tube was then vortexed for 10-15 seconds and then centrifuged at 12,000 x g for 15 seconds. The supernatant was then transferred to a fresh tube. The supernatant (20µl) was then added to a white maxisorp microwell plate (Nunc) followed by 100µl of luciferase assay reagent. The plate was then read immediately using a plate reader that can measure luminescence (Molecular devices).

4.2.10 MDCK growth on transwell inserts.

Double stable MDCK Tet-off cells were grown on 4.7 cm^2, 0.4µm pore size polycarbonate membrane transwell inserts (Costar) in DMEM containing 100U/ml penicillin, and 100µg/ml streptomycin (Gibco), 1µg/ml puromycin, 100µg/ml hygromycin, and an appropriate amount of doxycyline. Cells were seeded into transwell inserts at a density of 1 X 10^6 cells/well. Apical chambers contained 1ml medium, whereas basolateral chambers contained 1.5ml medium. Medium in each chamber was replaced every two-three days for five days, after which time monolayer integrity was demonstrated by measuring transepithelial resistance (section 3.2.10).

4.2.11 Measurement of endogenous cytokine expression in parental MDCK Tet-Off cells.

Conditioned medium from confluent cultures of MDCK Tet-off cells grown in $75 cm^2$ flasks, was analysed for the presence of endogenous LIF after twenty-four hours, using either the R&D mLIF ELISA (section 4.2.13) or the Bender Medsystem ELISA (section 3.2.7).

4.2.12 Measurement of polarised exogenous cytokine expression in MDCK Tet-off cells.

Once double stable MDCK Tet-off cell monolayer integrity was established (section 3.2.10) monolayers were incubated for twenty-four hours in a humidified $37°C$, 5% CO_2, 95% air mixture incubator. Conditioned medium was analysed for the production of LIF using the R&D mLIF ELISA or the Bender Medsystem ELISA.

4.2.13 R&D quantikine mLIF ELISA.

An ELISA to detect glycosylated mLIF was used in accordance with the manufacturers instructions (all reagents were supplied with the kit). To each well of the pre-coated microplate strips $50\mu l$ of assay diluent was added together with $50\mu l$ of standards and samples. Plates were sealed and incubated for two hours. Each well was then aspirated and washed with $400\mu l$ of wash buffer for a total of five washes. After the last wash the plates were inverted on clean paper towels to remove any remaining wash buffer. To each well $100\mu l$ of diluted anti-mouse LIF conjugate was added. The plates were then sealed and incubated for two hours at room temperature.

The aspiration was step was repeated as above. 100μl of substrate solution was then added per well and incubated until a suitable colour had developed. 100μl of stop solution was then added to plates ensuring thorough mixing. The optical density of each well was then determined at 450nm using a microplate reader (Anthos Labtech instruments).

4.2.14 Data analysis.

Results for polarity of secretion are presented as ± standard error of the mean (SEM). Analysis of variance followed by the Tukey's test was used for statistical comparison between means and was calculated by Graphpad Prism software.

4.3   Results.

4.3.1   Development of Caco-2 Tet-on (tetracycline inducible) cell lines.

In order to study directly the effect of the alternate signal peptides on the polarised secretion of LIF, it was necessary to create a number of cell lines that were able to produce LIF with known signal peptides. To control the amount of LIF expressed to within "normal" levels, LIF would be expressed under the control of an inducible promoter. Having observed the endogenous secretion of LIF in Caco-2 cells, these cells were used in order to create an inducible cell line, with the plan of expressing epitope-tagged LIF (this study was initiated before the limited utility of LIF-FLAG was evident).

Prior to performing any experiments to develop Tet-inducible cell lines using the BD biosciences Tet-system, it was necessary to perform a dose response curve with the CHO-AA8-Luc Tet-Off control cell line provided by BD Biosciences. This would serve two critical functions: 1) Testing the effective concentrations of doxycycline (Dox). 2) Testing the potential tetracycline contamination of the Biowest fetal calf serum (FCS) used to culture the Caco-2 cells, which may significantly lower the level of induction achieved in comparison to Tet-system approved FCS. Over the range of concentrations tested there was no significant difference between the Tet-approved FCS and Biowest FCS (Fig 4.3). The level of induction in the presence of the Tet-system approved FCS was 19 fold, whilst the level of induction in the presence of the Biowest FCS was 292 fold. The apparent increase in the induction observed for Biowest serum was not due to differences in the maximal induction but was a result of the differences in the background observed in the presence of

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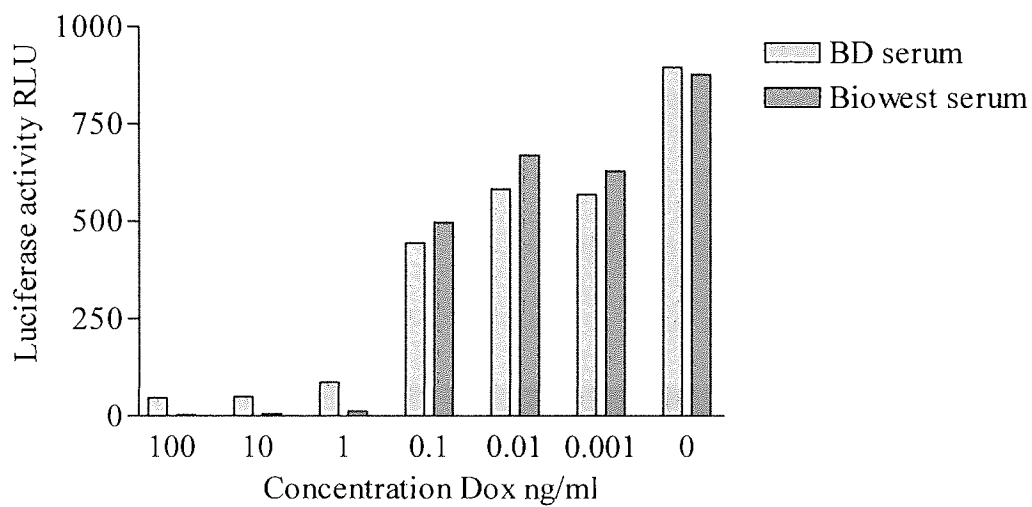
Figure 4.3 The induction of luciferase in CHO-AA8-Luc Tet-off cells was not significantly different in the presence of Tet-system approved FCS or Biowest FCS. CHO-AA8-Luc Tet-off cells were grown in the presence of either Tet-system approved FCS or Biowest FCS and tested for induction of luciferase over a range of Dox concentrations. Background RLU from cell lysis buffer alone has been subtracted. Data points represent samples from a single experiment.

doxycycline. The full (>10,000-fold) induction of luciferase outlined in the cell user manual was not observed with either type of serum. The apparent differences in induction compared to that identified in the manual could be due to a reduced efficiency of cell washing to remove doxycycline or could be due to the way in which BD Bioscience calculated their background. However, as there were no differences in the maximal expression of luciferase between the two types of serum and thus their tetracycline contamination it was decided that the Biowest FCS would be acceptable, for maintaining and testing inducible cell lines. Although almost maximal suppression of luciferase expression was observed at 100ng/ml doxycycline, it was decided that 1μg/ml would be sufficient for complete inactivation of expression. This level of doxycyline is far below cytotoxic levels for cell culture. Doxycycline has been shown to antagonize the proliferation of BA/F3 cells and Hep G2 cells at concentrations over 10μg/ml (Dr Mercia Spare personal communication)

4.3.1.1 Titrating G418 (Kill curves).

Prior to establishing stable Caco-2 cell lines, it was necessary to determine the optimal concentration of the selection agent G418 and the plating density for selection. The optimal concentration of G418 and optimal plating density would allow the cells to reach ~80% confluency before massive cell death occurred at approximately day five, and total cell death within two weeks. The concentration that had the required effect on cell survival was found to be 400μg/ml G418 (data not shown). Once this drug concentration was determined, it was necessary to determine the optimal plating density in the presence of the optimal drug concentration. Over the range of densities tested, 2×10^5 cells/9.6 cm^2 tissue culture well was found to be the optimal plating density for a single well of a six well plate (data not shown).

	Caco-2 Tet-On
No. of resistant clones.	24
No. of clones surviving selection with cloning discs	3
No. of clones exhibiting luciferase activity	3
No. of clones exhibiting inducibility	0

Table 4.1. Development of tetracycline inducible cell lines. Cells that exhibited resistance to selective antibiotics were cultured and assayed for inducibility of luciferase.

Having determined the optimal G418 concentration and plating density for Caco-2 cells, transfection and selection could begin in order to create stable Tet-On cell lines.

4.3.1.2 Screening stable Tet-On cell lines.

To ultimately create tetracycline-inducible Caco-2 cell lines, cells had to be first stably transfected with the Tet-on regulatory plasmid pTet-On (BD Biosciences). Once created these cells would be used to stably express a second plasmid: the response plasmid (pBI-L) expressing LIF-D or LIF-M under the control of a Tet-responsive element. Initially twenty-four hygromycin resistant clones were isolated, indicating uptake of the plasmid. Upon selection and replating of these cells using cloning discs, only three of these clones survived (Table 4.1). The low yield of surviving clones may reflect problems using the cloning discs or a more general difficulty in isolating stable Caco-2 cells. Performing a transient expression assay with the reporter plasmid pTRE2hyg-luc provides a quick indication of whether or not a cell line that gives a low background and high induction has been created. The Caco-2 cell clones were tested for transient inducible expression of luciferase following transient transfection with the plasmid pTRE2hyg-Luc. Clones were assayed for induction of luciferase in the presence and absence of doxycycline (1ug/ml). Although clones exhibited luciferase activity in comparison to the background (data not shown), none of the clones demonstrated inducibility. As such, the clones produced may not be suitable for the continued production of inducible Caco-2 cell lines.

4.3.1.3 Evaluation of mLIF quantikine ELISA.

As the Caco-2 Tet-on cells were not shown to be inducible in the previous section and since the epitope tagging of LIF proved problematic (chapter 2), an alternative strategy was desirable. Employing premade BD™ MDCK Tet-Off cells addressed both of these problems as these cells already stably expressed the Tet-Off regulatory plasmid and were unlikely to express endogenous LIF that would be confused with exogenous LIF in an ELISA since MDCK cells are canine. Experiments on Caco-2 cells and purified eukaryotic hLIF have already demonstrated the suitability of the Bender Medsystem ELISA for hLIF (chapter 3). To detect murine LIF in cells stably expressing mLIF constructs, an ELISA was obtained from R&D systems. The ELISA claims a detection range between 3-1,400 pg/ml mLIF (Fig 4.4). However as with the R&D hLIF ELISA the antibodies in this ELISA were also raised against the bacterial protein. The bacterial mLIF (section 2.2.4.2) and eukaryotic mLIF (section 2.2.5.5) were compared in this ELISA to the concentrations estimated by the R&D ELISA. The bacterial mLIF was detected accurately (within 5%) at the concentrations tested, suggesting a remarkable agreement in specific activities between recombinant LIF made by R&D and by ourselves. In contrast, the eukaryotic mLIF was estimated to be approximately 4-fold lower in concentration than the actual concentration tested (Table 4.2). Conditioned medium from MDCK cells was also tested in this ELISA and the Bender Medsystem ELISA but it did not detect any LIF protein (data not shown).

The apparent reduced sensitivity to eukaryotic mLIF will be discussed later. However, even if the reduced sensitivity is genuine, as there were no ELISAs
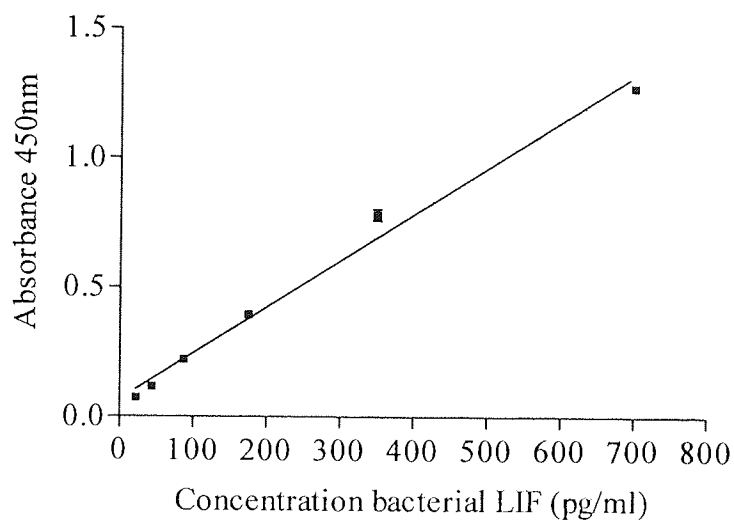
Figure 4.4 R&D quantikine mLIF ELISA detected approximately 20pg/ml bacterial mLIF. Linear regression showing detection of bacterial mLIF using R&D mLIF ELISA (R^2= 0.99 as calculated by Graphpad prism). Data points represent the mean of duplicate samples from a single experiment.

	Concentration (pg/ml) mLIF assayed by ELISA	Concentration (pg/ml) of mLIF estimated by ELISA	Fold difference
Bacterial mLIF	1000pg/ml	971pg/ml	1.03
Bacterial mLIF	500pg/ml	474pg/ml	1.06
Eukaryotic mLIF	1000pg/ml	258pg/ml	3.88
Eukaryotic mLIF	500pg/ml	88	5.70

Table 4.2. R&D quantikine mLIF ELISA detected bacterial and eukaryotic mLIF with different sensitivities. Bacterial or eukaryotic mLIF were placed into R&D quantikine mLIF ELISA and compared to values obtained for the mLIF standard provided. Values obtained are shown in pg/ml following extrapolation with the standard curve.

available that were specifically developed to detect eukaryotic mLIF proteins, this ELISA was therefore used for the analysis of cell lines expressing mLIF.

4.3.2 Development of MDCK Tet-off (tetracycline inducible) cell lines.

4.3.2.1 TEER measurements.

Firstly MDCK Tet-off cells were tested for their ability to produce confluent monolayers on transwells by measuring the TEER. MDCK cells express LIFR and gp130 and are responsive to stimulation with LIF (Buk *et al.* 2004). As these cells will be engineered to express mLIF or hLIF the effects of these proteins on monolayer integrity was also tested. Eukaryotic murine or human LIF (1ng/ml) was added to both sides of confluent monolayers and the effects on TEER were tested following a twenty-four hour incubation (Fig 4.5). MDCK cells produced TEERs in the region of 700-850 $\Omega.cm^2$ after five days growth on Transwells. No significant difference was observed following stimulation with either human or murine LIF in comparison to the control (no added LIF). To assess the reliability of the TEER measurements to verify the tightness of the monolayer, the ability of the monolayer to prevent the diffusion of eukaryotic mLIF was tested. Murine LIF (1ng/ml) was added to either the apical or basal side of the monolayer. Following a twenty-four hour incubation the media in the apical and basal chambers was tested for the presence of LIF using the mLIF ELISA. The mLIF ELISA did not detect mLIF in the basal compartment when mLIF was added to the apical compartment. However, when added to the basal compartment approximately 5% of the total amount of LIF detected in both chambers was found in the apical chambers (Fig 4.6). This result was unexpected since if the monolayer was leaky then passive diffusion would be expected to occur in both

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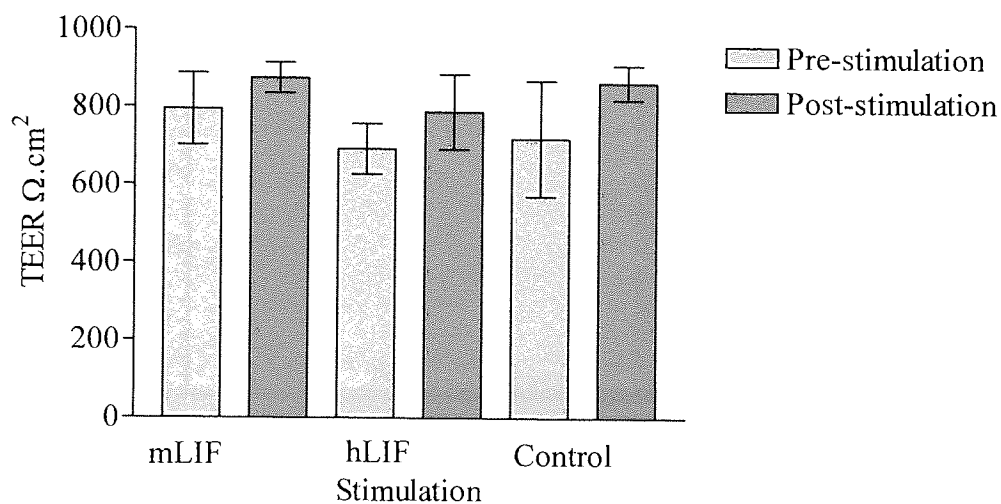
Figure 4.5 MDCK Tet-off cells formed confluent monolayers, which were not affected by stimulation with mLIF or hLIF. Eukaryotic mLIF or hLIF protein (1ng/ml) was placed into both the apical and basolateral chambers of independent transwells containing confluent layers of MDCK cells. Bars indicate the TEER before or following twenty-four hour incubation with hLIF or mLIF. Data points represent an average of three determinations/well for duplicate wells.
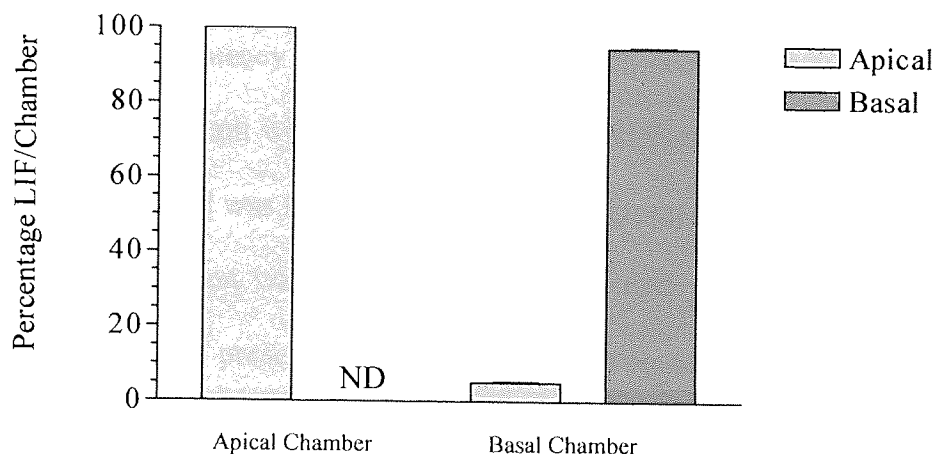
Figure 4.6 Transfer of eukaryotic mLIF across a confluent monolayer of MDCK cells occurred only in the Basal-Apical direction. Eukaryotic mLIF protein (1ng/ml) was placed into either the apical or basolateral chambers of independent transwells containing confluent layers of MDCK cells. Bars indicate the percentage of the total amount of LIF detected using the mLIF ELISA following a twenty-four hour incubation. Data points represent the mean of duplicate samples from a single representative experiment.

directions. The results may indicate that a more active transport process for LIF is operating in these cells (see discussion).

4.3.2.2 Titrating hygromycin (kill curves).

Prior to establishing double stable MDCK Tet-Off cell lines it was necessary to determine the optimal concentration of hygromycin for selection as well as the optimal plating density for selection. These optimal conditions would allow the cells to reach ~80% confluency before massive cell death occurred at approximately day five and total cell death within two weeks. The concentration that had the required effect on cell survival was found to be 100µg/ml hygromycin (data not shown). Once this drug concentration was determined, it was necessary to determine the optimal plating density in the presence of the optimal drug concentration. Over the range of densities tested, 4×10^4 cells/9.6cm^2 tissue culture well was found to be the optimal plating density for a single well of a six-well plate (data not shown). Having determined the optimal drug concentration and plating density for MDCK Tet-Off cells, transfection and selection could begin in order to create double stable Tet-Off cell lines.

4.3.2.3 Screening double stable Tet-off cell lines.

Following transfection, to identify all clones that produce LIF in response to induction with doxycycline, the clones were assayed for the production of luciferase and LIF. In the first round of transfections cells were transfected with the response

plasmid pBI-L-hLIF-D or pBI-L-hLIF-M. As there may have been potential problems with using cloning discs cloning cylinders were used instead.

Sixty wells (ten six-well plates) were plated, after transfection for each plasmid. Five clones containing the pBI-L-hLIF-D plasmid and four clones containing the pBI-L-hLIF-M plasmid were resistant to hygromycin. Of these, three MDCK Tet-Off-pBI-L-hLIF-D clones, and four MDCK Tet-Off-pBI-L-hLIF-M survived selection with cloning cylinders (Table 4.3). However, of these only one MDCK Tet-Off-pBI-L-hLIF-D clone (clone 6.1) demonstrated luciferase activity producing 640 RLU when switched on and 469 RLU when switched off. This clone subsequently died during the first few weeks of cell culture. When tested for LIF only the clone exhibiting luciferase activity produced detectable amounts of LIF, indicating that luciferase activity is a good predictor of LIF production (data not shown). The low number of clones isolated was probably due to an inefficient transfection (see discussion).

During evaluation of the hLIF clones, further transfections were carried out using the pBI-L-mLIF-D and pBI-L-mLIF-M plasmids. From the first transfection with the mLIF plasmids, it was possible to isolate nineteen mLIF-D clones and twenty-six mLIF-M clones. Of these, twelve mLIF-D and thirteen mLIF-M clones survived selection and replating with cloning cylinders. Only one mLIF-D clone (clone K) demonstrated luciferase activity. This clone also produced detectable amounts of LIF. However, this clone also died during the first few weeks of cell culture. Of the mLIF-M clones isolated, three (clones C, F, S) demonstrated luciferase activity (Fig 4.7), but only two (clones C, F) produced detectable amounts

	MDCK Tet-Off hLIF-D	MDCK Tet-Off hLIF-M	MDCK Tet-Off mLIF-D	MDCK Tet-Off mLIF-M
No. Of resistant clones.	5	4	39	26
No. of clones surviving selection with cloning cylinders	3	4	26	13
No. of clone exhibiting luciferase activity	1	0	9	3
No. of clones exhibiting inducibility	1	0	6	3

Table 4.3. Development of tetracycline inducible cell lines. Cells exhibiting resistance to selective antibiotics were cultured and assayed for inducibility of luciferase.

A
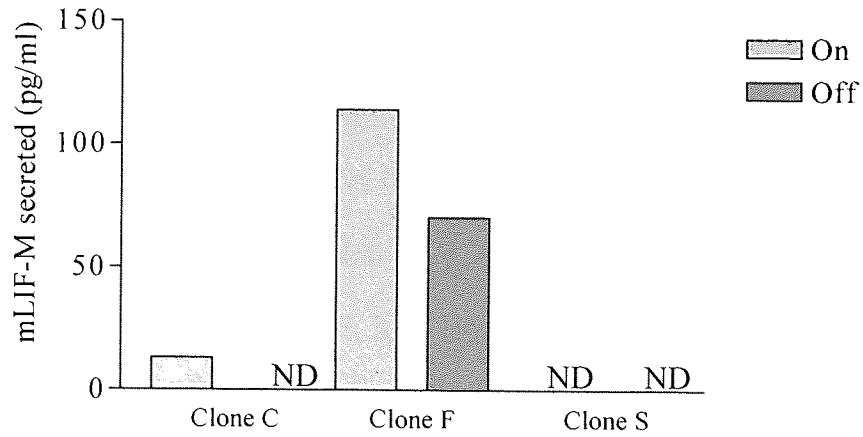
B

Figure 4.7 Production of mLIF-M and luciferase in the presence/absence 1μg/ml Dox. MDCK mLIF-M clones grown on six-well plates in the presence/absence of Dox 1μg/ml. A) LIF secreted (pg/ml), B) Production of luciferase. Data points represent samples from a single experiment.

of LIF (Fig 4.7). As a MDCK Tet-Off-pBI-L-mLIF-D cell line had not been successfully isolated, a further transfection was carried out using the pBI-L-mLIF-D plasmid. In this round the number of plates analysed was increased to twenty plates in the hope of isolating a larger range of clones. This time, twenty MDCK Tet-Off-pBI-L-mLIF-D clones were isolated. Of these fourteen survived selection and replating with cloning cylinders. Seven of these (4, 5, 8, 9, 10, 11, 19) demonstrated luciferase activity (Fig 4.8), but only five (4, 5, 8, 9, 19) produced detectable amount of LIF (Fig 4.8). Three inducible mLIF-D (4, 9, 19) and three inducible mLIF-M (clones C, F, S) clones were taken forward to examine the polarity of LIF secretion when grown on transwells (for comparison of induction of LIF and luciferase see table 4.4).

Comparison of the production of luciferase and LIF revealed that there is little correlation between the amount of luciferase produced and amount of LIF secreted between clones. For example, clone F produced <250 RLU, but produced >100pg/ml LIF. However, Clone C produced >500 RLU, but produced <25pg/ml LIF. Therefore, comparisons between clones expressing the alternate LIF signal peptides would not give any indication as to the relationship between the production of LIF mRNA for each isoform and the amount of LIF secreted (table 4.4). Screening of luciferase, does however provide an efficient method of predicting LIF production, since no clones produced detectable amounts of LIF in the absence of luciferase activity.
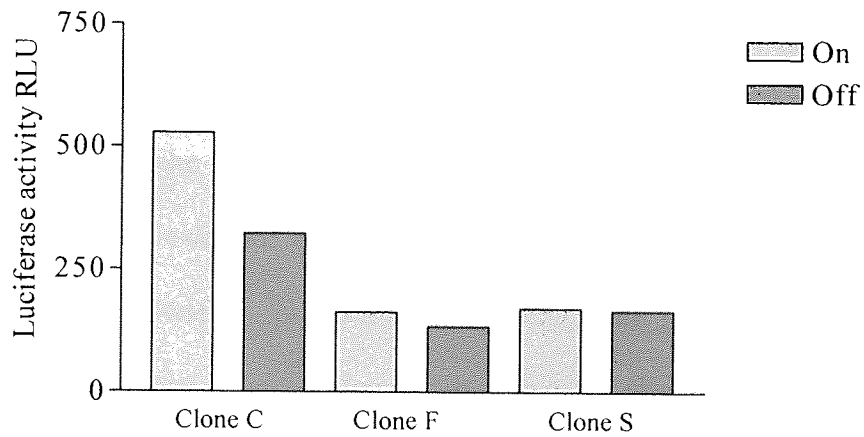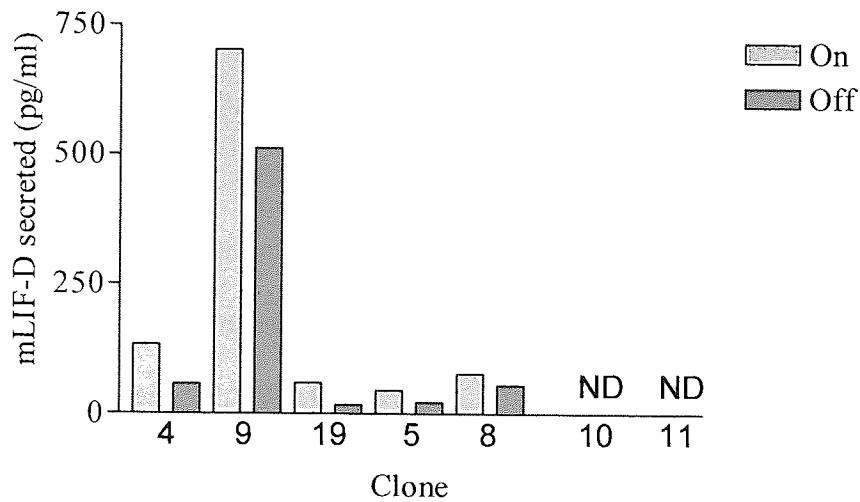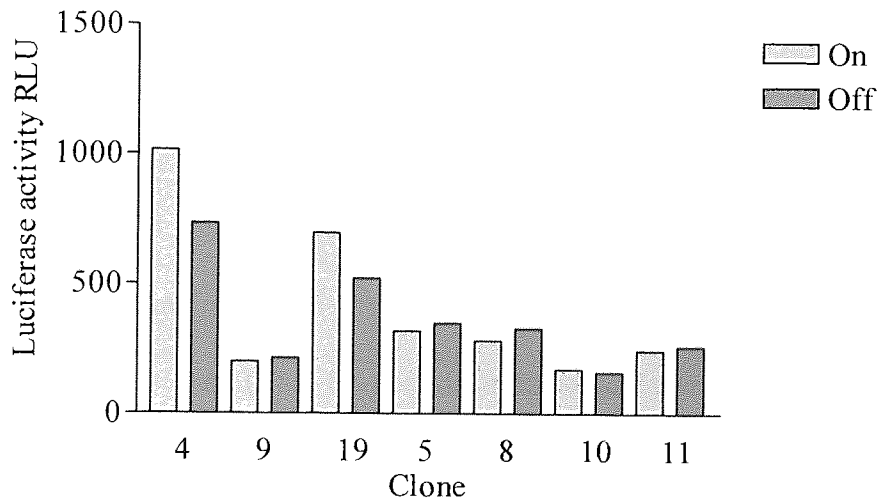
A

B

Figure 4.8 Production of mLIF-D and luciferase in the presence/absence of 1µg/ml Dox. MDCK mLIF-D clones grown on six-well plates in the presence absence of Dox 1µg/ml. A) LIF secreted (pg/ml). B) Production of luciferase. Data points represent samples from a single experiment.

4.3.2.4 Polarity of mLIF-D produced by MDCK Tet-Off cells.

To test the minimum and maximum levels of expression for the stable MDCK Tet-off mLIF-D cell lines, clones were analysed both in the fully switched on state (no doxycycline) or fully switched off (1μg/ml doxycycline). Since different clones expressed mLIF-D at different levels, this would allow a range of expression levels to be investigated and inform whether the level of expression was able to alter the polarity of LIF secretion. High producers of LIF may saturate their intracellular trafficking machinery and as a result, allow LIF secretion to occur at the opposite pole. Reaching saturation may be a mechanism that occurs naturally at high levels of LIF expression. By testing a range of LIF levels it may be possible to identify the level at which saturation occurs, and thus reveal a saturable mechanism controlling LIF secretion.

MDCK-Tet-off mLIF-D (4) produced the most LIF; when switched "on" this clone produced 90 ± 15ng ($61 \pm 1\%$) of LIF in the apical chamber and 56 ± 12ng ($39 \pm 1\%$) in the basal chamber (Fig 4.9). When switched "off" by the addition of doxycycline this cell line produced significantly less LIF and exhibited an altered pattern of secretion, 376 ± 10pg ($48 \pm 1\%$) in the apical chamber, and 412 ± 26pg ($52 \pm 1\%$) in the basolateral chamber (Fig 4.9). MDCK-Tet-off mLIF-D (9) when switched "on" produced 40 ± 11ng ($66 \pm 1\%$) in the apical chamber, and 21 ± 6ng ($34 \pm 1\%$) in the basal chamber. When switched "off", this cell line produced significantly less, 576 ± 118pg ($66 \pm 5\%$) in the apical chamber and 329 ± 138pg ($34 \pm 5\%$) in the basal chamber. Although the level of LIF secretion had been significantly lowered in the "off" state, the pattern of secretion in clone 9 in the "off"
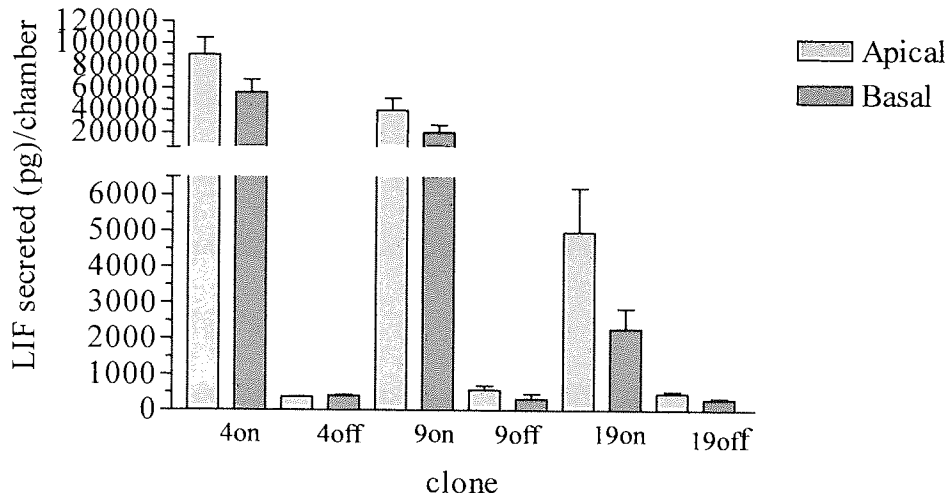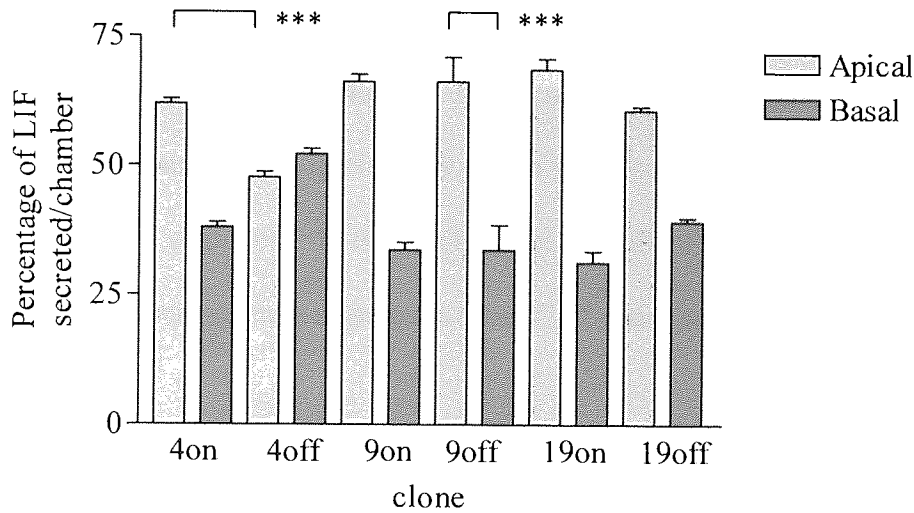
Figure 4.9 Stable MDCK Tet-off mLIF-D cells secreted mLIF in a polarised manner. Polarised MDCK cells grown on transwell filters were incubated with (off) or without (on) 1µg/ml doxycycline. Cell supernatants were collected from the apical and basolateral chambers and were analysed for mLIF by ELISA. (A) Amount (pg) of mLIF secreted/chamber. (B) Percentage of mLIF secreted/chamber. Values are expressed as SEM (n=3). Brackets indicate compared samples. *P<0.05; **P<0.01; ***P<0.001. For complete ANOVA see appendix 30, 31.

state is identical to the "on" state. MDCK-Tet-off mLIF-D (19) when switched on produced 5 ± 1ng (69 ± 2%) in the apical chamber and 2 ± 0.5ng (31 ± 2%) in the basal chamber. When switched "off" this cell line produced significantly less LIF 472 ± 64pg (61 ± 1%) in the apical chamber, and 307 ± 46pg (39 ± 1%) in the basal chamber. As in clone 9, clone 19 gives the same pattern of secretion in the "on" and "off" state (for comparison of induction of LIF and luciferase see table 4.4).

With the exception of clone 4 in the "off" state, all of the clones examined exhibited a similar pattern of LIF secretion, with the majority (>60%) of LIF secreted apically. Over the range of expression levels observed for two of the three clones tested the polarity of mLIF-D did not differ, suggesting that the pathway was not saturated between the levels observed. The highest level of LIF secreted was in clone 4 when fully switched "on" producing 56 ± 12ng in the basal chamber. The lowest amount of LIF produced was in clone 19,which when switched "off" produced 307 ± 46pg in the basal chamber. It is possible that for all of the clones analyzed, the trafficking of LIF was already saturated in the "Off" state (see discussion).

4.3.2.5 Polarity of mLIF-M produced by MDCK Tet-Off cells.

In order to compare any possible differences in the pattern of secretion between the LIF-D and LIF-M signal peptides, the MDCK Tet-off-mLIF-M clones were also examined over a range of expression levels. During the course of the experiments MDCK Tet-off-mLIF-M (C) stopped producing LIF completely, so had to be withdrawn. MDCK Tet-off-mLIF-M (F) when switched "on" produced the most LIF of all the mLIF-M clones analysed, 8 ± 1ng (78 ± 2%) in the apical chamber

and 2 ± 0.5ng ($22 \pm 2\%$) in the basal chamber (Fig 4.10). When switched off this clone produced significantly less LIF, 500 ± 27pg ($61 \pm 1\%$) in the apical chamber and 322 ± 22pg ($39 \pm 1\%$) in the basal chamber. MDCK Tet-off-mLIF-M (S), when switched on, produced 5 ± 1ng ($66 \pm 1\%$) of LIF in the apical chamber and 3 ± 0.5ng (34%) in the basal chamber. MDCK Tet-off-mLIF-M (S) did not produce a detectable amount of LIF when switched off, so comparison of lower levels of expression was not possible for this clone in this experiment. However, future experiments should titrate the concentration of doxycycline in order to find a concentration that produces a low level of LIF expression in this clone (for comparison of induction of LIF and luciferase see table 4.4).

With the exception of clone F in the "on" state, there is no significant difference in the pattern of secretion between the other two clones tested. The clones appear to secrete the majority of LIF ($>60\%$) in the apical direction, which is not dissimilar to the pattern seen with clones expressing mLIF-D (section 4.3.2.4). As such, there does not seem to be a difference between the two signal peptides in directing the polarity of secretion. As in the LIF-D clones, the levels of secretion observed with the LIF-M clones may also be saturating. The lowest total amount of LIF that was quantified, was 822 pg by MDCK Tet-Off-mLIF-M (F). This was approximately 2.7 fold higher than the highest level observed in Caco-2 cells when stimulated with IL-1β. In order to test for saturation it would be possible to alter the concentration of doxycycline to induce lower levels of expression in clone mLIF-M (S).

A
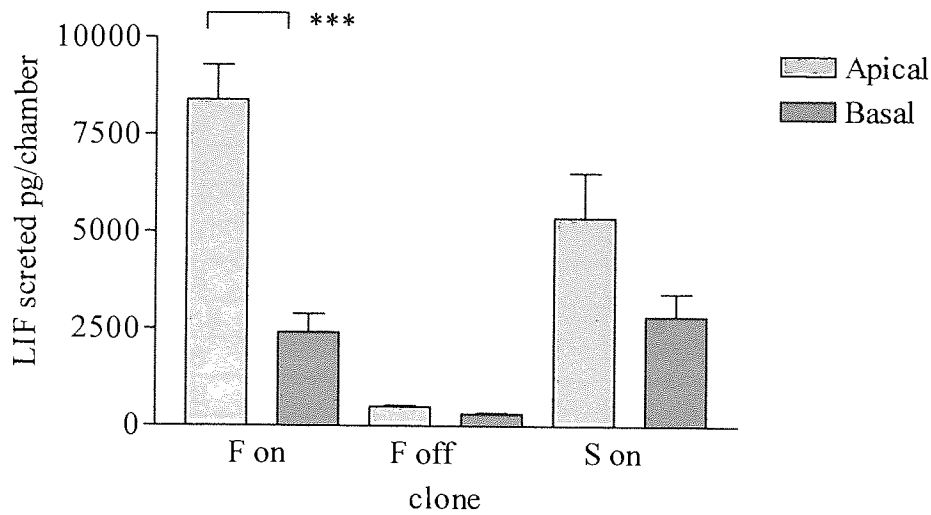
B
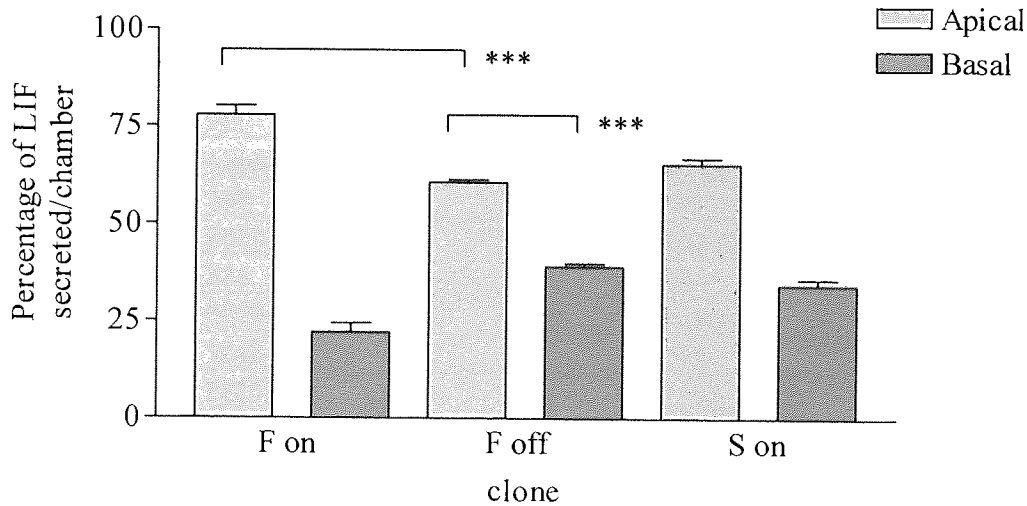
Figure 4.10. **Stable MDCK Tet-off mLIF-M cells secreted LIF in a polarised manner.** Polarised MDCK cells grown on transwell filters were incubated with (Off) or without (On) 1μg/ml doxycycline. Cell supernatants were collected from the apical and basolateral chambers and were analysed for mLIF by ELISA. (A) Amount (pg) of mLIF secreted/chamber (B) Percentage of mLIF secreted/chamber. Values are expressed as SEM (n=3). Brackets indicate compared samples. *P<0.05; **P<0.01; ***P<0.001. For complete ANOVA see appendix 32, 33.

A)

Clone On/Off	Luciferase activity (RLU)	Fold induction	Total amount of LIF (pg)	Fold induction
Fon	245	1.0	114	1.61
Foff	235		70	
Son	229	1.3	ND	0
Soff	171		ND	
9on	201	1.1	45	1.9
9off	215		23	
4on	1016	1.4	133	2.3
4off	736		57	
19on	698	1.3	78	1.4
19off	523		56	

B)

Clone On/Off	Luciferase activity (RLU)	Fold induction	Total amount of LIF (pg)	Fold induction
Fon	1318	4.6	10804	13.2
Foff	285		821	
Son	12895	65.0	8192	2730
Soff	198		ND	
9on	6599	19.7	60610	87.3
9off	334		694	
4on	50594	27.5	146031	185.3
4off	1840		788	
19on	22257	20.3	7257	9.3
19off	1099		779	

Table 4.4 Comparison of luciferase production and LIF secretion in MDCK-Tet-Off cells. A) Luciferase (RLU) and LIF (pg) production of MDCK-Tet-Off-pBI-L-mLIF-D and MDCK-Tet-Off-pBI-L-mLIF-M cells when grown on six-well plates. B) Luciferase (RLU) and LIF (pg) production of MDCK-Tet-Off-pBI-L-mLIF-D and MDCK-Tet-Off-pBI-L-mLIF-M cells when grown on transwells. The fold induction of LIF produced by clone S is calculated based on the limit of detection for the mLIF ELISA (3pg/ml) as no LIF was detected. Values are expressed as the average (n=3).

4.4 Discussion.

Caco-2 cells stably expressing the Tet-On regulatory plasmid (BD Biosciences) were created. However, despite demonstrating the production of luciferase when transiently transfected with a luciferase reporter they did not demonstrate inducibility. An alternative epithelial cell line that expressed the Tet-Off regulatory plasmid (BD biosciences) was obtained (MDCK Tet-Off). A number of double stable MDCK Tet-Off cells lines were created that expressed the mLIF-D or mLIF-M isoforms. However, over a range of expression levels, no differences were observed in the pattern of secretion between either murine LIF isoform, both mLIF-D and mLIF-M were secreted in a predominantly apical direction (60%).

To understand which signal peptide has given rise to the mature LIF protein that a cell secretes, the single LIF isoforms were expressed exogenously. Prior to developing MDCK Tet-off cell lines Caco-2 cells were used to create an inducible cell line to allow investigation into the effect of the LIF isoforms on the polarity of secretion. Of all the Caco-2 clones isolated, only a few survived selection using cloning discs. It is possible that selection using cloning discs is unsuitable for the selection of Caco-2 cell colonies, and this may have resulted in the death of most of the colonies picked. However, Yu *et al.* (2001) also reported a low yield of inducible clones when trying to produce stable inducible Caco2 cell clones using glass-cloning rings. It is therefore possible, that there may be a more general problem with isolating stable Caco-2 cell lines. Isolation of stable Caco-2 clones is not impossible since others have reported the isolation of stable Caco-2 cell line (Wick *et al.* 2002).

Of the three surviving Caco-2 cell Tet-on clones, all demonstrated a high level of luciferase activity when grown on six-well plates. However none of the clones demonstrated inducibility when grown on six-well plates. In view of the results indicating an increase in induction when MDCK-Tet-Off cells were grown on transwells, this may also be true for the Caco-2 clones and requires further investigation. As LIF-FLAG proteins proved insufficient to separate exogenous LIF from endogenous LIF, and Caco-2 cells were not inducible, systems for exogenous expression were developed in MDCK cells. MDCK cells confer an advantage of not presenting a mouse or human background and as such allow exogenous proteins to be separated from any endogenous LIF background using species specific ELISAs.

An ELISA that claimed to be capable of detecting eukaryotic mLIF was purchased from R&D. In order to test the ability of this ELISA to detect eukaryotic LIF, the eukaryotic LIF produced in section 2.3.5 was tested. Purified eukaryotic mLIF produced in section 2.3.5 demonstrated a 2.7-fold lower EC50 compared to bacterial mLIF and 3-fold lower than the eukaryotic hLIF in BA/F3 mLIFR-mgp130 bioassay (section 2.3.6). Some of this reduction in activity relative to eukaryotic hLIF could be attributable to the fact that murine LIF has a lower affinity for the mLIFR-mgp130 receptor complex than does the human LIF. This would manifest itself as a shift in the curve to the right toward the higher concentrations of LIF. However the eukaryotic mLIF also fared worse than the bacterial mLIF, which may be expected to contain more aggregates, misfolded proteins and degraded proteins due to the absence of posttranslational modification (Taupin *et al.* 1997).

In the R&D mLIF ELISA the concentration of eukaryotic mLIF was underestimated by approximately 4-fold. As with the R&D hLIF ELISA, this may represent the specificity of the antibodies toward the protein to which they were raised. However, the data from the ELISA for eukaryotic mLIF (four-fold lower activity compared to bacterial mLIF) and the bioassay for eukaryotic mLIF (2.7 fold lower EC50 compared to bacterial mLIF) are in good agreement suggesting that this ELISA is probably adequate for detecting eukaryotic mLIF. As no alternate ELISA was available to detect eukaryotic mLIF proteins this ELISA was used for analysis of cell lines expressing mLIF. Even if the absolute amounts of mLIF detected are underestimated with the mLIF R&D ELISA, the determination of the proportion secreted apically and basolaterally will not be affected.

The explanation for the difference between the bacterial and eukaryotic mLIF protein in the BA/F3 mLIFR mgp130 assay is less clear, and may result from the quality of the preparation. It appears that since more protein was produced with the eukaryotic mLIF (2.4mg/l) than with the eukaryotic hLIF preparation (0.5mg/l), it is possible that higher LIF production could have resulted in the misfolding and aggregation of the eukaryotic mLIF. To determine this, further preparations should be produced and compared to the bacterial mLIF and the preparation of mLIF used here.

Morphologically MDCK cells exhibit apical microvilli, and junctional complexes and when grown on permeable membrane supports can generate a transepithelial electrical resistance. It is possible that cytokines and other signalling molecules are able to disrupt the integrity of epithelial monolayers. MDCK cells express LIFR and gp130 and are responsive to stimulation with LIF, and as these cells

were engineered to express mLIF or hLIF the effects of these proteins on monolayer integrity was also tested. However, neither murine nor human LIF at high concentrations demonstrated an ability to disrupt the integrity of the epithelial monolayer when compared to the control. The expression of LIFR and gp130 in MDCK cells suggests that LIF may be a physiological regulator of kidney epithelial function. Indeed ureteric bud cells secrete LIF, which then leads to conversion of mesenchyme to epithelial cells during kidney development (Barasch et al. 1999). Adult roles in homeostasis or injury/repair could follow from the earlier developmental roles.

To test the effectiveness of using TEER as a marker of monolayer integrity, MDCK cell monolayers were tested for their ability to prevent the diffusion of eukaryotic LIF. When LIF was added to the basolateral side of cells, 5.2% of the total amount of LIF was detected in the apical compartment. No passage of LIF across the MDCK monolayer could be detected when LIF was added to the apical chamber. The specific basolateral-apical diffusion of eukaryotic LIF in MDCK cells suggests that an active transport process is occurring. One possible explanation for this may relate to the polarised distribution and trafficking of CIMPR and its ability to bind LIF. In MDCK cells CIMPR traffics between the TGN and the endocytic organelle of the basolateral domain and is found predominantly (90%) on the basolateral surface (Prydz et al. 1990).

Cell lines were generated from MDCK Tet-off cells in which, LIF expression was regulated by a tetracycline inducible promoter. Isolated clones revealed induction of LIF secretion and production of luciferase in the absence of doxycycline,

but the levels of induction were not positively correlated. It is possible that translation from the bidirectional promoter is not equal. Similar discrepancies were also noted by Yu *et al.* (2001) whilst developing a Tet-off system using the pBI-L vector in Caco-2 cells. As such, the use of luciferase in determining the level of expression underlying the level of secretion for each isoform was not possible.

When placed on transwells MDCK clones exhibited a greater fold induction as well as increases in the absolute levels of luciferase activity and LIF secretion than when grown on six-well plates (Table 4.4). When grown on transwells cells were plated and allowed to grow for five days before samples were collected as opposed to 48 hours on six-well plates. This may have allowed greater cell densities, due to increased growth and cell packing to occur, and thus allowed increases in the amount of LIF and luciferase produced. In addition cells grown in this manner may also have become fully polarised which may lead to more efficient secretion of proteins, as has been noted for Caco-2 cells (Molmenti *et al.* 1993). In addition Low *et al.* (2000) demonstrated that upon loss of cell polarity, epithelial cells redirect membrane trafficking pathways to intracellular cognate apical and basolateral compartments. These compartments appear not to be degradative, lysosomal compartments, indicating that proteins may be stored in them until cell contacts are re-established. Indeed vacuolar apical compartments (VACs) are rapidly exocytosed as a whole from MDCK cells after reestablishment of cell-cell contacts (Low *et al.* 2000). The storage of proteins may explain the reduction in the efficiency of secretion in comparatively sparse populations of MDCK cells in the six-well plates.

Exogenous mLIF produced in MDCK Tet-Off cells is secreted predominantly in an apical direction (>60%) irrespective of the signal peptide present. This would suggest that the LIF-M signal peptide does not direct the secretion of LIF basolaterally toward the ECM. The presence of LIF in the ECM of these cells has yet to be tested. The level of LIF secreted did not appear to influence the pattern of secretion over the range of expression tested. The lowest total amount of LIF secreted was 779pg, which is approximately 2.5 fold higher than the highest level of endogenous LIF secreted in Caco-2 cells (Section 3.3.6). Lower levels of LIF expression must be tested before concluding that the intracellular trafficking machinery is already saturated and the distribution of LIF observed is simply overspill into an alternative pathway. Conditioned medium from unstimulated MDCK cells demonstrates LIF-like activity in BA/F3 mLIFR-mgp130 and appears to be restricted to the apical chamber (personal communication Ann Vernallis). This finding is in good accordance with the predominantly apical release of exogenous LIF proteins and may indicate a common trafficking pathway for LIF-like proteins in this cell line. On the one hand the possibility that MDCK cells naturally produce LIF is encouraging as it suggests that the cells naturally express the machinery capable of trafficking LIF. On the other hand, if the cell is already producing LIF (as opposed to other LIFR/gp130 ligands) then additional protein may more easily saturate the trafficking machinery making the low expression of exogenous protein even more prudent. Since ELISAs for canine LIF have not been developed, positively identifying the LIF-like activity as LIF and properly quantifying the LIF is currently not feasible.

Many of the clones isolated demonstrated a high level of LIF secretion in the absence of doxycycline. However, many of the clones also suffered from high

background (with the exception of clone S) in the presence of doxycycline. The manufacturers claim that in general at least thirty clones may need to be screened in order to generate a cell line that exhibits a low background. However they also suggest that as many as a hundred clones may need to be screened. One solution that may allow the creation of a cell line that exhibits a suitably low background may simply be to screen more clones. Additional and/or alternate vectors are available that claim to effectively reduce basal expression. The pTet-tTs vector (BD Biosciences) expresses the tetracycline controlled transcription silencer (tTs). In the absence of doxycycline, tTs binds to the TRE preventing gene expression. The additional level of protection provides even tighter control over gene expression but is only available with the Tet-on system, so is not suitable for the MDCK cell line. BD Bioscience's new pTRE-Tight vector contains a modified TRE that claims to reduce basal expression while maintaining high induction capability. An alternative to isolating further clones with the existing pBI-L vectors, which may also exhibit reduced backgrounds, would be to use this alternate vector. This strategy may allow investigation into lower levels of LIF expression.

The fact that BD Biosciences offer new expression vectors with apparently lower backgrounds, suggests that the Tet-Off system used here may be an inadequate system to generate clones with a suitably low background.

Chapter 5   Conclusions and future directions.

The initial aim of this study was to investigate the factors influencing the sorting of LIF in polarised epithelial cells. The influences of factors such as cell type, cytokine stimulation, expression level, transcytosis and whether the alternate LIF signal peptides influence the sorting of LIF were addressed. The mechanisms underlying the polarised secretion of LIF and IL-6 remain to be investigated, hence future experimental approaches will be discussed below.

The polarity of LIF secretion is dependent upon a number of factors. Cell type is an important factor, and has a significant effect on the pattern of LIF secretion observed. Caco-2 and MDCK cells displayed different patterns of LIF secretion. In Caco-2 cells, the majority of endogenous LIF was secreted basolaterally, whereas in MDCK cells the majority of exogenous LIF was secreted apically. The patterns observed may be a result of the different mechanisms for protein sorting in these cell lines. Caco-2 cells use both direct and indirect (trancytosis) pathways to deliver proteins to the cell surface, whereas MDCK cell lines use a direct pathway to deliver proteins to the cell surface. In addition, the potential targets for LIF could also differ between the two cell types and, as such, require different patterns of secretion. Potential interactions with other cells, such as mucosal cells in the intestine are not well described and were not examined in the present experiments on isolated cell lines, however they merit future investigation.

For Caco-2 cells, the actions of paracrine factors are able to influence the pattern of secretion observed for both LIF and IL-6. The pattern of secretion of LIF

and IL-6 was regulated by IL-1β. The proportion of LIF secreted apically was higher with apical stimulation than with basolateral stimulation. However, LIF was still secreted predominantly at the basolateral surface. A similar trend was observed with IL-6. However, a greater shift in the pattern of IL-6 secretion following apical stimulation was observed, with the majority of IL-6 secreted at the apical surface. For both proteins the amount of protein secreted was highest at the basolateral membrane, when stimulated basolaterally. The increased cytokine production by Caco-2 cells treated with IL-1β *in vitro* has implications for the *in vivo* situation. However, the *in vivo* situation is probably more complex, relying on the interactions of multiple paracrine factors.

The initial action of pro-inflammatory cytokines, such as IL-1β, can lead to the production of additional cytokines, e.g. LIF and IL-6, which could have inflammatory or protective functions. IL-6 receptor subunit gp80 is present at the apical and basolateral poles of Caco-2 cells, whereas, gp130 is found predominantly at the basolateral pole. Basolateral stimulation with IL-6 is found to be a more potent stimulator of NFκB than apical stimulation. NFκB is a potent pro-inflammatory nuclear transcription factor and its activity is increased in inflamed tissue, such as the inflamed intestinal mucosa. One particular effect of basolateral stimulation with IL-6 is the apical expression of the adhesion molecule ICAM, which is involved in neutrophil-epithelial interactions (Wang *et al.* 2003). It is unknown if Caco-2 cells express LIFR and it is therefore unknown whether LIF exerts autocrine functions.

Components of trafficking pathways can become saturated, which may result in additional protein spilling over into alternative pathways. When expressing

proteins exogenously, it is important to consider the level at which saturation may occur. The identification of the level at which saturation of LIF trafficking pathways occurs requires further work. Current work in the laboratory is focusing on the low LIF producer (clone S), to identify any differences in the polarity of LIF secretion at low expression levels, in comparison to the high expression observed in other clones. Since saturation may occur at high expression levels during processes such as inflammation, elucidation of this mechanism may further our understanding of LIF trafficking.

Many epithelial cells are able to endocytose ligands using receptors such as polymeric immunoglobulin receptor (pIgR), or calcium independent mannose 6-phosphate receptor (CIMPR) at one cell surface and deliver them to the opposite cell surface (transcytosis). For example, in MDCK cells the pIgR is directed to the basolateral cell surface where it can bind ligands such as IgA and following internalisation, deliver them to the apical surface (Orzech *et al.* 2000). Further work is required to elucidate the mechanism responsible for the basolateral to apical transport of LIF in Caco-2 and MDCK monolayers. If CIMPR binding to LIF carbohydrate moieties is involved in this process, then no diffusion of bacterial (unglycosylated) LIF should be detected. This may provide a mechanism for the indirect targeting of LIF to the apical surface in these cell types.

The alternate murine signal peptides do not appear to influence the pattern of LIF secretion in MDCK cells. With both the LIF-D- and LIF-M signal peptide, 60% of the LIF was secreted apically. The effects of the alternate human signal peptides are yet to be tested. It is possible that the reduced production of LIF under control of

the hLIF-M signal peptide reported here and by Voyle *et al.*(1999) could affect the pattern of secretion simply by reducing expression levels.

Trafficking pathways identified in epithelial cells are also present in non-overtly polarised cell types, such as fibroblasts. Therefore, mechanisms involved in the trafficking of LIF in polarised epithelial cells may be relevant to the trafficking of LIF in fibroblasts. The association of LIF-M with the ECM of fibroblasts as observed by (Rathejen *et al.* 1990) suggested that LIF-M may traffic differently from LIF-D. In epithelial cells, it was hypothesised that LIF-M would be secreted toward the ECM, which is located basolaterally in these cells. Since the secretion patterns for the LIF isoforms were identical in MDCK cells, it can be concluded that the signal peptides do not influence the pattern of secretion in at least this cell type. A role in fibroblasts cannot be ruled out since fibroblasts may traffic LIF differently. The matrix association of LIF-M may be dependent on co-trafficking with the LIF-ECM binding protein itself. Consequently, trafficking of LIF in epithelial cells, which may lack the ECM binding protein, may not mimic the trafficking of LIF in fibroblasts. Many of the LIF constructs generated and validated in this thesis could be used to study LIF trafficking in fibroblasts.

5.1   Future experimental approaches.

Not much is known about LIF and IL-6 trafficking. The ability of IL-1β to alter the pattern of LIF and IL-6 secretion demonstrated here has raised important questions as to how paracrine factors are able to influence the polarity of secretion.

The molecular mechanism for agonist induced changes in trafficking are not known, but are likely to involve the vesicle docking and fusion machinery.

The establishment and maintenance of epithelial cell polarity depends upon the precise targeting of proteins to the apical and basolateral plasma membranes. The fidelity of protein targeting in the secretory pathway depends on the specificity of vesicle fusion. The N-ethymalemide sensitive factor (NSF) attachment protein receptor (SNARE) membrane fusion machinery is essential for most, if not all, vesicle fusion. Target (t)-SNAREs generally localise to distinct target membranes, where they mediate the fusion of specific classes of vesicles. Membrane fusion can only occur with matching combinations of vesicle associated (v) SNAREs and t-SNAREs, suggesting SNAREs contribute to specificity. When v- and t-SNAREs interact, the "zippering up" of the helical domains occur to form stable trans-SNARE complexes, which lock the two membranes together and allow fusion to occur. For example, t-SNARE proteins from the syntaxin and SNAP-25 family interact with SNARE domains of the v-SNARE (Vesicle associated membrane protein) VAMP. The availability of syntaxin for SNARE fusion complex formation is highly regulated and is likely to control vesicle fusion. The conserved autonomously folding amino-terminal structure in syntaxin molecules, may serve as an auto-inhibitory regulatory domain. Thus, syntaxin can adopt a closed configuration that prevents the formation of the core fusion complex (Logan *et al.* 2003).

It is thought that additional proteins are required to prevent t- and v-SNAREs from coming together inappropriately. A second family of proteins, the sec-1/munc18 (SM) proteins play a prominent role in vesicular trafficking. Generally, SM proteins

interact with their respective syntaxins with high specificity and affinity, and may confer specificity to SNARE-dependent trafficking. The chaperone protein sec1/Munc18 can bind to the closed conformation of syntaxin, however, controversy exists over whether binding promotes the closed conformation or destabilises it. Dissociation or conformational changes in n-sec/Munc18 may open up the structure to facilitate SNARE-complex formation. Specificity of granule/vesicle exocytosis is unlikely to be determined by a single family of proteins, but is a result of coordinated interactions of a multimeric-signalling complex (Logan *et al.* 2003).

Rab isoforms localise to membranes and are important in directing distinct vesicles/granules to their appropriate destination. Their roles include Golgi vesicle budding, recruitment of the cytoskeleton, protein-protein tethering and vesicle docking (Logan *et al.* 2003). Rab proteins ensure specificity of docking, like SNAREs, each Rab protein has a characteristic distribution on cell membranes. Although SNARE proteins are central to the process of membrane fusion, the first point of contact between a vesicle and target membrane, is defined, by a poorly understood tethering process. The GTP-dependent interaction of the tethering complex with Rab is thought to be important. Rabs cycle between GTP (active) and GDP (inactive) states and function as molecular switches at sites where they are localised. Rab mutants that are defective in GTP hydrolysis, or have a lower affinity for GTP than GDP provide useful tools for examining the importance of different Rab proteins. Low-density-lipoprotein receptor (LDLR) and P75 neurotrophin receptor (P75NTR) have been used as markers of basolateral and apical transport. Overexpression of a Rab3 mutant inhibited cell surface transport of LDLR but not P75NTR, indicating that Rab3 may be involved in basolateral transport. In addition

Rab13 mutants affected neither LDLR nor P75NTR (Yamamoto *et al.* 2003). Further studies are needed to elucidate the molecular mechanism linking Rabs to polarised vesicular transport.

Great variability exists in protein targeting behaviour among epithelial cell types, and may contribute to the differential pattern of secretion observed between different cell types. Differences in expression and/or subcellular localisation of syntaxins may be part of this mechanism. In MDCK cells, syntaxin 3 and 4 are exclusively localised to the apical and basolateral plasma membranes respectively. In addition MDCK cells express syntaxin 2 and 11 in an apparent non-polarised fashion (Low *et al.* 1996). Li *et al.*(2002) demonstrated that epithelial cell types, along the renal tubule, differ in the expressed complement of SNAREs, as well as their subcellular localisation. This suggests modulation of expression and localisation is used by epithelial cells as a mechanism to achieve the observed plasticity of sorting phenotypes.

Expression levels of SNAREs must be tightly regulated together with cellular requirements for trafficking pathways that involve a given SNARE. Activation of macrophages with LPS induces rapid synthesis and secretion of TNFα. In addition, a subset of t-SNAREs and their accessory proteins (synatxin-4 /SNAP23/Munc18c), is substantially increased by LPS in a temporal pattern coinciding with peak TNFα secretion. Increases in quantity at the plasma membrane allows for increased vesicular traffic and secretion of cytokines in activated macrophages (Pagan *et al.* 2003). Up-regulation of SNARE complex components by pro-inflammatory cytokines demonstrates potential mechanisms by which stimulation of epithelial cells

could result in increased secretion of IL-6 and LIF. Global up-regulation would not be enough to account for the behaviour observed, in which the side of stimulation favoured secretion through that side, rather increased activity of apical or basolateral secretion machinery would be required.

Differential stimulation of Caco-2 cells with IL-1β resulted in altered patterns of LIF and IL-6 secretion. It possible that differential expression of syntaxins could contribute to altered patterns of LIF and IL-6 secretion. Syntaxin 2 splice variants (2A, 2B, 2C, 2D) exhibit differential expression patterns and subcellular localisations. Syntaxin 2A and 2B differ only in their C-terminal domains and in MDCK and Caco-2 cells syntaxin 2A is found predominantly at the apical surface, whereas syntaxin 2B is found to be unpolarised (Quinones *et al.* 1999). Interestingly, studies of the localisation of endogenous syntaxin 2 in epithelial cells (using antibodies that do not distinguish between the variants) have reported distribution to both apical and basolateral plasma membrane domains. By altering the localisation of syntaxins protein targeting could also be regulated.

Paracrine factors, acting at cell surface receptors, could affect protein targeting via alternate mechanisms. Pancreatic acinar cells synthesise and release large amounts of zymogen enzymes necessary for digestion. These enzymes are packaged into large dense core zymogen granules (ZGs) that are stored and released at the apical pole. Precise temporal and spatial control of ZG secretion is likely to involve regulation of SNARE machinery. It is thought that additional proteins such as munc18 are required to prevent t- and V-SNAREs from coming together inappropriately. One role of Munc18c may be as an inhibitor of syntaxin interactions

with SNAP23 and VAMP, and this could be released by PKC phosphorylation (PKC could be activated selectively by particular receptors). In pancreatic acinar cells syntaxin 4 and SNAP23 are t-SNAREs, which are located basolaterally and are capable of binding to the v-SNARE VAMP2 that is located on the granule, to form a SNARE complex. Munc18c binds to syntaxin 4 blocking binding to SNAP23 and VAMP2 and may be displaced by direct phosphorylation. It has been demonstrated that supermaximal stimulation with Cholecystokinin (CCK) causes an aberrant targeting of ZGs to the basolateral membrane. Dissociation of Munc18c from the plasma membrane by CCK was dependant upon activation of PKC. This interaction may allow free syntaxin 4 to bind SNAP23 and granule VAMP2 to allow the formation of the exocytic complex and secretion of ZGs. Given that IL-1β is able to induce altered patterns of LIF and IL-6 secretion, it is possible that disruption of regulatory proteins such as munc18c, may play a role in the changes observed in the polarity of LIF/IL-6 secretion (Gaisano *et al.* 2001).

It was observed, that IL-1β acting at one pole of Caco-2 cells, influenced the secretion of LIF/IL-6 at the opposite pole. Indeed, it is possible that paracrine factors acting on one side of a cell could affect protein targeting at the opposite pole. Wang *et al.*(2004), investigated the membrane domain specificity of agonist induced membrane recruitment of the A2b receptor and the involvement of SNARE proteins in the trafficking of the A2b receptor. Adenosine acting through the A2b receptor induces predominantly apical chloride and IL-6 secretion in T84 cells, irrespective of the side of stimulation. A small proportion of A2b is found on the apical and basolateral surfaces of T84 cells, but the majority is found intracellularly. In unstimulated cells, A2b receptor expression is higher at the basolateral surface.

However, upon apical or basolateral adenosine stimulation there is a two to three-fold increase in the recruitment of the receptor to the apical surface.

To investigate whether the recruitment of the A2b receptor involves trafficking through vesicles and whether the SNARE complex was involved in this process, cell fractions were isolated by differential centrifugation. Upon stimulation, the A2b receptor was enriched in the vesicle fraction containing VAMP-2 (v-SNARE), indicating that recruitment to the apical surface involves trafficking through vesicles. In contrast, the A2b receptor was not detected in the vesicles from unstimulated cells. VAMP-2 was enriched in vesicles and was found predominantly in the subapical compartment. Further analysis of the type and distribution of SNARE proteins, revealed the presence of SNAP-23 (t-SNARE). SNAP-23 was observed in both apical and basolateral domains and was found to be enriched in the plasma membrane domain. Interestingly, transcript levels of VAMP-2 and SNAP-23 were not altered by adenosine treatment. Immunoprecipitation of SNAP-23 from cells stimulated with adenosine demonstrated a complex of VAMP-2, SNAP-23 and the A2b receptor, indicating that exocytic vesicles containing A2b receptor and VAMP2 dock with membrane associated SNAP-23.

Cells stimulated at the apical or basolateral surface demonstrated a seven-fold and thirty-fold increase in cAMP levels respectively as well as phosphorylation of PKA. This data suggests that adenosine may stimulate membrane receptors, resulting in increased cAMP, which in turn, can initiate recruitment of additional receptors to the membrane (Wang *et al.* 2004). It has been shown that cAMP mediates protein trafficking to the apical, but not the basolateral, cell surface by modulating the

sialylation of proteins and vesicle budding from the TGN (Jilling and Kirk 1996). In this respect it is possible that increases in cAMP, as a result of basolateral stimulation with adenosine, were only able to increase the recruitment of A2b to the apical surface.

Data presented in this thesis demonstrates alterations in the expression of cytokines, as a consequence of pro-inflammatory cytokines. SNARE protein regulation and pathologic fusion processes could lead to human disease. Additional research into SNARE proteins in polarised epithelial cells, in response to pro-inflammatory cytokines could lead to a fuller elucidation of the molecular mechanisms involved in pathologic membrane fusions. Pro-inflammatory cytokines such as IL-1β, IL-6 and LIF are increased in tissues from inflammatory bowel disease (IBD) patients (Molmenti *et al.* 1993). Intestinal epithelial cells may serve as targets for these locally produced cytokines, which may result in alterations in epithelial cell function or integrity. The effect of IL-1β on the differential secretion of LIF and IL-6 could also be examined in the context of SNARE activity. In addition, the effects of intermediary signalling molecules such as cAMP and PKC could also be elucidated. The identification of essential molecules in the cascade of events leading to exocytosis is critical in the search for novel therapeutics aimed at modulating mediator secretion from cells.

By determining which t-SNAREs and accessory proteins are up-regulated in response to IL-1β in Caco-2 cells, it may be possible to identify the mechanism by which differential targeting is accomplished. It will also be important to determine the localisation of endogenous t-SNAREs and accessory proteins, as well as studying

which v-SNAREs co-localise with LIF containing vesicles in both control and IL-1β

stimulated cells. Since PKC and PKA have been suggested as mediators of

vectorially directed secretion, inhibition of their activity may further unravel the

complexities involved in agonist induced polarised trafficking.

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Appendices

**Appendix 1. Transforming chemically competent cells, protocol (Invitrogen).**

Transforming Chemically competent cells

1. Briefly centrifuge the ligation reaction and place on wet ice.

2. Remove one 500μl tube of DH5α cells and thaw on wet ice.

3. Place the required number of 1.5ml microcentrifuge tubes on wet ice.

4. Gently mix the cells with the pipette tip and aliquot 50 μl into each centrifuge tube.

5. Pipet 5μl (1-10ng DNA) of each ligation reaction directly into the competent cells and mix by gently tapping.

6. Pipet 5μl pUC19 control DNA into 100μl competent cells and mix.

7. Incubate the vial on ice for 30 minutes.

8. Heat shock for exactly 20 seconds in t e37°C water bath.

9. Remove vial from water bath and place on ice for 2 minutes.

10. Add 950μl of pre-warmed LB media to each vial.

11. Shake at 37°C for exactly 1 hour at 225 rpm.

12. Spread 100μl from each transformation vial on separate LB agar plates containing appropriate selective antibiotic (cells may have to be diluted 1:10 to obtain well space colonies).

13. Invert the plates and incubate at 37°C overnight.

14. Select colonies and analyze by plasmid isolation, PCR or sequencing.

Appendix 2. QIAprep spin miniprep kit protocol.

Protocol: QIAprep Spin Miniprep Kit Using a Microcentrifuge

Aston University

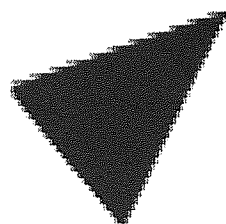Illustration removed for copyright restrictions

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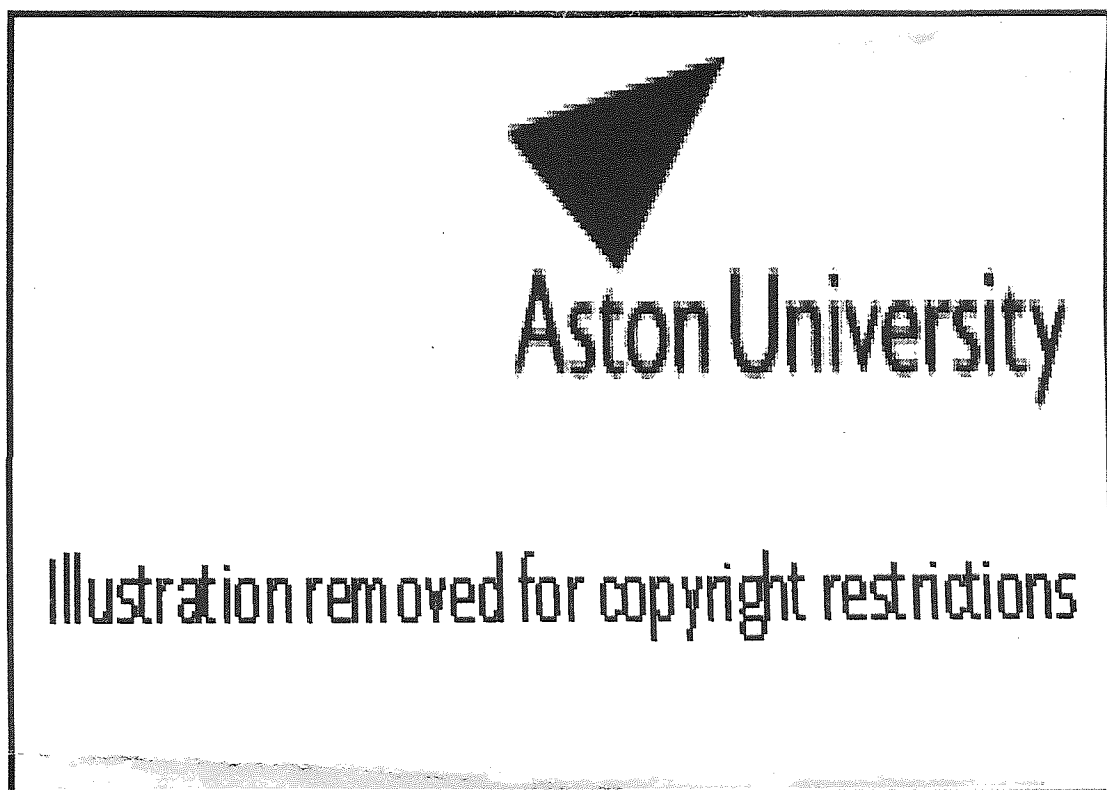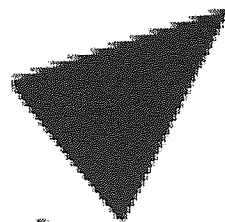
Aston University

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Appendix 3. Endofree plasmid maxi kit protocol.

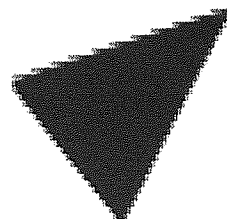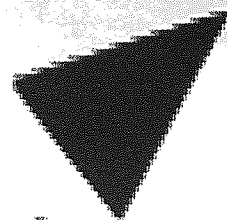## Protocol: Plasmid or Cosmid DNA Purification Using the EndoFree Plasmid Maxi Kit

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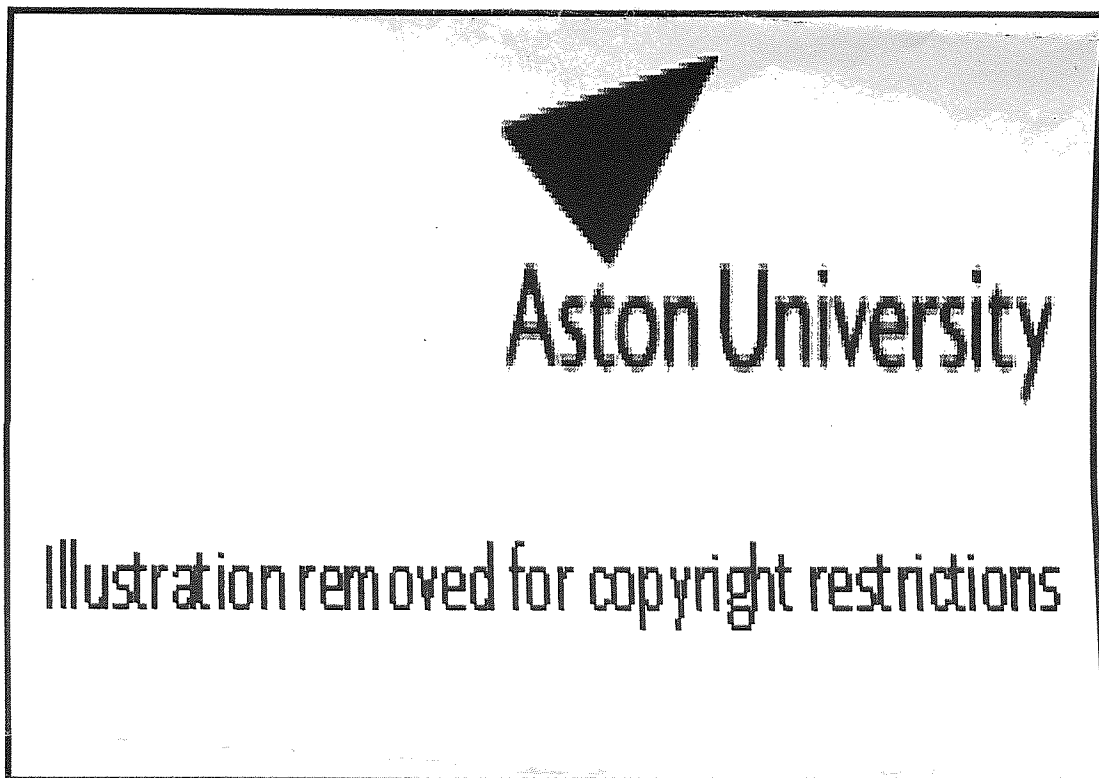Illustration removed for copyright restrictions

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Appendix 4. QIAquick gel extraction kit protocol (Qiagen).

QIAquick Gel Extraction Kit Protocol
using a microcentrifuge

Aston University

Illustration removed for copyright restrictions
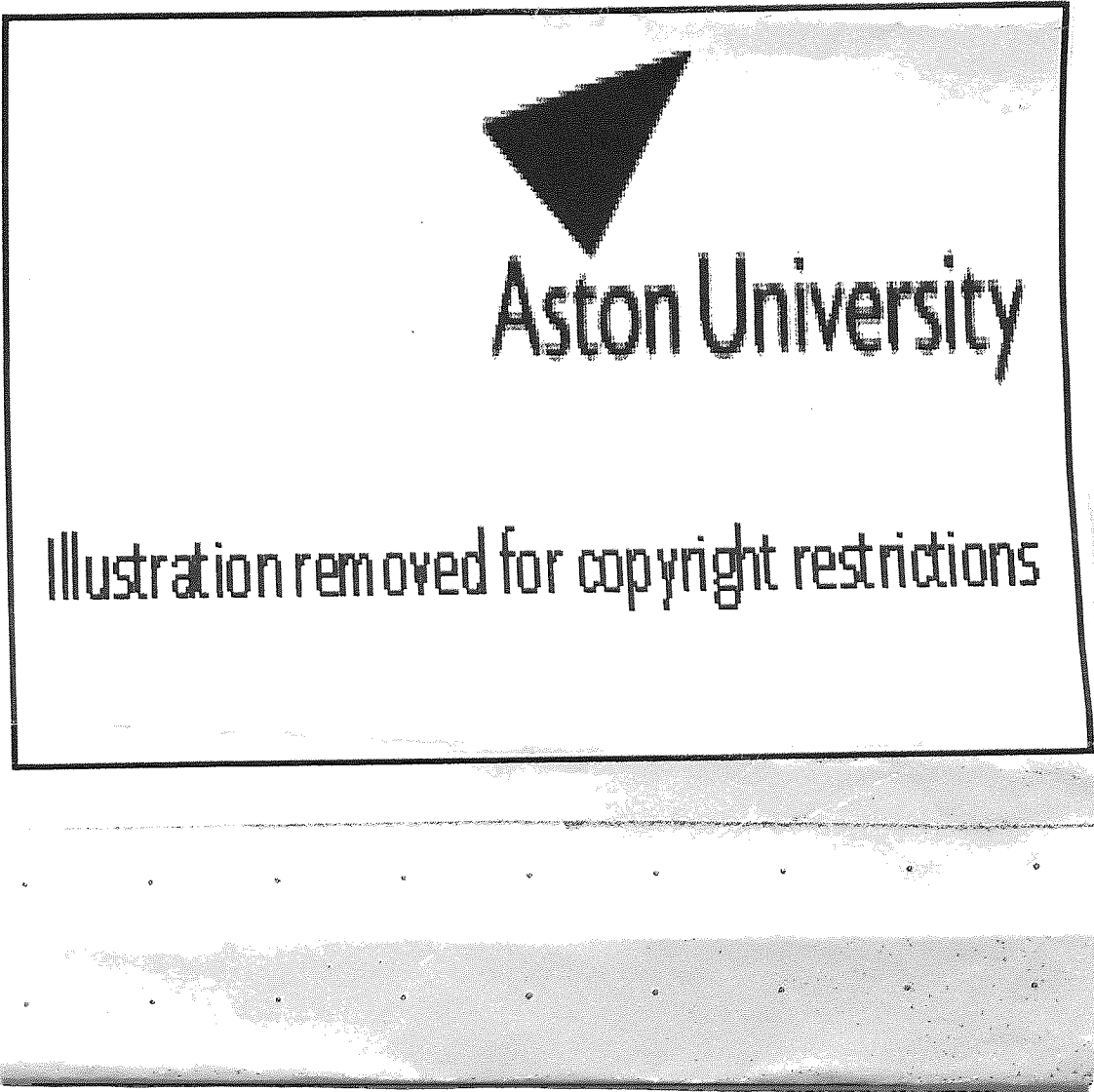
Illustration removed for copyright restrictions

Appendix 5. QIAquick PCR purification kit protocol (Qiagen).

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

Illustration removed for copyright restrictions

Primer target	Primer sequence
mLIF sequence	5'⁵GAGCTGTATCGGATG³'
	3'⁵TTGAGCTTGACCTGG³'
hLIF sequence	5'⁵AAGGTACACGACTATG³'
	3'⁵TTCTCTATTACACAGCC³'
pcDNA3.1 sequence	5'⁵CGCAAATGGGCGGTAGGCGTG³'
	3'⁵TAGAAGGCACAGTCGAGG³'

Appendix 6. Table of sequencing primers. The mLIF forward (5') and reverse (3') sequencing primers were used in the sequencing of all mLIF inserts. The hLIF forward (5') and reverse (3') sequencing primers were used in the sequencing of all hLIF inserts. The pcDNA3.1 forward (5') and reverse (3') sequencing primers were used in the sequencing of all inserts contained within the pcDNA3.1 plasmid. All primers are shown in the 5'-3' orientation.

Constuct	Primer
pGex2T-mLIF-FLAG	5' 5'TAGGATCCCCTCTTCCCATCACCCCT3'
	3'5'ATGAATTCCTACTTGTCATCGTCGTC CTTGTAGTCGAAGGCCTGGACCACCACA3'
pcDNA3.1-mLIF-D-Fc	5' 5'TAGGATCCGCCACCATGAAGGTCTT GGCCGCA3'
	3' 5'ATGAATTCCGAAGGCCTGGACCACC3'
pcDNA3.1-mLIF-D-FLAG-Fc	5' 5'TAGGATCCGCCACCATGAAGGTCTT GGCCGCA3'
	3' 5'ATGAATTCCCTTGTCATCGTCGTCCT TGTAGTCGAAGGCCTGGACCACC3'
pcDNA3.1-hLIF-D	5' 5'TAGGATCCATGAAGGTCTTGGCGGC AGGAGTTGTG3'
	3' 5'CAGAATTCCTAGAAGGCCTGGGCCA ACAC3'
pcDNA3.1-hLIF-D-FLAG	5' 5'TAGGATCCATGAAGGTCTTGGCGGC AGGAGTTGTG3'
	3' 5'CAGAATTCCTACTTGTCATCGTCGTC CTTGTAGTCGAAGGCCTGGGCCAACAC3'
pcDNA3.1-hLIF-D-FLAG-no stop codon	5' 5'TAGGATCCATGAAGGTCTTGGCGGC AGGAGTTGTG3'
	3' 5'CAGAATTCCCTTGTCATCGTCGTCCT TGTAGTCGAAGGCCTGGGCCAACAC3'
pcDNA3.1-hLIF-M-no stop codon	5' 5'TAGGATCCTCTGGAAGCGTGTGGTC TGCGCTAGGAGTTGTGCCCCTG3'
	3' 5'CAGAATTCCGAAGGCCTGGGCCAAC AC3'

**Appendix 7. Table of oligonucleotides used in the creation of LIF constructs.**
The forward (5') and reverse (3') primers were employed in the PCR amplification of inserts that were subsequently used to create the constructs listed in the table.
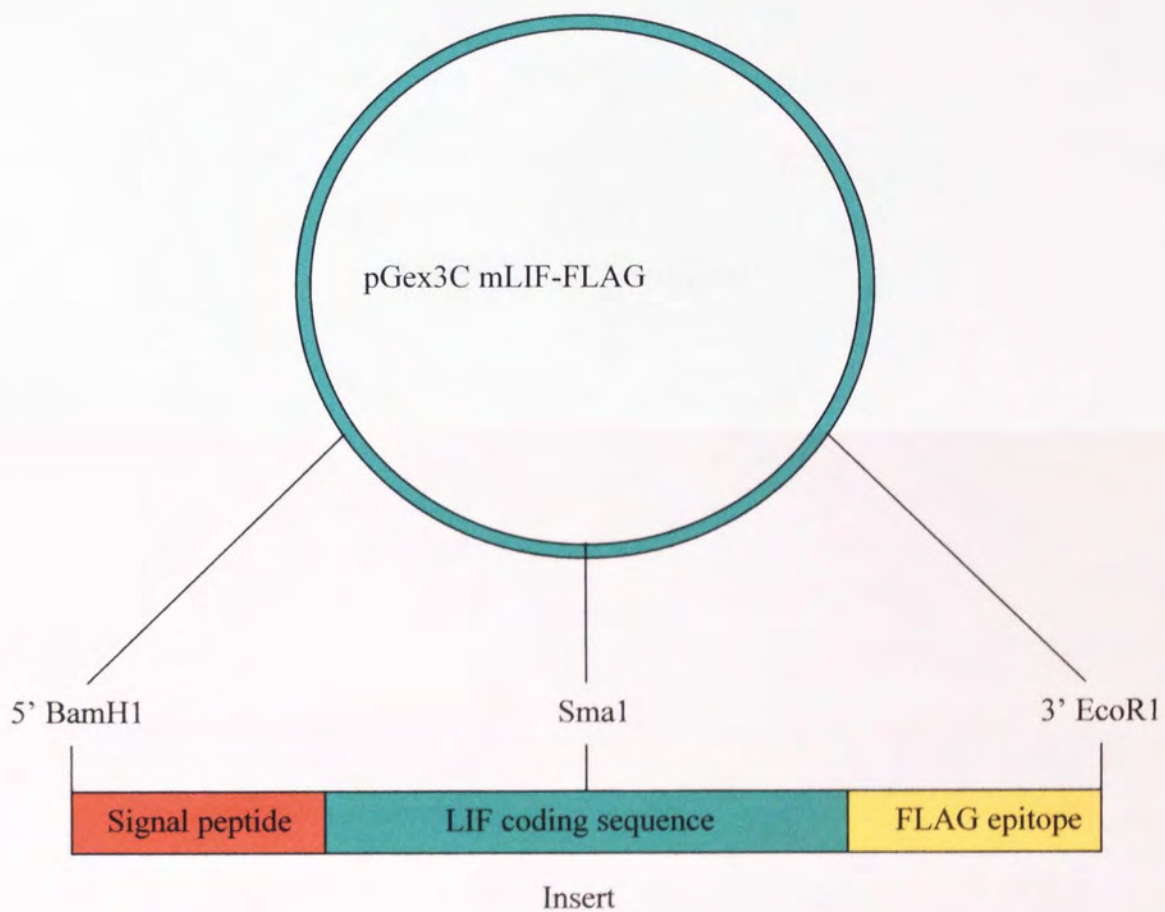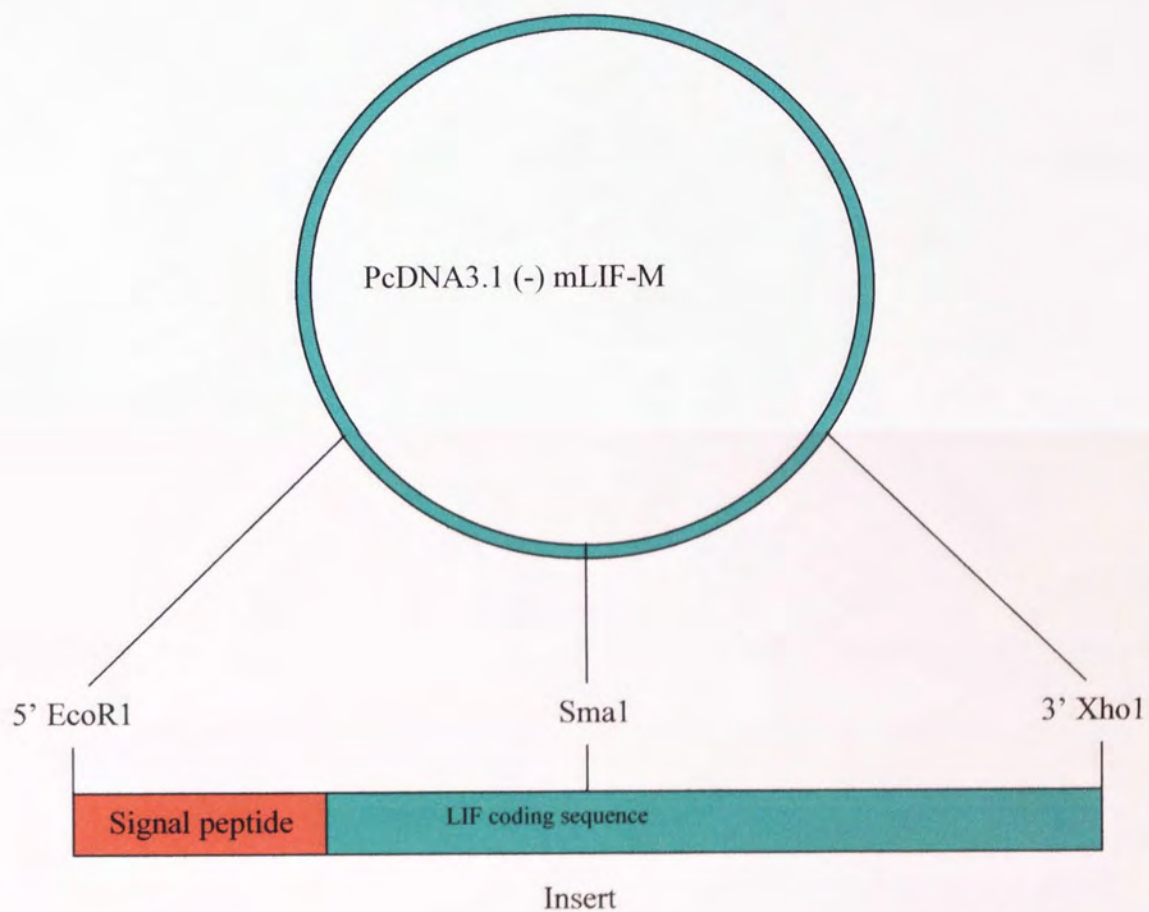
Appendix 8 Schematic diagram of pGex3C-mLIF-FLAG (EJH1). Showing restriction endonuclease sites, and components of insert.
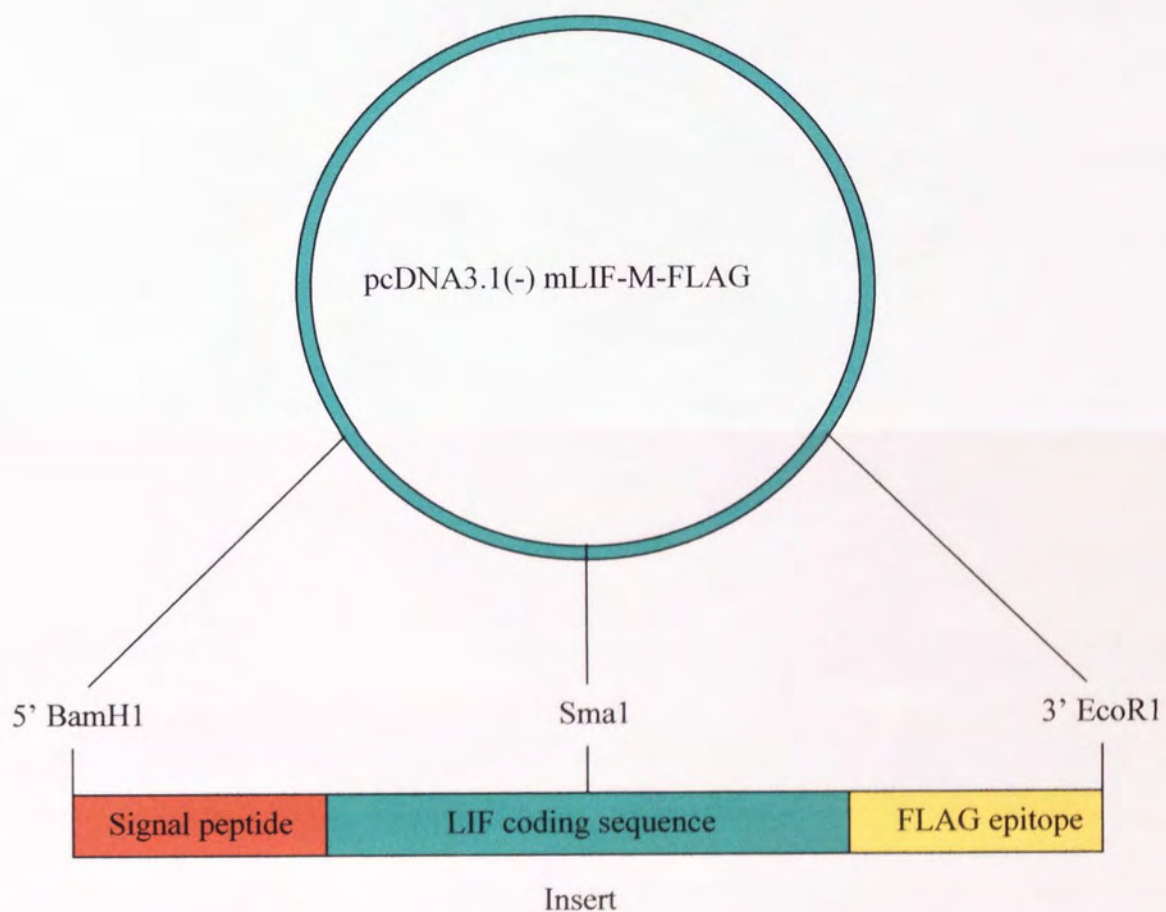
Appendix 9 Schematic diagram of pcDNA3.1(-) mLIF-M-FLAG (EJH2). Showing restriction endonuclease sites, and components of insert.
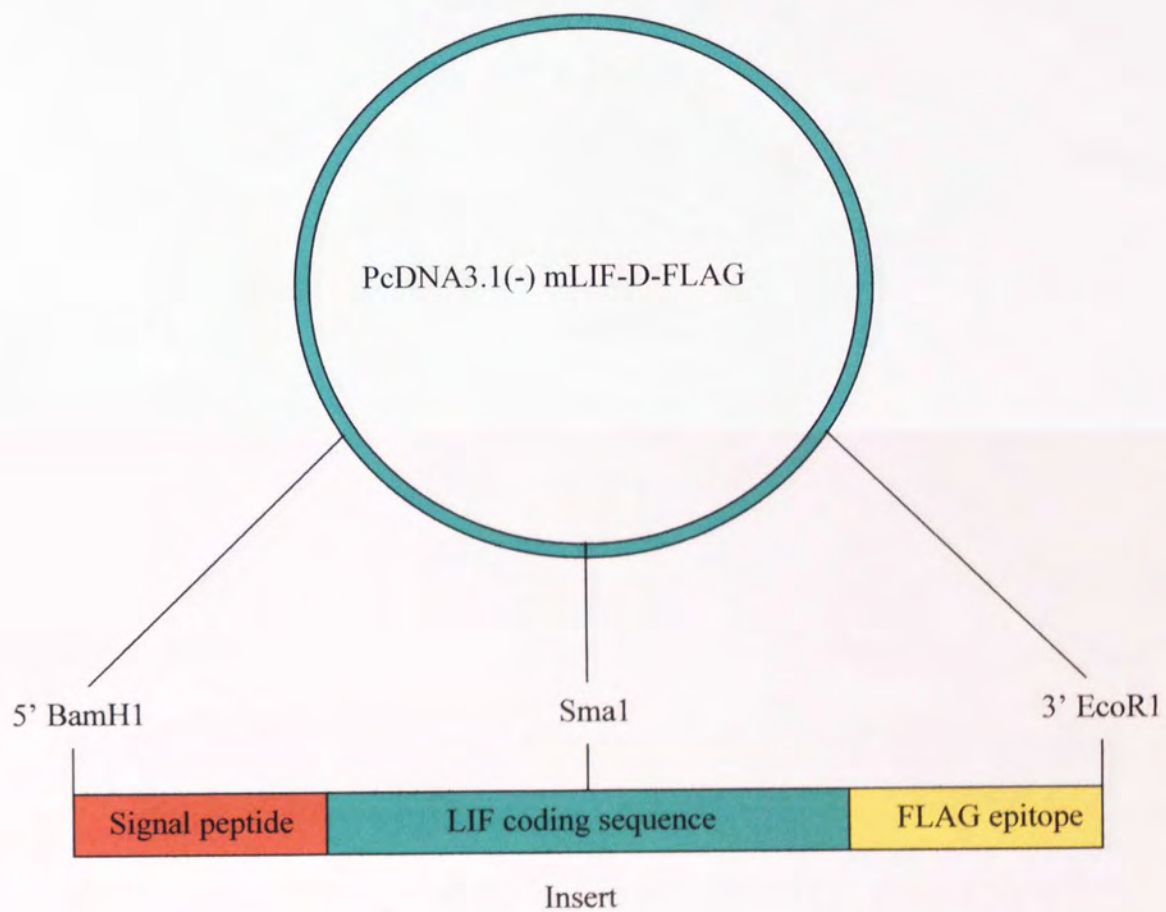
Appendix 10 Schematic diagram of pcDNA3.1(-) mLIF-D-FLAG (EJH3). Showing restriction endonuclease sites, and components of insert.

PcDNA3.1 (-) mLIF-M

5' EcoR1   Sma1   3' Xho1

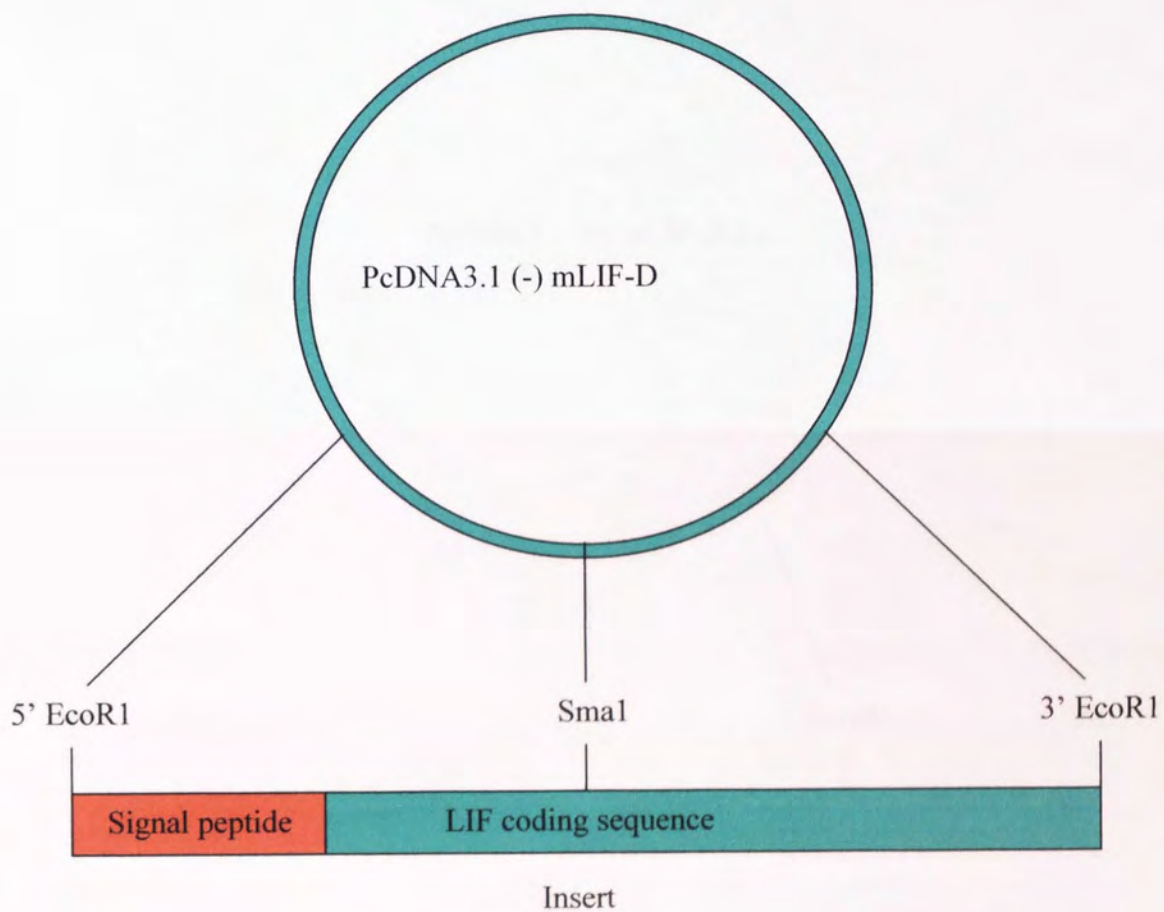| Signal peptide | LIF coding sequence |

Insert

Appendix 11 Schematic diagram of pcDNA3.1(-) mLIF-M (EJH4). Showing restriction endonuclease sites, and components of insert.

Appendix 12 Schematic diagram of pcDNA3.1(-) mLIF-D (EJH5). Showing restriction endonuclease sites, and components of insert.

PcDNA3.1 (+) mLIF-D-Fc

5' BamH1   Sma1   EcoR1   3' Xho1

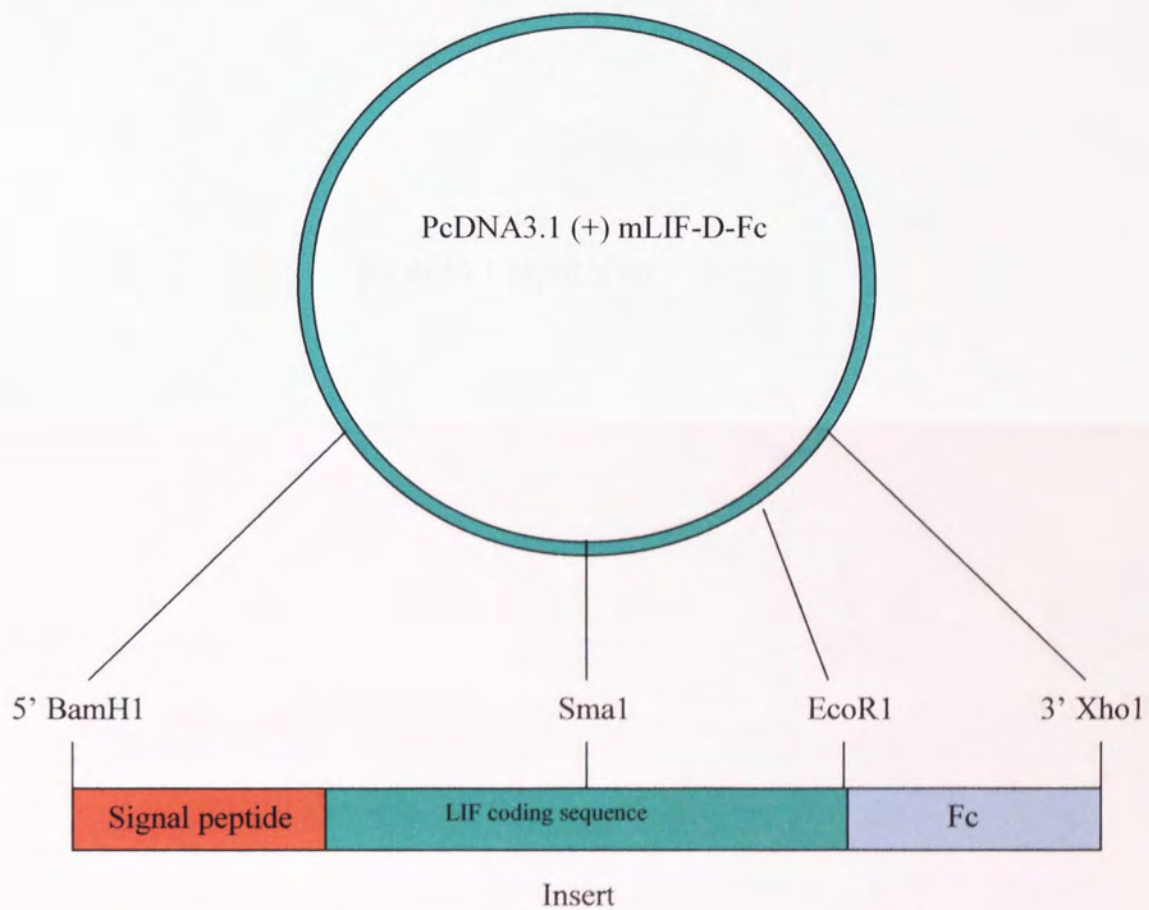| Signal peptide | LIF coding sequence | Fc |

Insert

Appendix 13 Schematic diagram of pcDNA3.1(-) mLIF-D-Fc (EJH6). Showing restriction endonuclease sites, and components of insert.

Appendix 14 Schematic diagram of pcDNA3.1(+) hLIF-D (EJH7). Showing restriction endonuclease sites, and components of insert.

PcDNA3.1 (+) hLIF-D

5' BamH1   Sma1   3' EcoR1

Signal peptide   LIF coding sequence

Insert

Appendix 15 Schematic diagram of pcDNA3.1(-) hLIF-D-FLAG (EJH8). Showing restriction endonuclease sites, and components of insert.
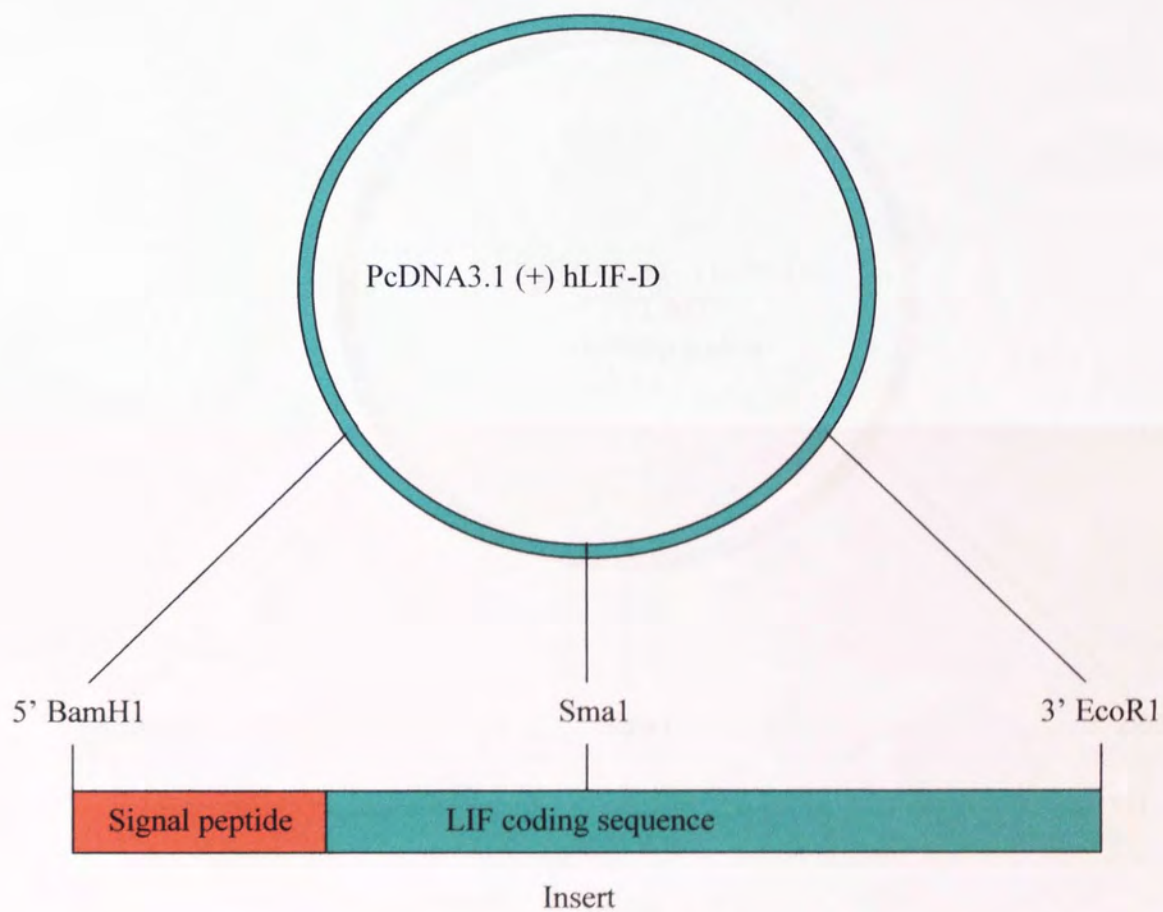
Appendix 16 Schematic diagram of pcDNA3.1(+) hLIF-D-FLAG-no stop codon (EJH9). Showing restriction endonuclease sites, and components of insert.

Appendix 17 Schematic diagram of pcDNA3.1(+) hLIF-M-no stop codon (EJH10). Showing restriction endonuclease sites, and components of insert.

Appendix 18 Schematic diagram of pcDNA3.1(+) mLIF-M (EJH11). Showing restriction endonuclease sites, and components of insert.
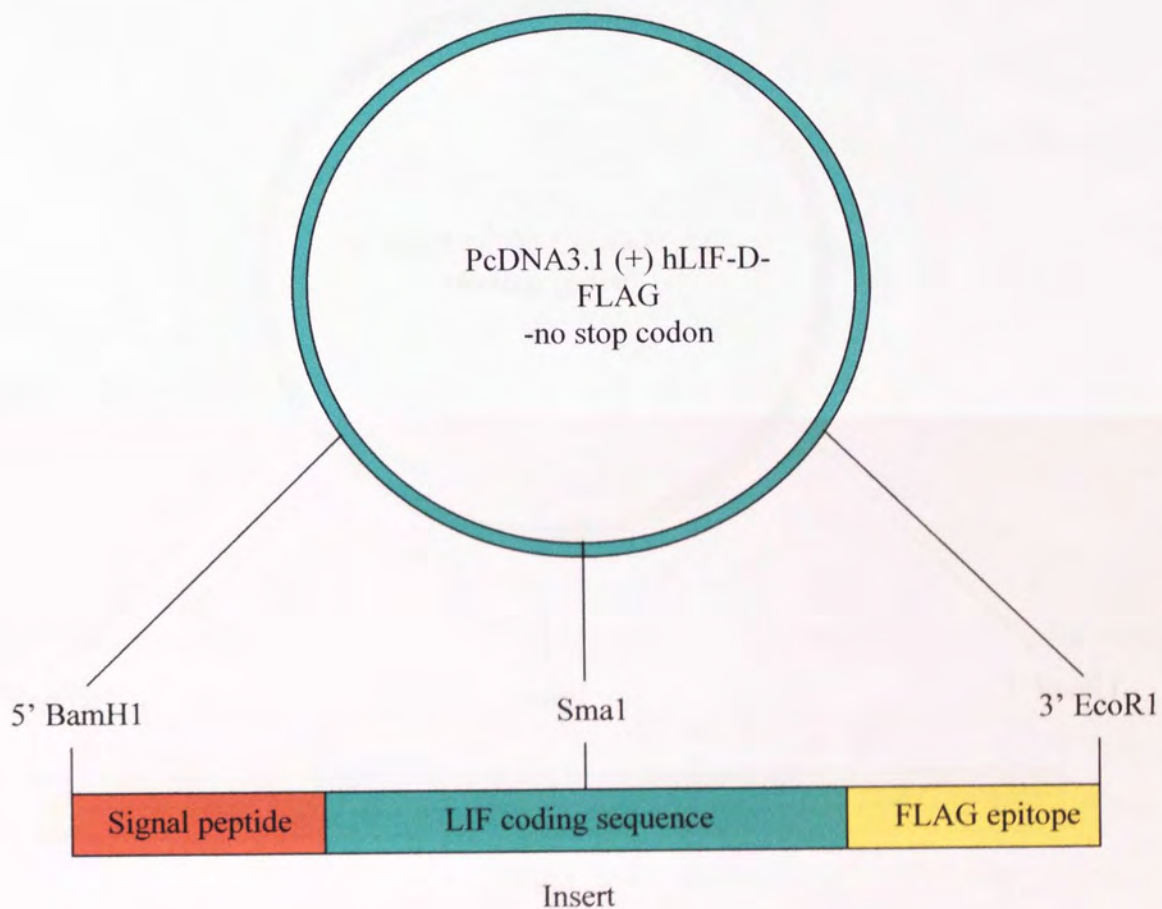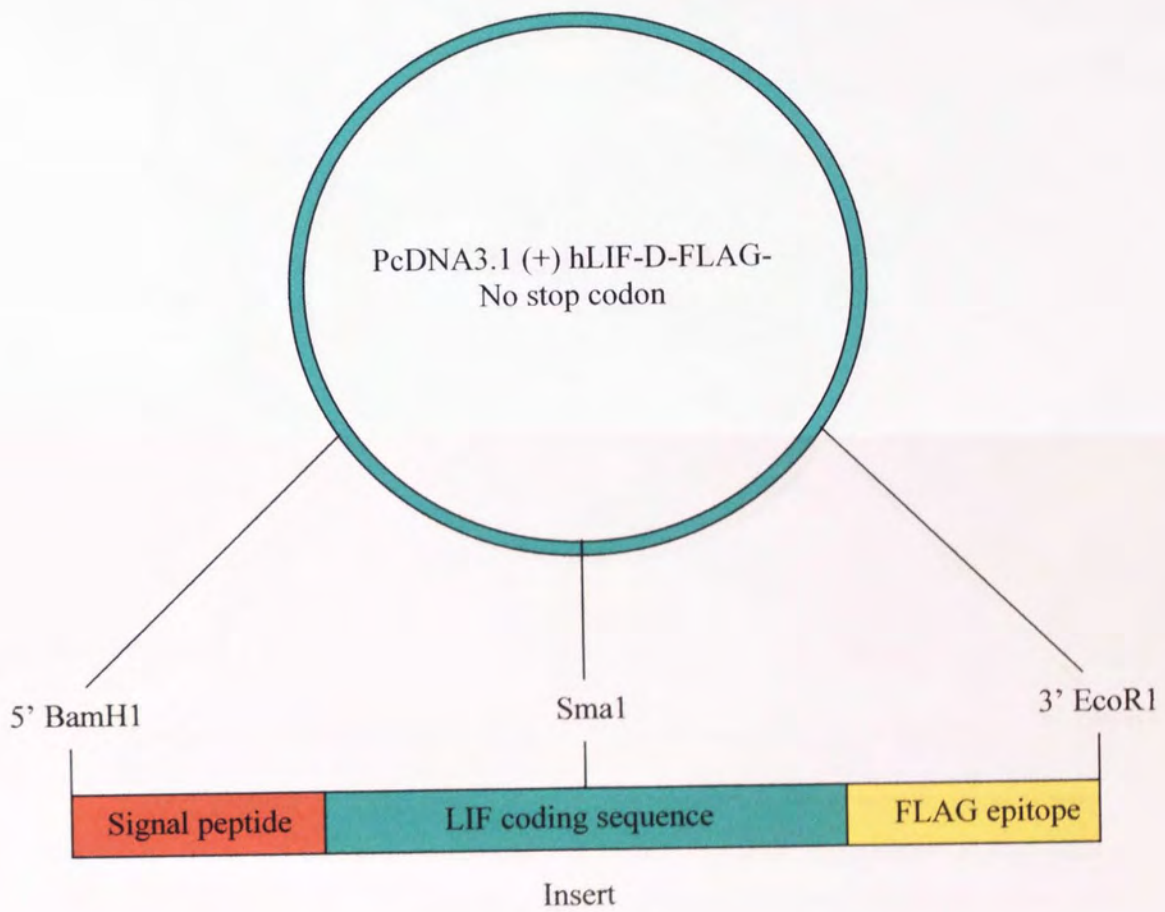
Appendix 19 Schematic diagram of pcDNA3.1(+) hLIF-M-FLAG (EJH12). Showing restriction endonuclease sites, and components of insert.

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Appendix 20 Schematic diagram of pcDNA3.1(+) hLIF-D-no stop codon (EJH13). Showing restriction endonuclease sites, and components of insert.

Appendix 21 Schematic diagram of pcDNA3.1(+) hLIF-D-FLAG-Fc (EJH14). Showing restriction endonuclease sites, and components of insert.

Appendix 22 Schematic diagram of pcDNA3.1(+) hLIF-D (EJH15). Showing restriction endonuclease sites, and components of insert.
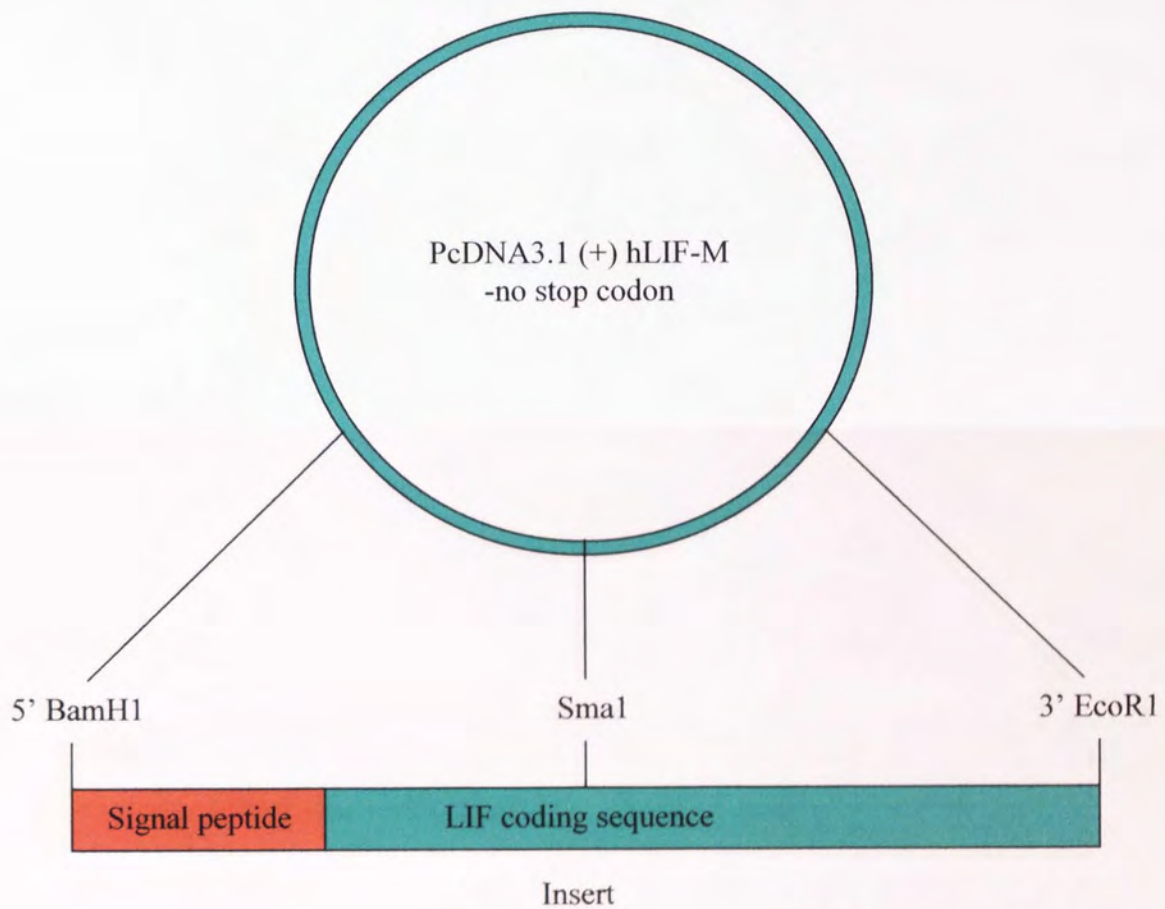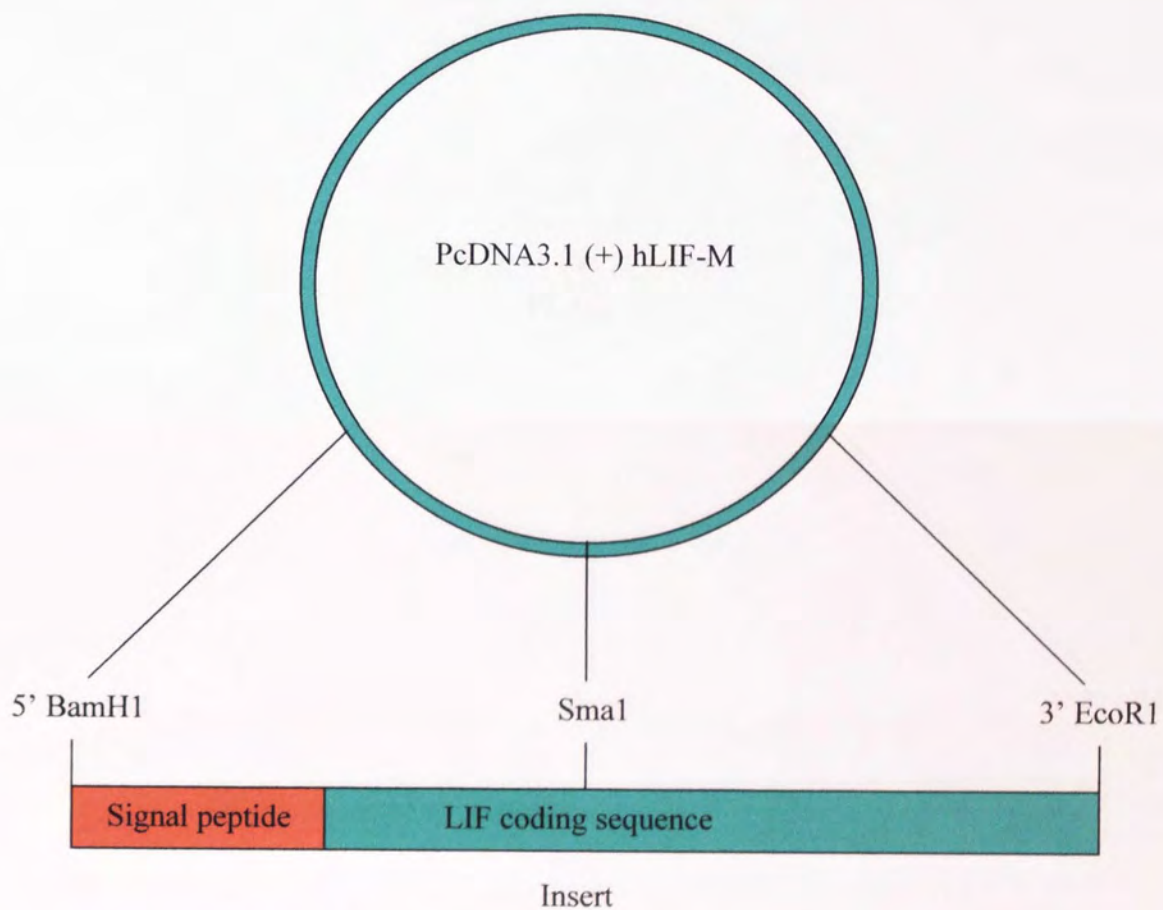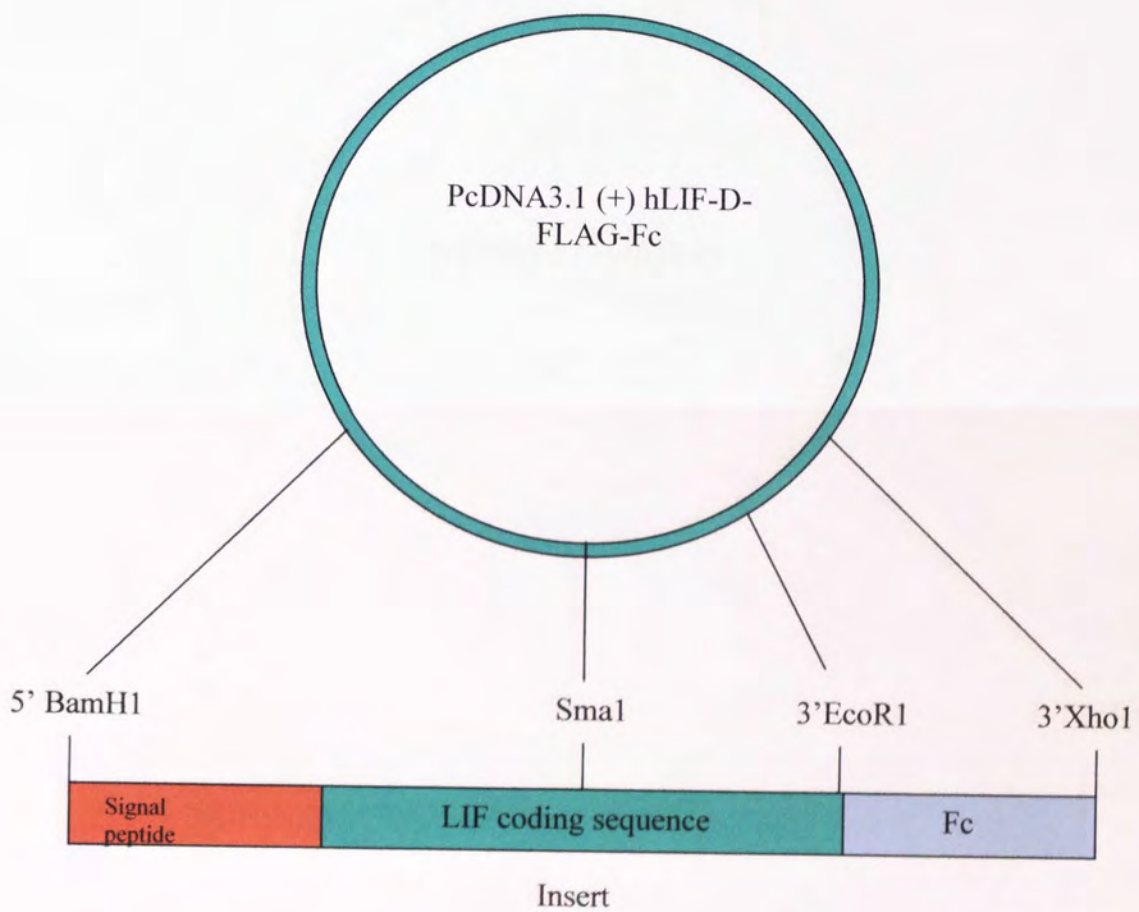
pBI-L mLIF-M

5' Nhe1   Sma1   3' HindIII

Signal peptide | LIF coding sequence

Insert

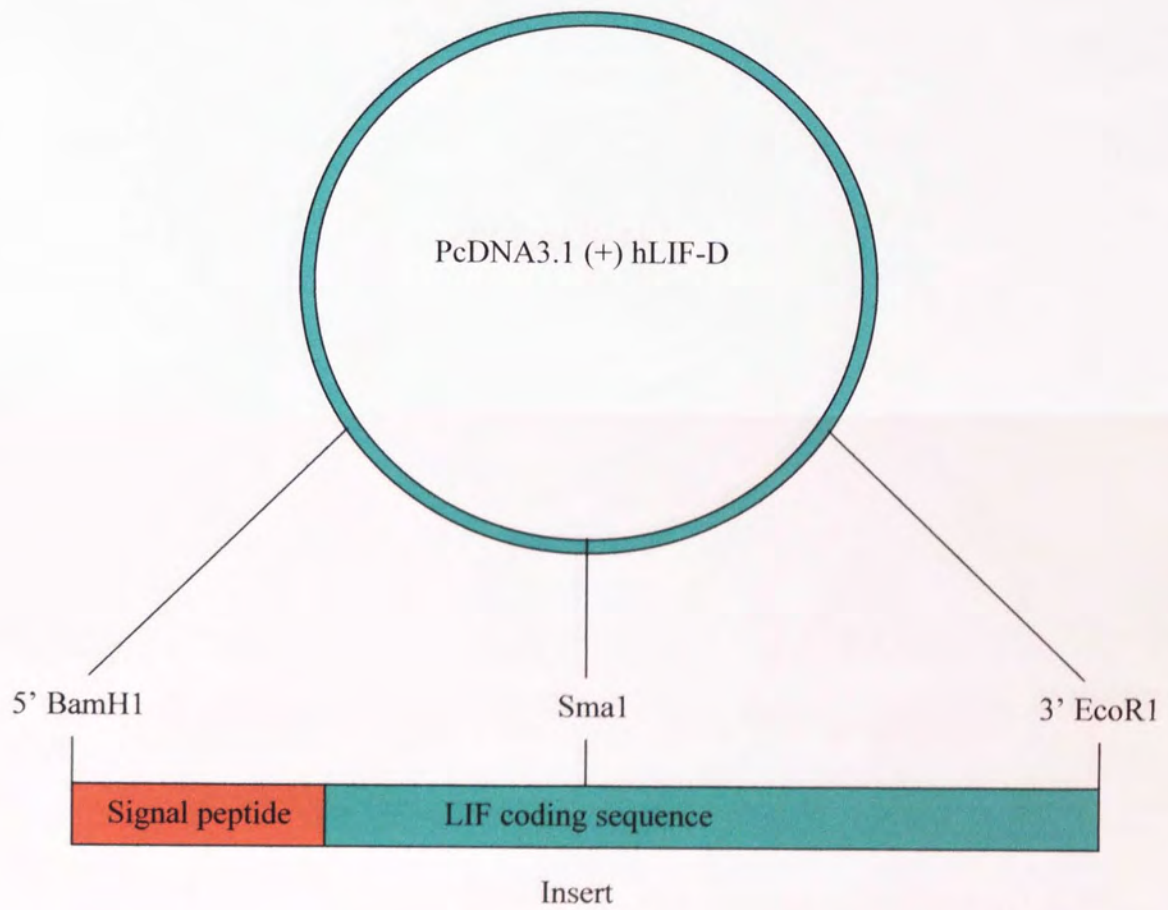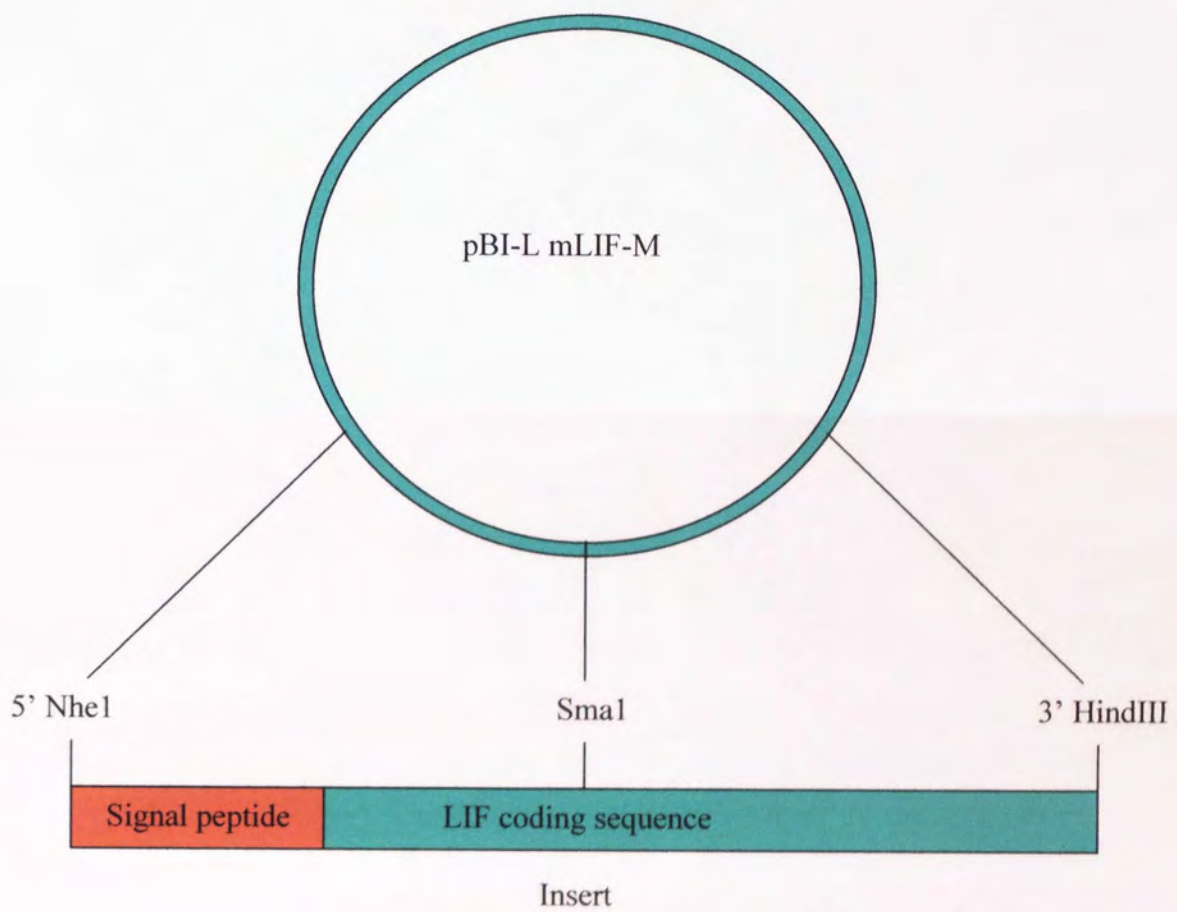**Appendix 23 Schematic diagram of pBI-L-mLIF-M (EJH16).** Showing restriction endonuclease sites, and components of insert.
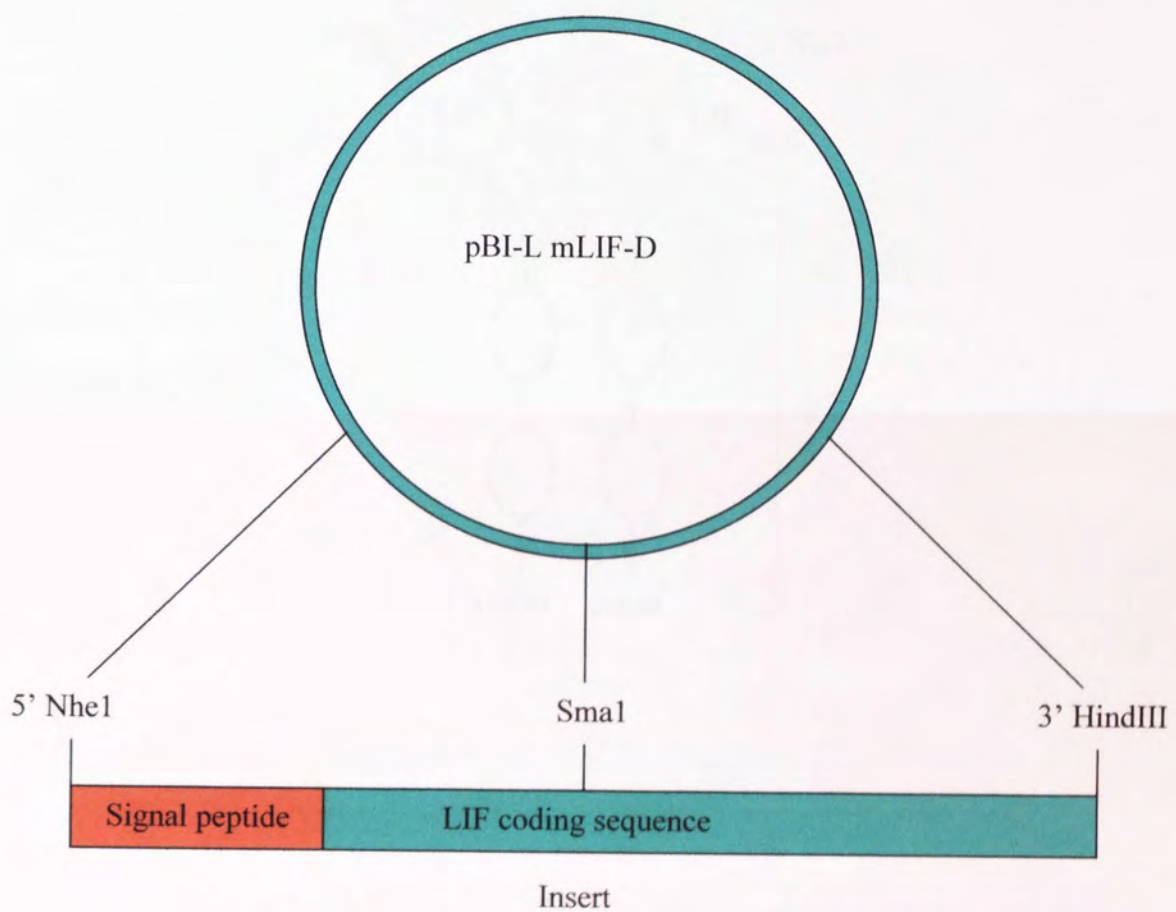
Appendix 24 Schematic diagram of pBI-L-hLIF-D (EJH17). Showing restriction endonuclease sites, and components of insert.
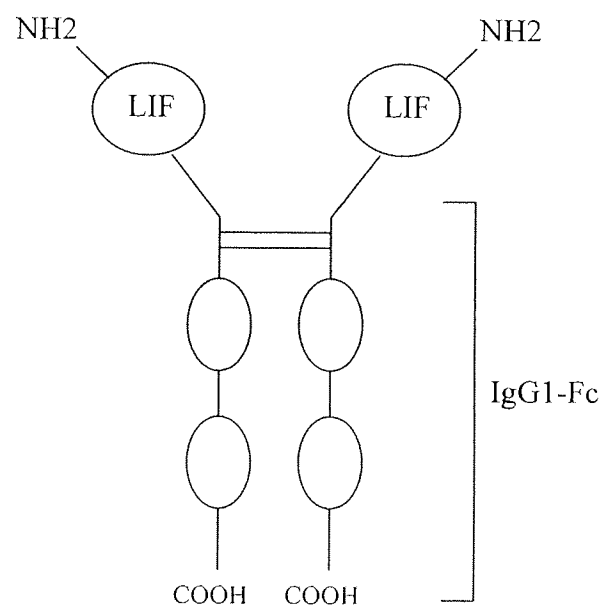
Appendix 25 Diagrammatical representation of LIF-Fc

	DualA	DualB	BasalA	BasalB	ApicalA	ApicalB	ControlA
DualB	>0.05						
BasalA	>0.05	<0.01					
BasalB	>0.05	>0.05	>0.05				
ApicalA	>0.05	<0.05	>0.05	>0.05			
ApicalB	>0.05	>0.05	>0.05	>0.05	>0.05		
ControlA	>0.05	<0.001	>0.05	<0.05	>0.05	>0.05	
ControlB	>0.05	<0.01	>0.05	<0.05	>0.05	>0.05	>0.05

Appendix 26. ANOVA followed by Tukey's post test (Graphpad prism). Concentration LIF secreted from caco-2 per chamber. A=Apical, B=Basolateral.

	DualA	DualB	BasalA	BasalB	ApicalA	ApicalB	ControlA
DualB	<0.001						
BasalA	<0.001	<0.001					
BasalB	<0.001	<0.001	<0.001				
ApicalA	<0.01	<0.001	<0.001	<0.001			
ApicalB	<0.01	<0.001	<0.001	<0.001	<0.001		
ControlA	>0.05	<0.001	<0.001	<0.001	>0.05	<0.001	
ControlB	<0.001	>0.05	<0.001	<0.001	<0.01	>0.05	<0.001

Appendix 27. ANOVA followed by Tukey's post-test (Graphpad Prism). Percentage LIF secreted from caco-2 per chamber. A=Apical, B=Basolateral.

	DualA	DualB	BasalA	BasalB	ApicalA	ApicalB
DualB	>0.05					
BasalA	>0.05	<0.05				
BasalB	>0.05	>0.05	>0.05			
ApicalA	>0.05	<0.05	>0.05	>0.05		
ApicalB	>0.05	<0.05	>0.05	>0.05	>0.05	

Appendix 28 ANOVA followed by Tukey's post-test (Graphpad Prism).
Concentration IL-6 (pg) secreted from caco-2 per chamber. A=Apical, B=Basolateral.

	DualA	DualB	BasalA	BasalB	ApicalA	ApicalB
DualB	<0.01					
BasalA	>0.05	<0.01				
BasalB	<0.01	>0.05	<0.001			
ApicalA	<0.05	>0.05	<0.01	>0.05		
ApicalB	>0.05	<0.05	>0.05	<0.01	>0.05	

Appendix 29. ANOVA followed by Tukey's post-test (Graphpad Prism). Percentage IL-6 secreted from caco-2 per chamber. A=Apical, B=Basolateral.

	4onA	4onB	4offA	4offB	9onA	9onB	9offA	9offB	19onA	19onB	19offA
4onB	<0.05										
4offA	<0.001	<0.001									
4offB	<0.001	<0.001	>0.05								
9onA	<0.001	>0.05	<0.05	<0.05							
9onB	<0.001	<0.05	>0.05	>0.05	>0.05						
9offA	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05					
9offB	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05	>0.05				
19onA	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05			
19onB	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05		
19offA	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05	
19offB	<0.001	<0.001	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Appendix 30. ANOVA followed by Tukey's post-test (Graphpad Prism). Concentration mLIF-D secreted per chamber. A=Apical, B=Basolateral.

	4onA	4onB	4offA	4offB	9onA	9onB	9offA	9offB	19onA	19onB	19offA
4onB	<0.001										
4offA	<0.01	>0.05									
4offB	>0.05	<0.01	>0.05								
9onA	>0.05	<0.001	<0.001	<0.05							
9onB	<0.001	>0.05	<0.05	<0.001	<0.001						
9offA	>0.05	<0.001	<0.001	<0.05	>0.05	<0.001					
9offB	<0.001	>0.05	<0.05	<0.001	<0.001	>0.05	<0.001				
19onA	>0.05	<0.001	<0.01	<0.01	>0.05	<0.001	>0.05	<0.001			
19onB	<0.001	>0.05	<0.01	<0.001	<0.001	>0.05	<0.001	>0.05	<0.001		
19offA	>0.05	<0.001	<0.05	>0.05	>0.05	<0.001	>0.05	<0.001	>0.05	<0.001	
19offB	<0.001	>0.05	>0.05	<0.05	<0.001	>0.05	<0.001	>0.05	<0.001	>0.05	<0.001

Appendix 31. **ANOVA followed by Tukey's post-test (Graphpad Prism).** Percentage mLIF-D secreted per chamber. A=Apical, B=Basolateral.

	FonA	FonB	FoffA	FoffB	SonA
FonB	<0.001				
FoffA	<0.001	>0.05			
FoffB	<0.001	>0.05	>0.05		
SonA	>0.05	>0.05	<0.01	<0.01	
SonB	<0.001	>0.05	>0.05	>0.05	>0.05

Appendix 32. ANOVA followed by Tukey's post-test (Graphpad Prism).
Concentration mLIF-M secreted per chamber. A=Apical, B=Basolateral.

FonB	<0.001				
FoffA	<0.001	<0.001			
FoffB	<0.001	<0.001	<0.001		
SonA	<0.001	<0.001	>0.05	<0.001	
SonB	<0.001	<0.01	<0.001	>0.05	<0.001
	FonA	FonB	FoffA	FoffB	SonA

Appendix 33.   ANOVA followed by Tukey's post-test (Graphpad Prism). Percentage mLIF-M secreted per chamber. A=Apical, B=Basolateral.