Leptin regulation of inflammatory responses in human gingival fibroblasts

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Abstract

Obesity and type 2 diabetes mellitus (T2DM) are positively associated with the destructive, chronic inflammatory disease periodontitis. The adipokine leptin is elevated in obesity and T2DM, and promotes inflammatory responses. Gingival fibroblasts are implicated in the pathogenesis of periodontitis because inflammatory stimuli can drive these cells towards destructive, inflammatory responses. The aim of this study was to identify whether leptin promotes inflammatory responses in gingival fibroblasts, focussing on the extracellular matrix-degrading matrix metalloproteinases (MMPs), and their inhibitors (TIMPs).

Primary human gingival fibroblasts (hGFs) were isolated from gingival tissue and cultured in vitro. RT-PCR, ELISA and flow cytometry were used to assess MMP, TIMP and TLR expression; hGF signalling (±chemical pathway inhibitors) was assessed by Western blotting.

hGFs constitutively expressed numerous MMPs and TIMPs. Leptin increased the expression of MMP-1, MMP-3, MMP-8 and MMP-14, but not TIMPs, in hGFs. Leptin and interleukin-1 or the TLR2 agonist pam2CSK4 synergistically increased MMP-1 and MMP-3 production by hGFs; in contrast, leptin and Escherichia coli LPS regulated MMP production in a donor-dependent manner. TLR4 was detected on the surface of hGFs from all donors tested, suggesting that differential responses to E. coli LPS were not due to absent cell surface TLR4. Overall, these results suggest that leptin promotes an ECM-degrading hGF response, which is further enhanced under inflammatory conditions. Leptin activated multiple intracellular signalling pathways, but the MAPK pathway and ERK in particular, regulated leptin-stimulated MMP-1 expression in hGFs. Genome-wide expression profiling revealed that leptin enhanced the expression of genes in hGFs whose products function in inflammation; similarly, leptin enhanced the inflammatory gene expression profile induced by IL-1 in hGFs. Leptin+interleukin-1 did not promote collagen degradation in human gingival connective tissue explants.

In conclusion, leptin enhances wide-ranging inflammatory responses in hGFs in a context-dependent manner. This response could be a mechanistic link between obesity, T2DM and periodontitis.
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2M</td>
<td>α2-macroglobulin</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>AIR</td>
<td>acute inflammatory reaction</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
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<td>AP-1</td>
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<td>bovine serum albumin</td>
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<td>CAL</td>
<td>clinical attachment loss</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>CEJ</td>
<td>cemento-enamel junction</td>
</tr>
<tr>
<td>CGS</td>
<td>Cambridge Genomic Services</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>cytokine receptor homology</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>CSF</td>
<td>colony-stimulating factor</td>
</tr>
<tr>
<td>CT</td>
<td>connective tissue</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variance</td>
</tr>
<tr>
<td>DAB</td>
<td>4-(dimethylamino)benzaldehyde</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DMB</td>
<td>dimethyl-methylene blue</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMEM+</td>
<td>Dulbecco’s modified eagle medium supplemented with 10 % v/v FBS</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EHOM</td>
<td>engineered human oral mucosa</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>EMPRIN</td>
<td>extracellular MMP inducer</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3’-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCF</td>
<td>gingival crevicular fluid</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GRB2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>hGF</td>
<td>human gingival fibroblast</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box-1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>hepatic stellate cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>H+E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2, 3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopaedia of genes and genomes</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NAC</td>
<td>no antibody control</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>OHP</td>
<td>hydroxyproline</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>OSM</td>
<td>oncostatin M</td>
</tr>
<tr>
<td>PA</td>
<td>plasminogen activator</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PD</td>
<td>pocket depth</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>PTGS2</td>
<td>prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence units</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RSI</td>
<td>root surface instrumentation</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDD</td>
<td>sub-antimicrobial dose doxycycline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SFM</td>
<td>serum-free DMEM</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signalling 3</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBS</td>
<td>TRIS-buffered saline</td>
</tr>
<tr>
<td>TBS/T</td>
<td>TBS Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TLDA</td>
<td>TaqMan low density array</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>4PL</td>
<td>four parameter logistic</td>
</tr>
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</table>
Chapter 1. Introduction

In the 1950s and 1960s two mutant mice strains arose which displayed similar phenotypes (Friedman and Halaas, 1998). Homozygote mutants of both strains were extremely obese (weighing approximately three-times wild-type animals), suffered from diabetes, but showed signs of starvation (Myers et al., 2008). Using parabiosis experiments (sharing the blood circulation between two mice) Friedman deduced that the obese strain (ob/ob) had a mutation in a soluble factor that regulated feeding and adipose tissue mass (Coleman, 2010). These experiments also suggested that the other mouse strain (db/db) had a mutation in the receptor for the ob gene product. The product of the ob gene was subsequently identified in 1994 by positional cloning techniques and named leptin (Zhang et al., 1994). Similarly, the leptin receptor, and product of the db gene, was identified in 1995 (Tartaglia et al., 1995).

1.1 Biological importance of leptin

Leptin is a 16 kDa protein hormone that regulates adipose tissue mass by reducing food intake; a function controlled primarily by the hypothalamus (Friedman and Halaas, 1998). Leptin is secreted by adipocytes in proportion to total adipose tissue mass (Maffei et al., 1995). Therefore, increased circulating leptin levels indicate abundant adipose tissue energy stores, while decreased circulating leptin levels signal the opposite (Kelesidis et al., 2010). In non-obese humans, fasting blood leptin concentrations are usually < 30 ng/ml and are significantly higher in women compared to men (Considine et al., 1996). This is attributed to the difference in adipose tissue mass between the sexes (Maffei et al., 1995). As evidenced by the obese phenotypes of ob/ob and db/db mice, and humans with mutations in the leptin gene (LEP), the primary function of leptin is to inform the brain of energy reserves and to regulate energy expenditure (Figure 1.1) (Myers et al., 2008). In the central nervous system (CNS), leptin activates intracellular signalling pathways in different types of neurons within the hypothalamus that express the leptin receptor (Coppari and Bjorbaek, 2012). This activates complex pathways involving the production of downstream neurotransmitters and hormones that reduce feeding, increase the metabolic rate, and increase insulin sensitivity. As such, leptin is considered to be a satiety factor (Kelesidis et al., 2010).

Mice and humans that lack leptin signalling also exhibit reduced reproductive capacity and immunocompetence (Kelesidis et al., 2010). These physiological defects are reported in cases of malnourishment and are hypothesised to be reduced to improve an
individuals’ chance of survival during a period of starvation (Matarese et al., 2005). Therefore, leptin is hypothesised to be a link between nutrition, reproduction and immunity. In support of this hypothesis the leptin receptor has been detected on cells of the haematopoietic lineage including lymphocytes and macrophages/monocytes (Uddin et al., 2010), which suggests that leptin can act directly on these cells. Indeed, a recent study found that the reduced capacity for T lymphocyte activity observed in fasting mice could be rescued by leptin directly signalling to T lymphocytes (Saucillo et al., 2014). Interestingly, the leptin receptor is expressed on other cells types including human endothelial cells, fibroblasts and macrophages (Sierra-Honigmann et al., 1998; Glasow et al., 2001; Gruen et al., 2007). Similarly, immunohistochemistry (IHC) analyses have found that leptin is present in peripheral tissues (Vernooy et al., 2010). The role of leptin on these cells and at these sites is only just beginning to be unravelled (Section 1.4) (Figure 1.1), and suggests that the biological importance of leptin is more wide-ranging than initially thought.

1.2 Intracellular signalling pathways stimulated by leptin
Since leptin and the leptin receptor were identified and sequenced in the 1990s much research has been undertaken to understand how leptin binds to the leptin receptor, the intracellular signalling pathways this interaction activates, and how these pathways relate to the physiological functions of leptin in vivo. (Myers et al., 2008)

1.2.1 Structural features of leptin and the leptin receptor
Structural investigations demonstrated that leptin is a member of the four α-helix bundle cytokine family (Kline et al., 1997). The four α-helix bundle cytokine family has several sub-families and leptin shares structural features with members of the interleukin (IL)-6 cytokine family (Fantuzzi and Faggioni, 2000). However, unlike the other members of the IL-6 cytokine family, leptin has a unique structural feature called a cysteine knot (Haglund et al., 2012). Interestingly, Haglund et al. (2012) found that the ability of leptin to activate intracellular signalling in vitro was reduced when the disulphide bond responsible for the cysteine knot was absent (due to reducing conditions or molecular substitution of cysteine residues). Whether or not this structural feature is important in vivo is not yet known.

The leptin receptor is structurally related to members of the type I cytokine receptor family; a classification based on structural similarity and the presence of cytokine receptor homology (CRH) domains (Fantuzzi and Faggioni, 2000). This cytokine
receptor family includes gp130, leukaemia inhibitory factor (LIF) receptor and granulocyte colony-stimulating factor (CSF) receptor (Tartaglia et al., 1995). Several leptin receptor isoforms have been identified; these isoforms are generated by alternative splicing and are categorised in humans as long or short, depending on the length of the intracellular domain (Kelesidis et al., 2010). Soluble leptin receptor is detected in the circulation and in humans is thought to be secreted by ectodomain shedding; in contrast mice express a secreted leptin receptor isoform that lacks a transmembrane region (Ge et al., 2002). Soluble leptin receptor was shown to inhibit leptin-mediated intracellular signalling in leptin receptor-transfected human embryonic kidney cells (Schaab et al., 2012), and therefore soluble leptin receptor is thought to regulate the availability of free leptin. Electron microscopic analysis of leptin/leptin receptor crystals, and studies using leptin receptors with specific sequence alterations, have shown that leptin binds to the CRH2 domain found on the extracellular region of the leptin receptor (Peelman et al., 2004).

1.2.2 Leptin signalling pathway activation

The intracellular region of the leptin receptor has no intrinsic kinase activity (Kelesidis et al., 2010). However, the intracellular membrane proximal region of the short and long leptin receptor isoforms contains binding sites for Janus kinase 2 (JAK2) and Src family kinases (SFKs); and both JAK2 and SFKs are constitutively bound to the leptin receptor (Coppari and Bjorbaek, 2012) (Figure 1.2). Fluorescence resonance energy transfer (FRET)-based studies using living cells have shown that a dimeric form of the leptin receptor is required to activate intracellular signalling, and that the long leptin receptor isoform is found as a dimer constitutively (Couturier and Jockers, 2003). This supports the hypothesis that a 2:2 ratio of leptin/leptin receptor is required for leptin to initiate intracellular signalling; JAK2 and SFKs activate by transphosphorylation between leptin receptor monomers and phosphorylate several tyrosine residues (Y985, Y1077, Y1138) on the intracellular region of the long leptin receptor isoform membrane distal to the JAK2 binding site (Mancour et al.; Coppari and Bjorbaek, 2012). The short leptin receptor isoforms lack much of the intracellular region of the leptin receptor, including these tyrosine residues, but can still facilitate some intracellular signalling via JAK2 (Bjorbaek et al., 1997). However, in vivo studies using truncated leptin receptors in mice have shown that the metabolic and endocrine functional outcomes of leptin signalling require the phosphorylation of the tyrosine residues found in the long leptin
receptor isoform (Myers, 2010). As such, much of the research on leptin signalling has focussed on the long leptin receptor isoform.

Phospho-tyrosine residues on the leptin receptor form binding sites for Src homology (SH)2 domain-containing proteins which are then activated by JAK2 or SFKs (Coppari and Bjorbaek, 2012). In vitro and in vivo approaches using mutant leptin receptors (lacking Y985, Y1077 and/or Y1138) have demonstrated that each of these phosphorylated residues favours the binding and activation of different signalling pathway intermediates (Myers, 2010). For example, Y1077 is necessary for maximum signal transducer and activator of transcription (STAT) 5, STAT1 and STAT6 activity (Gong et al., 2007; Coppari and Bjorbaek, 2012). Leptin-receptor bound STATs are phosphorylated and activated by JAK2 (Myers et al., 2008). Activated STATs form dimers which translocate to the nucleus, where STATs enhance gene expression by binding to interferon (IFN)-γ-activated sequences or IFN-stimulated response elements in the promoter regions of a wide range of genes (Bezbradica and Medzhitov, 2009). Y1138 is necessary for STAT3 activity and it is this arm of the leptin signalling pathway which is responsible for many of the hormonal functions of leptin that regulate body weight (Myers, 2010). The phosphatase Shp2 binds to phosphorylated Y985 and acts as a binding site for growth factor receptor-bound protein 2 (GRB2); GRB2 drives the activation of the mitogen-activated protein kinase (MAPK) signalling pathway (Coppari and Bjorbaek, 2012). GRB2 contains SH3 domains which the guanine nucleotide exchange factor Son of Sevenless (SOS) binds to (Rojas et al., 2011). This relocation of SOS to the plasma membrane brings it in contact with its substrate, Ras. SOS-activated Ras then activates upstream proteins in the MAPK signalling pathway (Mor and Philips, 2006). The MAPK signalling pathway consists of a kinase cascade whereby an active upstream kinase phosphorylates and activates a downstream kinase. There are several arms of the MAPK signalling cascade, named according to the terminal MAPK molecule (extracellular signal-related kinase (ERK), p38, c-Jun N-terminal kinase (JNK)). Each of these terminal MAPK molecules can phosphorylate, and thereby activate, several transcription factors including c-Fos, c-Jun, CCAAT-enhancer-binding proteins (C/EBP) and activating transcription factor (ATF) 2. The gene targets upregulated by these transcription factors span a broad range of functions. Additionally, MAPK signalling can enhance the activity of STATs and the nuclear factor κB (NF-κB) signalling pathway (Morris and Rui, 2009).
Leptin also activates phosphoinositide 3-kinase (PI3K), Akt/protein kinase B, mammalian target of rapamycin and NF-κB signalling (Zhou et al., 2011; Coppari and Bjorbaek, 2012). It is thought that leptin-mediated PI3K activity requires JAK2 but not the phosphorylation of tyrosine residues on the leptin receptor (Tong et al., 2008). Therefore, PI3K activity may occur downstream of both short and long leptin receptor isoforms. Leptin-mediated PI3K activity regulates cellular growth (Jaffe and Schwartz, 2008; Shan et al., 2008; Valerio et al., 2009; Uddin et al., 2010), motility (Gruen et al., 2007), glucose sensitivity (Coppari and Bjorbaek, 2012) and neurone action potentials (Hill et al., 2008).

1.2.3 Regulation of the leptin signalling pathway

The leptin signalling pathway is fairly well described in neurones and transfected cell lines, but less is known about how leptin signalling varies between cell types (Coppari and Bjorbaek, 2012). As the leptin signalling pathway is complex, there are gaps in our understanding about how the different arms of the leptin signalling pathway are regulated and how they interact. Negative regulators of leptin signalling (suppressor of cytokine signalling 3 (SOCS3), protein tyrosine phosphatase 1B (PTP1B)) have been described (Morris and Rui, 2009). PTP1B and presumably other phosphatases negatively regulate leptin signalling by dephosphorylating, and thereby inactivating, the leptin receptor and associated proteins; the mechanisms of SOCS3 inhibition of leptin signalling are not fully understood (Coppari and Bjorbaek, 2012). Positive regulators of the leptin signalling pathway such as SH2B adaptor protein 1 have been identified but exactly how they function is unclear (Morris and Rui, 2009). Mechanisms of interaction between cytokine signalling pathways include cross-talk/feedback, amplification of downstream pathways and convergence of multiple pathways to a single downstream molecule (Bezbradica and Medzhitov, 2009). It is likely that all of these mechanisms occur during leptin signalling.

The signalling pathways activated by leptin are similar to those activated by other agonists of the type I cytokine receptor families (Tartaglia, 1997). Therefore, the magnitude of activity of these shared intracellular signalling pathways could be altered if a cell is exposed to leptin in combination with other stimuli. For example, there is crosstalk between the insulin and leptin signalling pathways in the CNS (Carvalheira et al., 2005). Specifically, Carvalheira et al. (2005) found that insulin and leptin signalling converged at PI3K, but diverged downstream at Akt in the rat hypothalamus. Similarly, crosstalk at the level of STAT signalling has been described between leptin and
oestrogen in the CNS (Gao and Horvath, 2008). Leptin can also crosstalk with other stimuli at the level of intracellular signalling in cells outside of the CNS. For example, leptin and insulin-like growth factor-1 synergistically increase Akt and ERK signalling in breast cancer cells, thereby increasing the potential of these cells to invade and migrate (Saxena et al., 2008). In human articular chondrocytes leptin and IL-1 synergistically increase the expression of MMPs by amplifying MAPK signalling (Hui et al., 2012). There are multiple possibilities, thus there is scope for further research in this area because cellular microenvironments are variable. For example, the cellular microenvironment may change depending on local energy supplies, or during infection and inflammation. By understanding the intricacies of leptin signalling in different contexts it may be possible to identify how variation, dysregulation and defects in leptin signalling and therefore function, can contribute to human disease.

1.3 Obesity, diabetes and leptin resistance
The prevalences of metabolic disorders, such as obesity and type 2 diabetes mellitus (T2DM), are increasing at an alarming rate globally, presumably due to changes in diet and lifestyle (Malik et al., 2013). Central (or abdominal) obesity and impaired insulin sensitivity (as observed in T2DM) are both features of metabolic syndrome (Bullon et al., 2009). Definitions of metabolic syndrome vary but usually require an individual to have elevated blood pressure and dyslipidaemia, as well as signs of insulin resistance and obesity (Hotamisligil, 2006; Pischon et al., 2007). Between 5 – 20 % of the world population is estimated to have metabolic syndrome (Alberti et al., 2006). Obesity and T2DM are a costly burden to health services and are associated with increased morbidity and mortality in affected populations (Malik et al., 2013). Systemic markers of oxidative stress and chronic inflammation have been detected in individuals who have metabolic syndrome. As such, mediators of oxidative stress and inflammation have been suggested to play a mechanistic role in the relationship between metabolic syndrome and other conditions associated with oxidative stress and chronic inflammation, such as periodontitis and cardiovascular disease (Hotamisligil, 2006; Bullon et al., 2009).

Individuals with T2DM have a reduced ability to metabolise blood glucose and, therefore, T2DM is characterised by the World Health Organisation (2006) as having a fasting blood glucose level of > 7 mM. In contrast, obesity is determined in several ways; such as by measuring the waist to hip ratio, by assessing body fat composition
and distribution, and by calculating the body mass index (BMI) (Alberti et al., 2006). BMI is calculated using the formula:

\[ BMI = \frac{\text{weight (kg)}}{(\text{height (m)})^2} \]

Adults with a BMI > 25 are generally considered overweight and those with a BMI > 30 are considered obese, although ethnic and gender differences complicate these broad definitions (Alberti et al., 2006). Both genetic and environmental factors contribute to the risk of obesity and T2DM, and therefore, these disorders have a complex molecular basis (Kolb and Mandrup-Poulsen, 2010). For example, mutations in the leptin or leptin receptor genes in rodents and humans result in extreme obesity and endocrine dysfunction (Clement et al., 1998). However, most obese individuals have increased circulating leptin levels (Coppari and Bjorbaek, 2012). In contrast, individuals with a lower adipose tissue mass (e.g. due to lipodystrophy) have reduced circulating leptin concentrations (Petersen et al., 2002). This is not surprising given the relationship between adipose tissue mass and leptin production (Maffei et al., 1995). However, this appears to contradict the evidence that increased levels of leptin stimulate the neuroendocrine pathways that control appetite and metabolism in an effort to reduce adipose tissue mass (Friedman and Halaas, 1998). Obese individuals with increased circulating leptin concentrations often remain obese, and even gain more weight, suggesting that the body develops a resistance to leptin (Coppari and Bjorbaek, 2012). This has similarities to the resistance to insulin that develops in T2DM (Hotamisligil, 2006). Regarding leptin, resistance makes sense from an evolutionary perspective as starving was presumably a greater cause of death than being in energy excess; this suggests that the dominant function of leptin is to signal starvation, not energy excess (Friedman and Halaas, 1998). Interestingly, circulating leptin levels are uncoupled from adiposity and energy balance in mammals in two normal physiological processes, namely hibernation (Kronfeld-Schor et al., 2000) and pregnancy (Tessier et al., 2013).

Studies using mouse models identified that during obesity leptin-stimulated neurons increase the production of SOCS3 and PTP1B, which may reduce leptin signalling (Section 1.2) (Munzberg et al., 2004; Gamber et al., 2012). However, increased CNS concentrations of leptin and proinflammatory cytokines in addition to endoplasmic reticulum stress are all implicated in the development of leptin resistance in neurons (Coppari and Bjorbaek, 2012). It is hypothesised that there is a threshold concentration of leptin above which there is no increase in the strength of leptin signalling, e.g. the
leptin signalling pathway is saturated (Mantzoros et al., 2011). This relates to the idea that leptin signals starvation, and not energy excess (Friedman and Halaas, 1998). Interestingly, there is evidence that essentially identical neurones differentially respond to leptin (Williams et al., 2010). Pro-opiomelanocortin neurones (which regulate leptin-mediated insulin sensitivity) activate leptin signalling in a manner distinct to the leptin-sensitive γ-aminobutyric acid neurones (which regulate body weight) (Coppari and Bjorbaek, 2012). Furthermore, the differential ability of leptin to regulate insulin sensitivity and body weight have been determined in both in vivo studies in mice (Jager et al., 2011), and clinical trials in humans who are hypoleptinaemic (Petersen et al., 2002). These studies suggest that the different functional responses of leptin are regulated independently of each other, due to cell- or signalling-specific mechanisms.

Most of the current studies of leptin resistance have investigated CNS leptin resistance (Myers et al., 2008; Morris and Rui, 2009; Coppari and Bjorbaek, 2012). Peripherally, there is some evidence to support leptin resistance in adipose tissue (Mantzoros et al., 2011), skeletal muscle (Steinberg et al., 2002) and cardiomyocytes (Radin et al., 2011). One complication of studying leptin sensitivity in peripheral tissue, and/or using in vitro models, is that it is difficult to determine the relative effects of central leptin signalling compared to direct leptin action on peripheral cells.

There is currently a large global population of hyperleptinaemic individuals; including those who are obese, and those treated with exogenous leptin as a therapy (Kelesidis et al., 2010). Central leptin resistance has been well studied but the mechanisms underlying this phenomenon appear to be cell-type and signal dependent (Coppari and Bjorbaek, 2012). Even less is understood about peripheral leptin sensitivity and resistance. It is plausible that peripheral leptin-responsive sites have a different sensitivity to leptin compared to the CNS. Could some peripheral sites remain sensitive to leptin, or only be exposed to leptin, during hyperleptinaemia? What are the functional implications of this? Further studies investigating the sensitivity of non-CNS sites to leptin are warranted, particularly at those sites relevant to energy metabolism, endocrine function, reproduction and immunocompetence.

1.4 Leptin as a regulator of immunity, inflammation and wound healing

Mice and humans that lack leptin signalling exhibit reduced immunocompetence (Kelesidis et al., 2010). Leptin and the leptin receptor are structurally homologous to IL-6 cytokines and type I cytokine receptors respectively (Section 1.2) (Fantuzzi and Faggioni, 2000). IL-6 family cytokines have wide-ranging local and systemic functions,
and are particularly well studied in regard to inflammation, immunity and wound healing (Taga and Kishimoto, 1997a). Leptin was hypothesised to have similar functions, and numerous studies have provided evidence to support this hypothesis (Matarese et al., 2005).

1.4.1 Leptin and leukocyte recruitment

Intraperitoneal administration of leptin in a mouse model of chronic obstructive pulmonary disease causes an increased infiltration of neutrophils, T cells and dendritic cells (DCs) in bronchoalveolar lavage (BAL) fluid (Vernooy et al., 2010), suggesting that leptin promotes leukocyte infiltration into inflamed lungs. However, Vernooy et al (2010) found that the levels of the chemokines CXCL1 and CCL2 in BAL were increased in ob/ob and db/db mice compared to wild-type controls, suggesting that in this model leptin-mediated leukocyte infiltration was not due to enhanced lung CXCL1 or CCL2 production. Leptin dose-dependently increases chemokine receptor CCR7 expression on human colon DCs in Crohn’s disease, and this increase in chemokine receptor expression is responsible for enhanced DC migration (Al-Hassi et al., 2013). This suggests that leptin may promote chemotaxis by increasing the expression of chemokine receptors on leukocytes. Leptin increases the in vitro production of CCL2 by activated, myofibroblast-like human hepatic stellate cells (HSCs) (Aleffi et al., 2005), increases CXCL8 expression by synovial fibroblasts (Tong et al., 2008), and increases CCL3/CCL4/CCL5 expression by murine macrophages (Kiguchi et al., 2009). These studies suggest that leptin may promote leukocyte chemotaxis by promoting the production of chemokines by tissue-resident cells. Intriguingly, leptin itself acts as a chemoattractant for murine peritoneal monocytes/macrophages and the human THP-1 monocytic cell line in vitro (Gruen et al., 2007), however, no mechanistic explanation for this finding was described. Overall, leptin appears to promote leukocyte chemotaxis which may function to promote tissue inflammatory responses.

1.4.2 Leptin regulation of leukocyte function

Monocytes, macrophages, DCs, mast cells, eosinophils and natural killer cells all express the leptin receptor (Fernandez-Riejos et al., 2010). Only the short isoform of the leptin receptor is expressed in neutrophils (Zarkesh-Esfahani et al., 2004), although this appears sufficient to drive leptin-mediated neutrophil survival (Bruno et al., 2005). Similarly, leptin promotes the survival and proliferative ability of monocytes (Sanchez-Margalet et al., 2003) and DCs (Mattioli et al., 2009). Leptin also has an indirect effect on the circulating numbers of leukocytes, by affecting haematopoiesis in the bone
marrow and foetal liver (Fernandez-Riejos et al., 2010). Overall, these findings suggest that leptin acts as a growth factor for innate leukocytes.

Leptin increases the differentiation and activation of monocytes (Sanchez-Margalet et al., 2003; Fernandez-Riejos et al., 2010). For example, leptin increases the surface expression of the innate pattern recognition receptor (PRR) Toll-like receptor (TLR)-2 on monocytes (Jaedicke et al., 2013), and regulates the production of monocyte-derived proinflammatory cytokines (tumour necrosis factor (TNF)-α/IL-6/IFN-γ) (Zarkesh-Esfahani et al., 2001). Additionally, leptin promotes the secretion, but not expression, of the cytokine IL-18 by primary human monocytes (Jitprasertwong et al., 2014). Jitprasertwong et al. (2014) found that this leptin-dependent effect on IL-18 processing required the activity of the intracellular protease caspase-1. Interestingly, leptin synergises with the Gram negative bacterial outer membrane molecule lipopolysaccharide (LPS) to enhance the expression of TNF-α by innate leukocytes (Shen et al., 2005; Jaedicke et al., 2013), which suggests that leptin could enhance inflammation in hyperleptinaemic populations.

Leptin has a direct role in DC priming in a way that favours a proinflammatory Th1 immune response (Fernandez-Riejos et al., 2010), suggesting that leptin indirectly regulates adaptive immune responses. Several studies have shown that leptin promotes lymphocyte proliferation, activation and function in a subset-specific manner (Martin-Romero et al., 2000; Farooqi et al., 2002; Papathanassoglou et al., 2006; Saucillo et al., 2014). For example, leptin positively regulates the growth and survival of CD4+ T cells and B cells, but has no similar effect on Treg cells (Fernandez-Riejos et al., 2010).

Leptin enhances T cell activation and polarises responses, which is consistent with the known activities of related cytokines (Sanchez-Margalet et al., 2003). Overall, these studies support a widespread potential of leptin to modulate cells of both the innate and adaptive immune system, and suggest that leptin functions in immune homeostasis and enhance immune responses in hyperleptinaemia.

1.4.3 Leptin regulation of ECM remodelling

Leptin increases the expression and extracellular matrix (ECM)-degrading activity of matrix metalloproteinase (MMP)-2 by a murine cardiac muscle cell line in a p38 MAPK-dependent manner, while simultaneously reducing the mRNA expression of the MMP inhibitor tissue inhibitor of metalloproteinases (TIMP)-1 and increasing the production of collagen (Schram et al., 2010). Leptin suppresses the production of the
collagenase MMP-1 by a HSC line (Cao et al., 2007), but increases MMP expression in primary murine trophoblast cells (Schulz et al., 2009). These studies suggest that leptin regulates ECM remodelling, however it appears that the effect of leptin on this function differs between cell types, and depends on which MMPs are studied.

A glioma cell line stimulated with leptin has an increased ability to migrate compared to unstimulated cells (Yeh et al., 2009). Yeh et al. (2009) found that this ability depends on the collagenase MMP-13 and is regulated by p38 MAPK and NF-κB. Similarly, primary rat cardiac fibroblasts have an enhanced ability to migrate across wounds, likely due to increased expression of membrane integral MMP-14 and increased MMP-2 activity after leptin stimulation (Schram et al., 2011). These studies suggest that leptin promotes cell migration and invasion by promoting ECM degradation, in addition to regulating chemokine/chemokine receptor production. Schram et al. (2011) also showed that leptin increases actin polymerisation in these fibroblasts in a RhoA/Rho-associated protein kinase-dependent manner, suggesting that leptin also enhances cell motility by regulating intracellular motility equipment. Interestingly, leptin synergises with the proinflammatory cytokines IL-1 and TNF-α to increase MMP-1 and MMP-13 expression by human articular chondrocytes and to increase cartilage degradation (Hui et al., 2012). In this study, the increased MMP mRNA expression detected required JAK/STAT, MAPK and PI3K signalling pathways. Additionally, Hui et al (2012) showed that white adipose tissue from articular joint fat pads also drives cartilage degradation, and that this effect is in part due to leptin. This study supports a role for leptin in enhancing ECM degradation during inflammation in cartilage, and suggests that leptin may be mechanistically relevant to the positive clinical association between obesity and osteoarthritis (Grotle et al., 2008). Overall, the current evidence supports a role for leptin in regulating tissue-resident cell-mediated ECM remodelling. Whether this is beneficial or deleterious may depend on the context of exposure to leptin. Further studies investigating the ability of leptin to promote ECM degradation by other tissue-resident cell types are warranted, particularly at those sites where deleterious ECM remodelling can occur during inflammation. Could leptin synergise with other inflammatory mediators to promote ECM degradation? Array-based investigations may help to identify the complete profile of ECM-degrading enzymes, inhibitors of these enzymes and ECM components that leptin regulates, while tissue-based or in vivo models may help to determine the functional ability of leptin to regulate ECM remodelling.
1.4.4 Leptin regulation of the vasculature and fibrosis

Leptin drives the proliferation and migration of both vascular smooth muscle cells (Shan et al., 2008) and human umbilical vein endothelial cells (HUVECs) (Park et al., 2001). Leptin stimulates vascular endothelial growth factor (VEGF) production in a mammary tumour cell line (Zhou et al., 2011) and HSCs (Aleffi et al., 2005). Leptin-stimulated HUVEC supernatants also have increased gelatinolytic MMP (MMP-2/MMP-9) activity (Park et al., 2001). Park et al (2001) also showed that leptin enhances the formation of capillary-like tubes by HUVECs, and promotes angiogenesis in the rat cornea angiogenesis model. These studies support a role for leptin in promoting angiogenesis, which may be relevant to wound healing in the resolution of inflammation. Administration of leptin in a mouse model of vascular injury increases the formation of a neointimal layer in the femoral artery (Shan et al., 2008). The neointima is caused by proliferation of vascular smooth muscle cells and aids the healing of vascular injuries, but also narrows the blood vessel lumen which is a risk for vessel occlusion (Jeremy and Thomas, 2010). In a model of ischaemic stroke, ob/ob mice were more susceptible to permanent vascular occlusion than wild-type animals (Valerio et al., 2009). Valerio et al. (2009) observed that the administration of leptin prior to the induction of stroke reduced the extent of neuronal damage observed. The conflicting findings presented in these studies could be due to the different models of injury used and the complex responses to leptin but, nonetheless, do support a role for leptin in regulating the wound healing response of the vasculature. The extent of CCl₄ or diet-induced liver fibrosis is reduced in db/db mice compared to wild-type animals, suggesting that leptin promotes fibrotic responses in these models (Aleffi et al., 2005; Choi et al., 2010).

1.4.5 In vivo studies of immunological functions of leptin

In starved mice, which therefore have reduced circulating leptin levels, administration of leptin restores immune function (Mantzoros et al., 2011). Leptin administration in ob/ob mice protects against fatal Gram positive bacterial infections (Hsu et al., 2007), supporting a role for leptin in immunocompetence. However, mice which lack leptin receptor signalling via STAT3 survive a similar infectious challenge better than wild-type controls (Gove et al., 2009; Mancuso et al., 2011), suggesting that leptin signalling through STAT3 enhances the fatality of infections. These studies appear to contradict each other but may just serve to highlight the complexity of leptin signalling in vivo. In humans, leptin replacement therapy in a child that expressed no leptin protein due to
genetic mutation increased the potential for their T cells to be activated \textit{ex vivo} (Farooqi \textit{et al.}, 2002). However, in a study of women who had low circulating concentrations of leptin due to starvation, the administration of recombinant leptin had no effect on the \textit{in vitro} potential for T cell activity (Chan \textit{et al.}, 2006). Additionally, high doses of recombinant leptin given to lean, obese and obese+diabetic humans had no effect on the production of inflammatory cytokines (Chan \textit{et al.}, 2005). These studies support a role for leptin in regulating immunocompetence in individuals that are starving, but suggest that above a (possibly quite low) threshold of circulating leptin concentration that leptin no longer has the ability to modulate certain immune and inflammatory responses. This is consistent with the hypothesis that leptin receptor signalling can be saturated (Section 1.3) (Mantzoros \textit{et al.}, 2011). However, \textit{ob/ob} and \textit{db/db} mice have lower amounts of auto-antibodies and reduced T cell proliferation compared to wild-type animals in the antigen-induced arthritis mouse model (Busso \textit{et al.}, 2002). Similarly, \textit{db/db} mice are protected from both colitis and hepatitis (Gove \textit{et al.}, 2009). In this study, Gove \textit{et al.} (2009) found that the expression of cytokines, but not disease severity, was regulated in a STAT3-dependent manner, suggesting that different arms of the leptin signalling pathways regulate diverse inflammatory and immune-related functions. Additionally, recombinant leptin accelerates auto-immune diabetes in the non-obese diabetic mouse model (Matarese \textit{et al.}, 2002). These studies suggest that leptin enhances auto-immune diseases and pathogenic inflammatory conditions, at least in these models.

Leptin regulates numerous events relevant to inflammation, immunity and wound healing, affecting both leukocytes and tissue-resident cells. Both obesity and T2DM, where elevated circulating leptin levels are often observed, are associated with a low-grade, systemic chronic inflammatory state (Hotamisligil, 2006; Coppari and Bjorbaek, 2012). Therefore, leptin could be a molecular link underlying the associations between obesity, T2DM and diseases where excessive/chronic inflammation is implicated in the pathogenesis like periodontitis (Pischon \textit{et al.}, 2007; Preshaw, 2008; Bullon \textit{et al.}, 2009). Further studies of leptin action, alone or in an inflammatory setting, on cells and tissues relevant to these diseases would help to address this hypothesis, and determine whether leptin has a protective or pathogenic role.

1.5 \textbf{The periodontium: structure and function}

The periodontium is a collection of tissues in the oral cavity that function to support and protect the teeth. Nowhere else in the human body are mineralised tissues (the teeth) exposed to the external environment, essentially protruding through the epithelium. As
such, the bone, connective tissues and epithelium of the periodontium contain several distinctive features which make this anatomical region unique. (Bartold and Narayanan, 2006)

1.5.1 Alveolar bone and the periodontal ligament

The alveolar bone, an extension of the maxilla and mandible, comprises sockets holding teeth. A thin layer of connective tissue called the periodontal ligament (PDL) is found between the teeth roots and alveolar bone, which helps to support and protect both the teeth and bone from the forces that occur during mastication. Loss of integrity of the PDL and alveolar bone resorption compromise tooth function and can result in tooth loss. The PDL contains a dense ECM consisting predominantly of type I, III and V collagens which form fibres attaching the root cementum to the alveolar bone. Fibroblasts are the predominant cell type in the PDL, but endothelial cells and neurons are also present. (Bartold and Narayanan, 2006)

PDL fibroblasts are thought to function in maintaining the integrity of the PDL by producing and remodelling ECM components (Beertsen et al., 1997). In vitro studies using primary human PDL fibroblasts have demonstrated that these cells recognise and respond to numerous stimuli, including mechanical stimuli and immunological mediators (Xiang et al., 2009; Um et al., 2011; Zhu et al., 2011; Du et al., 2012). Stimulated PDL fibroblasts produce a range of molecules that function in ECM remodelling, as well as inflammatory responses (Jonsson et al., 2011).

1.5.2 Gingival tissue

The alveolar bone is covered superficially by a mucosal tissue called the gingiva (Figure 1.3). Gingival tissue is composed of a connective tissue layer, also called the lamina propria, which is separated by a type IV collagen-containing basal lamina to an epithelial layer. The lamina propria is characterised by a dense type I and III collagen-rich ECM which, similar to the PDL, provides resistance against mechanical forces. Unlike most other mucosal tissues, the gingiva rarely contains a submucosal layer; instead collagen fibres within the lamina propria attach to the underlying alveolar bone directly. Tissue-resident immune cells (predominantly macrophages), endothelial cells, neurons and occasionally adipocytes are present in gingival connective tissue. (Bartold and Narayanan, 2006)

Similar to the PDL, the predominant cell type within gingival connective tissue is the fibroblast; their primary function is to produce and remodel ECM components (Bartold
and Narayanan, 2006). Gingival fibroblasts, like PDL fibroblasts, respond to a wide-range of molecules by producing inflammatory mediators (Section 1.11). Several studies have performed comparisons of primary PDL and gingival fibroblasts in vitro and found differences in the type and extent of responses between the cells (Jonsson et al., 2011). For example, after being stretched gingival fibroblasts and PDL fibroblasts express a different panel of integrins (Bolcato-Bellemin et al., 2000); similarly, in comparison to PDL fibroblasts, gingival fibroblasts are generally more responsive in regulating ECM synthesis after treatment with an enamel matrix protein derivative which stimulates growth responses (Haase and Bartold, 2001). Interestingly, gingival fibroblasts, unlike PDL fibroblasts, increase the expression of TLR2 after stimulation with TLR2 agonists, which suggests that these cell types may respond to infection in different ways (Faria Morandini et al., 2012).

The epithelium which covers the external surface of the gingiva consists of three distinct types based on location and structure, namely the gingival, sulcular and junctional epithelium. The gingival epithelium covers the external surfaces of the gingiva, from the muco-gingival border to the free gingival margin. The gingival epithelium is thick and keratinised, which helps to protect the gingiva from damage during mastication, and acts as an innate barrier against the invasion of oral microbes. The sulcular epithelium covers the gingiva from the free gingival margin into the gingival sulcus, essentially facing the crown of the tooth. Unlike gingival epithelium, sulcular epithelium tends not to be keratinised. The third type of epithelium in the gingiva is the junctional epithelium. Junctional epithelium is found at the base of the gingival sulcus between the sulcular epithelium and the tooth surface (at the cemento-enamel junction (CEJ) in health). It is a non-keratinised and thin epithelium. In fact, the junctional epithelium can be only a few cells thick. This structure helps to make the junctional epithelium fairly permeable, and in health there is an outward movement of fluid and cells. (Bartold and Narayanan, 2006)

1.5.3 Gingival crevicular fluid

The fluid secreted from the junctional epithelium makes up the gingival crevicular fluid (GCF), which is a serum and tissue exudate and contains molecules involved in host defence, namely complement, cytokines and antibodies. Additionally, the junctional epithelium appears to be the site of leukocyte egress into the gingival sulcus. This action may be directed by the production of chemokines by junctional epithelium cells. Most of the leukocytes found in GCF are neutrophils, but macrophages and lymphocytes have
also been detected. Although GCF contains molecules and cells of the innate and adaptive immune systems, the lack of a keratinised barrier in the gingival sulcus is a potential weakness available to exploit by oral microbes. (Delima and Van Dyke, 2003)

1.5.4 Host-microbe interactions in the periodontium

Over 500 species of microorganisms have been identified in dental plaque on the surfaces of the tooth and tooth root. Most of these microorganisms are commensals; several species are pathogenic. The attachment of early colonising bacteria to the teeth is the first step in the formation of mixed-species ‘plaque’ biofilms, which survive due to synergistic and cooperative relationships. (Darveau, 2010)

Initially, the biofilm forms on supra-gingival surfaces, which are sequestered from host cells by the mineralised or keratinised external barrier surfaces and superficial antimicrobial peptides (Delima and Van Dyke, 2003). However, if the supra-gingival biofilm is allowed to mature, through poor oral hygiene for example, it can extend to sub-gingival surfaces (the gingival sulcus) (Darveau, 2010). The sub-gingival biofilm contains different types of microorganisms compared to the supra-gingival biofilm; with a shift towards mostly anaerobic, gram negative, motile and potentially pathogenic species (Zijinge et al., 2010). The high proliferative rate of gingival epithelial cells, and therefore the high turnover of the gingival epithelium, limits the extent of attachment, colonisation and invasion of microorganisms in the periodontium (Bartold and Narayanan, 2006). However, the lack of an innate physical barrier in the gingival sulcus leaves the epithelial cells and the underlying lamina propria exposed directly to microbes and their secreted products (Madianos et al., 2005).

The expression of PRRs (e.g. TLRs, NLRs) is widespread in periodontal cells including resident and recruited leukocytes (Madianos et al., 2005). TLR1 - TLR10 have been detected by IHC in human gingival tissue sections (Beklen et al., 2008). LPS-stimulated gingival epithelial cells upregulate the production of proinflammatory cytokines (e.g. IL-1β, IL-6), chemokines and MMPs (Kraus et al., 2012). LPS from the periodontitis pathogen Porphyromonas gingivalis increases the production and activity of the gelatinases MMP-2 and MMP-9 in a gingival fibroblast and U937 macrophage coculture model (Kuo et al., 2012). Interestingly, LPS from Escherichia coli and P. gingivalis alters the gene expression patterns of THP-1 monocytes in similar and unique ways (Barksby et al., 2009). Gingival fibroblasts also respond to TLR agonists (Section 1.11). Inflammatory responses in gingival cells are also driven through the recognition
of microbial metabolites (e.g. formylmethionine), or by the activation of protease-activated receptors by microbial proteases (Slots, 2005; Guo et al., 2010). Therefore, cells found in the gingiva recognise and respond to a range of microbial stimuli (although LPS has been most studied), and this interaction is likely to play a role in the initiation and propagation of microbial-derived inflammation in the gingiva.

The acute inflammatory and immune responses generated by the sub-gingival biofilm are usually successful in inhibiting the invasion of microorganisms into the periodontium; however, these host defence mechanisms are unable to clear the biofilm (Darveau, 2010). Some of the species within the biofilm produce molecules that modulate the ability of the host to kill microbes (Madianos et al., 2005). For example, poorly immunogenic polysaccharide capsules cover immunogenic epitopes, while bacterial-derived proteases degrade cytotoxic molecules such as complement (Delima and Van Dyke, 2003). Additionally, bacteria can regulate the activity of complement (Guo et al., 2010). The binding of P. gingivalis to complement receptor 3 drives the production of damaging proinflammatory cytokines and chemokines, while reducing the production of the immunoregulatory cytokine IL-12 (Hajishengallis, 2010). P. gingivalis also promotes the cleavage of complement component C5 into C5a and C5b. P. gingivalis further degrades C5b thereby inhibiting cell lysis via the membrane attack complex, while levels of C5a accumulate which may paralyse neutrophil and macrophage function (Popadiak et al., 2007). The large mass of the biofilm limits the extent to which it can be phagocytosed (Darveau, 2010). As such, neutrophils may be required to promote extracellular microbial killing, via the release of microbicidal molecules or neutrophil extracellular traps; these mechanisms of extracellular killing can further exacerbate the inflammatory response by promoting damage or death to periodontal cells and tissues (Vitkov et al., 2009; Scott and Krauss, 2012).

1.6 Periodontitis

The inability of the host to clear the sub-gingival biofilm drives a chronic inflammatory response, known as gingivitis (Darveau, 2010). Gingivitis is characterised by swollen and painful gums that may bleed under probing; these clinical signs are caused by the actions of inflammatory mediators and cells in the gingiva. Over 50 % of the UK population have gingival bleeding (White et al., 2011), but it is a reversible condition that can be ameliorated by the mechanical removal of the biofilm. However, if the sub-gingival biofilm is not removed, gingivitis can progress to an irreversible, destructive,
chronic inflammatory disease of the periodontium called periodontitis. (Chapple and Gilbert, 2002)

Periodontitis is characterised by the progressive destruction of gingival tissue and the underlying alveolar bone which results in reduced function, and eventually loss, of the teeth. The aetiological agent of periodontitis is the microbial biofilm that forms on periodontal surfaces. However, periodontitis is driven by the actions of tissue-degrading molecules and cells activated by the host immune and inflammatory responses. This destructive inflammatory state spreads throughout the periodontium, from the epithelia through the connective tissue, to the PDL and alveolar bone. (Darveau, 2010)

1.6.1 Clinical aspects of periodontitis

In the UK 45 % of dentate adults have evidence of current or historical periodontitis (pocket depths (PD) ≥ 4 mm), while 8 % have moderate to severe disease (PD ≥ 6 mm) (White et al., 2011). Currently 10 % of the UK population has periodontitis, and this prevalence is increasing (White et al., 2011). Interestingly, alveolar bone loss as observed in periodontitis is the most common osteopenia in humans (Liu et al., 2010). Periodontitis presents as either an aggressive or chronic, local or generalised disease in both adults and children (Chapple and Gilbert, 2002). Chronic periodontitis is the most common form of the disease, and is positively associated with increased age (White et al., 2011). Periodontitis has a complex pathogenesis and the severity of the disease depends upon microbial, immunologic, genetic and environmental factors that are not presently fully understood (Kornman, 2008).

The loss of alveolar bone, determined clinically by radiograph, is used to assist in the diagnosis of periodontitis; this resorption of the alveolar bone, and destruction of gingival ECM, causes recession of the gingiva (Chapple and Gilbert, 2002). Alveolar bone resorption coupled with the activation of epithelial cells, and the advancing plaque biofilm during periodontitis cause proliferation of the epithelium towards the root apex; this results in the loss of attachment of the junctional epithelium at the CEJ, destruction of the PDL, and potentially destabilisation of the tooth (Bartold and Narayanan, 2006). The loss of attachment and total depth of this periodontal ‘pocket’ are measured as part of the diagnostic periodontal examination (Chapple and Gilbert, 2002). At healthy sites, Chapple and Gilbert (2002) state that there should be no clinical attachment loss (CAL) and a PD of < 3 mm. However, these definitions vary widely across the global periodontal research population.
Although periodontitis is a disease that has evolved with humans over 1000s of years we are yet to develop an effective treatment for the inflammation (Newman et al., 2011). Current preventative strategies involve implementing good oral hygiene practise to minimise the build-up of the periodontal plaque biofilm (Chadwick et al., 2011). One routine treatment option is surgical debridement of the gingiva to reduce the depth of the periodontal pocket (flap surgery) and therefore, to reduce the area available for the sub-gingival biofilm to advance (Heitz-Mayfield et al., 2002). The gold standard treatment for periodontitis is the mechanical removal of the sub-gingival biofilm by root surface instrumentation (RSI) (Heasman et al., 2004). Only one host-modulating therapeutic has been licensed for use in Europe and the USA, namely sub-antimicrobial dose doxycycline (SDD) which has a potent MMP-inhibiting activity at doses that are not toxic to bacteria (Preshaw, 2008). Several clinical trials have shown that SDD improves the clinical outcome of periodontitis when used as an adjunct to RSI (Lee et al., 2004a; Lee et al., 2004b; Preshaw et al., 2004). While these treatments can stabilise active disease, the loss of alveolar bone in particular is irreversible and these treatments are time-consuming and expensive (Preshaw, 2008). Therefore, there is a need to better understand the pathogenesis of periodontitis to identify novel therapeutic targets, and to better understand which individuals are at risk of developing periodontitis.

1.6.2 The pathogenesis of periodontitis

Several bacterial species (P. gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythia, Treponema denticola, Fusobacterium nucleatum) are isolated more frequently from sites of periodontitis compared to health (Darveau, 2010). These species adhere to Sokransky’s criteria as microbial causes of periodontitis (Socransky, 1979). Pathogenic strains of these species, and/or those that utilise virulence factors, are more likely to evade host defences, to invade host cells and disrupt tissue integrity (Madianos et al., 2005). Several bacterial species, but most notably P. gingivalis, produce proteases, which target both pro and anti-inflammatory host mediators (Guo et al., 2010). Additionally, there is in vitro evidence that bacterial-derived proteases activate MMPs (DeCarlo et al., 1997), and may exacerbate host tissue destruction indirectly. Overall, this suggests that a change in the composition of the periodontal plaque biofilm plays a role in the pathogenesis of periodontitis.

Tissue destruction during the pathogenesis of periodontitis is currently attributed to host immune and inflammatory mechanisms (Darveau, 2010). Many studies have shown that the concentration and activity of proteases (particularly MMPs) increase in periodontitis.
(Section 1.9). However, it is not fully understood how these proteolytic enzymes, which are normally tightly controlled even in acute inflammation, are dysregulated in periodontitis (Section 1.10). Osteoclasts are the only known cell with the ability to demineralise bone, and are therefore implicated in the pathogenesis of periodontitis; osteoclast development and activation are promoted by receptor activator of NF-κB ligand (RANKL), macrophage-CSF and several cytokines (Liu et al., 2010). Elevated levels of RANKL (and an elevated RANKL/osteoprotegerin (OPG) ratio) have been identified in the GCF and saliva of patients with periodontitis (Taubman et al., 2007). Additionally, the inhibition of RANKL by OPG results in reduced alveolar bone loss in the ligature-induced periodontitis model in rats (Jin et al., 2007), which is in support of increased osteoclast activity during periodontitis.

Numerous clinical studies (mostly cross-sectional) have identified associations between periodontitis and potential risk factors; these risks may be due to genetic, environmental, or behavioural factors; or due to the presence of concurrent systemic disorders (Kornman, 2008). For example, attempts have been made to identify associations between periodontitis and polymorphisms in cytokine (Taylor et al., 2004) and MMP genes (Loo et al., 2011). Overall, these genetic association studies present conflicting results and suggest that the impact of individual genetic polymorphisms on the risk of periodontitis are only small and relevant to particular populations. Similarly, there is some evidence that the presence of chronic viral infections in the periodontium may determine the risk of developing periodontitis (Slots, 2005). There is strong evidence that smoking is a behaviour that increases an individual’s risk of developing periodontitis, and reduces the chance of effective therapy (Kornman, 2008). Cardiovascular disease, T2DM and obesity/overweight have all been positively associated with periodontitis (Section 1.7). Prospective clinical studies that could determine whether these risk factors are causal in the pathogenesis of periodontitis are lacking.

The pathogenesis of periodontitis is complex. The risk of developing periodontitis and the severity of disease varies between individuals, between sites within the same individual, and over time. However, the complex nature of the inflammatory and immune responses in humans means that the mechanisms underlying many of these clinical associations have not been fully explored.
1.7 Association of obesity and T2DM with periodontitis

Obesity and T2DM are associated with a systemic low-grade, chronic inflammatory state (Hotamisligil, 2006). As periodontitis is a chronic inflammatory disease (Darveau, 2010), it has been hypothesised that T2DM and obesity affect the pathogenesis of periodontitis (Preshaw et al., 2012).

1.7.1 T2DM and periodontitis

Many clinical studies show that T2DM is positively associated with periodontitis (Preshaw et al., 2012). However, in a small study by Davies et al. (2011), no significant differences were observed in a range of serum biomarkers relevant to diabetes (and obesity) in individuals with aggressive periodontitis compared to healthy controls, suggesting that the association between periodontitis and diabetes may depend on study design and the outcomes investigated. No strong associations have been found linking T2DM with changes in the plaque microbiota; instead, it is thought that elevated levels of systemic cytokines in T2DM promote periodontitis (Taylor et al., 2013). Also implicated, are advanced glycation end-products (AGEs), which accumulate in the gingiva of periodontitis patients with poorly controlled T2DM (Zizzi et al., 2012). The amount of AGEs found in the gingiva is associated with the length of time since the onset of T2DM, suggesting the duration of T2DM may determine the risk of developing periodontitis. AGEs reduce collagen production and increase MMP production by gingival fibroblasts, suggesting that AGEs may promote gingival tissue degradation in individuals with T2DM and supporting a mechanistic role for fibroblasts in the association between T2DM and periodontitis (Ren et al., 2009; Yu et al., 2012). T2DM is associated with various bone diseases, and increased alveolar bone loss is observed in the diabetic (leptin receptor mutant) Zucker rat strain (Liu et al., 2006). Interestingly, there is some evidence to suggest that treating periodontitis improves T2DM, possibly by reducing systemic levels of pro-inflammatory cytokines, and indicates a bidirectional relationship exists between these diseases (Taylor et al., 2013). Although a positive clinical association between T2DM and periodontitis has been well demonstrated, the temporal and mechanistic relationships between these two diseases are not yet understood.

1.7.2 Obesity and periodontitis

A relationship between obesity and periodontitis has been hypothesised since at least the 1970s when it was shown that the pathology of periodontitis was more severe in the Zucker (leptin receptor mutant) rat strain (Perlstein and Bissada, 1977). Since then
many clinical studies have investigated the link between obesity/overweight and periodontitis (Pischon et al., 2007). A recent systematic review and meta-analysis of the available literature on obesity/overweight and periodontitis has been performed (Suvan et al., 2011). Suvan et al. (2011) found that most of the clinical studies used a cross-sectional design with only a few case/control or cohort approaches so the evidence available was of fairly low quality. Overall, this meta-analysis found that overweight/obese individuals have an odds ratio (OR) of 2.1 for having periodontitis (Suvan et al., 2011). Additionally, Suvan et al. (2011) found that periodontitis was still significantly positively associated with overweight (OR 1.3) and obese (OR 1.8) categories individually; it was significant that the OR for obesity was higher than that of overweight suggesting that the risk of periodontitis increases as weight increases. Since this meta-analysis Suvan et al. (2014) have published their own clinical study investigating how BMI affects the outcome of treatment for chronic periodontitis. They found that obese individuals have more severe periodontitis (as measured by PD, plaque index and bleeding index) than non-obese controls both before and after treatment. Additionally, the treatment outcomes were poorer in obese individuals compared to controls suggesting that obesity affects the success of treatment for periodontitis (Suvan et al., 2014). Interestingly, they found that the poorer response to treatment in the obese group was comparable to that attributed to smoking, one of the risk factors most strongly linked to periodontitis (Kornman, 2008). Another recent study compared the outcomes of treatment for chronic periodontitis between obese and non-obese study groups (Altay et al., 2013). Altay et al. (2013) found that while treatment improved periodontitis and reduced systemic levels of IL-6 in both groups, these changes were not significant between the obese and non-obese groups. However, they found that systemic levels of TNF-α, leptin and insulin resistance were reduced only in the obese group. It would have been interesting to see how the measures of periodontitis and obesity compared to controls (non-obese and obese) with no periodontitis; unfortunately these groups were not investigated in this study.

Clinical interventional studies investigating any effects obesity has on periodontitis may be difficult to manage, given the complex nature of obesity and the difficulty in controlling food intake across a study population. Therefore, in vivo models have been utilised instead (Pischon et al., 2007). Using the ligature-induced periodontitis model in rhesus macaques, Branch-Mays et al. (2008) found that the severity and progression of periodontitis (as measured by gingival index, bleeding on probing (BOP), PD and CAL)
is reduced in animals that have a 30% calorie-restricted diet compared to normal diet control. This study suggests that nutritional intake determines the risk of developing periodontitis, although whether this is translatable to humans is unknown. Additionally, it would have been interesting to see if the severity and progression of periodontitis was increased in animals that were overfed. This approach was taken, to some extent, by Bendyk et al. (2009) in the bacteria-induced periodontitis model in mice. In this study mice were fed either an ω3-polyunsaturated fatty acid (PUFA) oil-rich diet or a control oil-rich diet. The animals that received the ω3-PUFA diet had reduced alveolar bone loss compared to the control oil-rich diet (Bendyk et al., 2009). This could have been due to the increased synthesis of anti-inflammatory lipid mediators such as lipoxins, which reduce bone loss in periodontitis (Serhan et al., 2003). However, this study lacked a normal chow diet-fed control which would have been a useful comparison.

T2DM and obesity are positively associated with periodontitis (Pischon et al., 2007; Taylor et al., 2013). However, the results to date show some variation. This is probably due to the complicated natures of T2DM, obesity and periodontitis, the various ways to measure the severity of these conditions, and the variety of questions addressed in the studies. The mainly cross-sectional design of the clinical studies means there is a lack of information on the temporal relationship between obesity, T2DM and periodontitis. While most of the studies have adjusted for some potentially confounding factors, such as age, gender, oral hygiene status and smoking, it is possible that other masked confounding factors may be behind these associations. Questions that are still currently unanswered are do obesity or T2DM promote periodontitis or vice versa; and if one condition does exacerbate the others are disease initiation, severity, progression and/or outcome affected? The identification of molecules that may be mechanistically relevant to these clinical associations, such as adipokines, may help to address these questions.

1.8 Association of leptin with periodontitis
Circulating levels of the adipokine leptin are elevated in obesity and T2DM (Section 1.3), and these conditions are positively associated with periodontitis (Section 1.7). Leptin functions as a cytokine and can promote inflammation and ECM remodelling (Section 1.4), which are functions relevant to the pathogenesis of periodontitis. In this thesis it is hypothesised that leptin could be a molecular link mechanistically underlying the associations between obesity, T2DM and periodontitis.
### 1.8.1 Clinical associations between leptin and periodontitis

Several clinical studies have investigated whether leptin concentrations correlate with periodontitis disease severity (Johnson and Serio, 2001; Bozkurt et al., 2006; Karthikeyan and Pradeep, 2007a; Karthikeyan and Pradeep, 2007b; Gangadhar et al., 2011; Ramesh et al., 2011; Yetkin Ay et al., 2011; Gundala et al., 2012). Study approaches were generally cross-sectional and assessed leptin concentrations in serum, GCF or gingival tissue homogenates from individuals that had varying severities of periodontitis. Most of these studies report that leptin concentrations are lower in GCF and gingival tissue, but higher in serum as the severity of periodontitis progresses (Johnson and Serio, 2001; Karthikeyan and Pradeep, 2007a; Karthikeyan and Pradeep, 2007b; Gangadhar et al., 2011). Unfortunately, most of these studies are of a poor quality. For example, none of these studies presented gender or BMI data for study groups (or where presented these demographics were significantly different between groups) (Johnson and Serio, 2001; Bozkurt et al., 2006; Karthikeyan and Pradeep, 2007a; Karthikeyan and Pradeep, 2007b; Gangadhar et al., 2011; Ramesh et al., 2011; Yetkin Ay et al., 2011; Gundala et al., 2012). Both gender and BMI are highly positively associated with leptin concentration, and therefore, the observed changes may be due to these factors and not periodontitis (Maffei et al., 1995; Considine et al., 1996). Other studies fail to comprehensively describe the statistical approach used for making comparisons between groups (Bozkurt et al., 2006; Karthikeyan and Pradeep, 2007a; Ramesh et al., 2011; Gundala et al., 2012). In yet other studies, the measurement of leptin from very small volumes of GCF, albeit using sensitive antibody-based techniques, seems implausible (Bozkurt et al., 2006; Karthikeyan and Pradeep, 2007b).

In a study where the results were adjusted for gender and BMI no difference was identified in the serum leptin concentration between individuals who had aggressive periodontitis compared to healthy controls (Davies et al., 2011). However, Davies et al. (2011) found that serum leptin concentrations were significantly higher in women compared to men. Similarly, a well-designed study by Teles et al. (2009) found no difference in serum leptin concentrations across different periodontal PDs. In the individual study groups no significant differences in gender and BMI were found, but across the whole study population leptin concentrations correlated highly with gender and BMI (Teles et al., 2009). In a study by Shimada et al. (2010), serum leptin concentrations were positively associated with chronic periodontitis (as measured by PD, CAL and alveolar bone loss) compared with controls with similar BMI and gender.
Additionally, after non-surgical treatment for periodontitis serum leptin concentrations were reduced, but were still significantly higher than healthy controls (Shimada et al., 2010). Another study investigated leptin concentrations in four different groups (chronic periodontitis, chronic periodontitis and obese, obese, healthy) (Zimmermann et al., 2013). They found increased serum levels of leptin in the chronic periodontitis and/or obesity groups compared to healthy controls. Overall, the poor quality of many studies investigating leptin and periodontitis and the conflicting results of the better quality studies, mean that an association between leptin and periodontitis (let alone a mechanistic link) has yet to be established.

1.8.2 Leptin in the periodontium

Several studies have used IHC to show that leptin and the leptin receptor are present in gingival tissues from humans (Ay et al., 2012), rats (Ide et al., 2011) and macaques (Li et al., 2014). Additionally, both leptin and the leptin receptor are present in human gingival tissue from healthy, gingivitis and periodontitis sites (Ay et al., 2012). Leptin has also been detected by enzyme-linked immunosorbent assay (ELISA) in gingival papillae, and the amount of tissue leptin decreased as the severity of periodontitis (characterised by PD and BOP) increased (Johnson and Serio, 2001). However, gender and BMI data were not presented by study group, which limits the conclusions that can be drawn from this study.

Leptin receptor is widely expressed in the gingiva, while leptin expression seems more limited to epithelial and endothelial cells (Li et al., 2014). In vitro studies have shown that THP-1-derived macrophages and human gingival fibroblasts (hGFs) express the long isoform of the leptin receptor (Kim, 2010; Park et al., 2013). These studies suggest that leptin is found in the periodontium and that resident cells respond to leptin. One question that remains is whether the source of periodontal leptin is local or systemic? It is likely that serum leptin leaches from the vasculature into the periodontal tissues, particularly during inflammation. Ectopic adipose tissue in close proximity to the periodontium (e.g. the bone marrow of the maxilla or mandible), or adipocytes and other leptin-producing cells in the periodontium could be local sources of leptin (Mantzoros et al., 2011).

Leptin treatment of stem cells isolated from the PDL results in decreased expression of the leptin receptor, increased alkaline phosphatase activity and a reduced potential for adipogenesis (Um et al., 2011). This suggests that leptin regulates periodontal cell
function. Interestingly, leptin synergises with LPS from the putative periodontal pathogen *Prevotella intermedia* to increase TNF-α secretion in THP-1-derived macrophages, while leptin alone increases CXCL8 production (Kim, 2010). This suggests that leptin may enhance inflammatory responses in the periodontium. There is little other information known regarding the possible functions of leptin in the periodontium, or in the pathogenesis of periodontitis (except for one study in hGFS (Section 1.11.6)).

### 1.9 The role of MMPs in periodontitis

MMPs are a family of zinc-dependent endopeptidases that together can degrade all components of the demineralised ECM (Nagase *et al.*, 2006). Therefore, MMPs are implicated in the pathogenesis of diseases where deleterious tissue remodelling occurs, including arthritis (Burrage *et al.*, 2006), and periodontitis (Birkedal-Hansen, 1993). The identification of MMP functions *in vivo* has been hampered by evidence (e.g. using MMP knockout mouse models) that there is functional redundancy and compensation between MMPs. Functional redundancy means that multiple MMPs can perform the same function. For example, knockout of single MMPs in mice results in normal, or eventually normal, development; however, development in double MMP knockouts is more severely affected and can be lethal (Oh *et al.*, 2004; Stickens *et al.*, 2004). Similarly, in single MMP knockout mice but not wild-type controls, the expression of other MMPs increases after myocardial infarction or during uterine involution postpartum (Rudolph-Owen *et al.*, 1997; Ducharme *et al.*, 2000), which supports functional compensation between MMPs. Interestingly, several studies have now reported non-redundant functions of MMPs in models of inflammation (Page-McCaw *et al.*, 2007).

#### 1.9.1 MMP structure and function

MMPs are grouped by substrate specificity and were originally named likewise. However, after the discovery of further members of the MMP family through sequence and structural bioinformatics searches of the human genome, and the discovery that MMPs have overlapping substrate specificities, MMPs were renamed numerically (Table 1.1). Mammalian MMPs are highly homologous, share the same basic structure and all contain a propeptide region and catalytic domain. The catalytic domain contains the active site in which a Zn$^{2+}$ ion is bound. The active site is well conserved between MMPs, and this similarity could be the reason why MMP inhibitors that targeted the active site caused wide-spread adverse events in clinical trials (Peterson, 2006; Murphy
and Nagase, 2008). The catalytic site of the gelatinases contains fibronectin type II repeats, which facilitate binding to collagen. The propeptide region regulates MMP activity; a highly conserved cysteine residue in the propeptide region interacts with the Zn$^{2+}$ ion in the active site, subsequently preventing MMP activity. The proteolytic cleavage of the prodomain disrupts the interaction between the conserved cysteine and Zn$^{2+}$ ion, promoting MMP activity. This activatory step is known as the ‘cysteine switch’. The initial proteolytic cleavage of the prodomain requires exogenous protease activity; subsequent activatory cleavage steps can occur auto-lytically. Interestingly, the interaction between the conserved cysteine and Zn$^{2+}$ ion can also be destabilised chemically, by reactive oxygen species (ROS) for example (Hannas et al., 2007). ROS-mediated MMP activation may be relevant to MMP activity in periodontitis as levels of lipid peroxidation (a marker of ROS) are elevated in the circulation of individuals with periodontitis compared to controls (Dahiya et al., 2013). It is currently thought that the haemopexin or regulatory domain, which is linked to the catalytic domain by a hinge region and is found in all mammalian MMPs except the matrilysins, determines MMP substrate specificity by facilitating protein-protein interactions. (Nagase et al., 2006; Page-McCaw et al., 2007)

MMPs are grouped into six main categories: collagenases, gelatinases, stromelysins, membrane-type MMPs, matrilysins and others (Table 1.1). Collagenolytic MMPs (MMP-1, MMP-8, and MMP-13) are the only known enzymes that can initiate the degradation of fibrillar collagens, resulting in the characteristic 1/4 and 3/4 collagen fragments. As a number of other MMPs further degrade collagen fragments, the initial cleavage step is thought to be critical in the regulation of collagen degradation. As fibrillar collagens are the predominant ECM component in the periodontal connective tissues, collagenase activity is implicated in periodontitis (Hannas et al., 2007). The gelatinases (MMP-2, MMP-9) have broad substrate specificity but are characterised for their degradation of partially hydrolysed fibrillar collagen (gelatin) and type IV collagen (which is found in periodontal basement membranes). The stromelysins (MMP-3, MMP-10, and MMP-11) and matrilysins (MMP-7, MMP-26) have a broad range of substrate specificities, similar to the gelatinases. The membrane-type MMPs (MMP-14 - MMP-17, MMP-24, and MMP-25) also have broad substrate specificity but differ to other MMPs by remaining in association with the cell membrane instead of being secreted. Four of the membrane-type MMPs (MMP-14 - MMP-16, MMP-24) are
transmembrane proteins, while MMP-17 and MMP-25 are associated with the membrane by a glycosylphosphatidylinositol-anchor. (Nagase et al., 2006)

1.9.2 MMPs are associated with periodontitis
Numerous studies have detected MMPs in the oral cavity and MMPs are usually found to be elevated in periodontitis compared to healthy controls (Hannas et al., 2007). MMPs can be detected in saliva; these MMPs are generally derived from GCF, and therefore from the gingival tissue (Makela et al., 1994). Several studies have found an increase in GCF MMP-8 and MMP-13 in periodontitis samples compared to controls (Kiili et al., 2002; Lee et al., 2004a; Pozo et al., 2005). Using IHC, Kiili et al. (2002) found that MMP-8 is predominantly associated with neutrophils, epithelial cells and plasma cells in gingival tissue, and GCF MMP-8 is presumed to be neutrophil-derived. This correlates with the extra-gingival location of neutrophils during periodontitis (Delima and Van Dyke, 2003). However, a less glycosylated form of MMP-8 is secreted by gingival tissue resident cells (Kiili et al., 2002), and the levels of tissue-MMP-8 may not have been taken into account in studies using ELISA instead of electrophoresis-based detection techniques. Routine mechanical treatment for periodontitis reduces the concentration of active MMP-8 in the GCF (Kiili et al., 2002; Pozo et al., 2005). Additionally, studies showing elevated GCF MMP-8 have taken samples from individuals with chronic periodontitis (Kiili et al., 2002; Lee et al., 2004a), while in juvenile periodontitis there is evidence that MMP-1 predominates in the GCF (Uitto et al., 2003). In a study by Soell et al. (2002) the proportions of inactive and active MMPs (MMP-1/MMP-2/MMP-3/MMP-9) were the same in GCF samples from periodontitis compared to health, suggesting that the absolute levels of MMP may be increased in periodontitis instead of the conversion rate of inactive to active MMP. Overall, these studies have led to the trial of GCF/salivary MMPs (and MMP-8 in particular) as diagnostic and prognostic biomarkers for periodontitis (Sorsa et al., 2006). However, there are limitations with measuring MMPs in GCF. For example, the small volumes of GCF acquired per site make measuring quantitative differences in MMP levels difficult. Additionally, levels of GCF MMPs vary between sites and between individuals.

IHC analyses have shown that MMP-2, but only low levels of other MMPs, are found throughout the healthy gingiva (Birkedal-Hansen, 1993; Seguier et al., 2001; Kiili et al., 2002; Dong et al., 2009). Gingival tissue levels of MMP-1, MMP-2, MMP-3, MMP-8 and MMP-13 are elevated in periodontitis (Seguier et al., 2001; Dong et al., 2009).
MMP-8 and MMP-9 localise to neutrophils and epithelial cells, while MMP-1 localises to the epithelium and the sites of leukocyte infiltration (where both leukocytes and fibroblasts stain positively) (Dong et al., 2009). While these studies do support the increased presence of MMPs in periodontitis, it is unclear whether gingival or GCF MMPs are mechanistically relevant to disease activity or outcome. Additionally, the measurement of MMPs by IHC is fairly subjective, and therefore, differences in MMP levels and their cellular sources throughout the gingiva have been reported between studies.

1.9.3 MMPs in the pathogenesis of periodontitis

The results of studies investigating the use of SDD as an adjunct therapy for periodontitis directly supports a functional role for MMPs in the pathogenesis of periodontitis (Preshaw, 2008). Doxycycline is a member of the tetracycline family of antibiotics, and members of this family have the ability to inhibit MMP activity (Preshaw et al., 2004). This function is attributed to their ability to chelate zinc or to suppress ROS activity (Preshaw, 2008). Doxycycline was chosen as it inhibits MMP-8 more selectively than other MMPs, including MMP-1 (Preshaw et al., 2004), and was therefore thought to be more relevant to periodontitis given the increased levels of MMP-8 observed in GCF (Kiili et al., 2002; Pozo et al., 2005). The addition of SDD to gold standard RSI treatment regimens for periodontitis results in a significantly larger reduction in PD, CAL and GCF MMP-8 and MMP-13 levels compared to gold standard treatment alone (Lee et al., 2004a; Lee et al., 2004b; Preshaw et al., 2004). These studies suggest that MMPs (and in particular MMP-8) play a deleterious role in the pathogenesis of periodontitis.

There is increasing evidence to suggest that the degradation of ECM components by MMPs during inflammation is a key regulatory part of this physiological process. For example, cleavage of ECM components may assist in path clearing through tissues and epithelial migration (by disrupting intercellular adhesions and basement membranes). Similarly, fragments of ECM components and molecules bound to ECM components can be released by MMPs, and act as signalling molecules (Xu et al., 2001). MMPs can proteolytically cleave inflammatory mediators, an action that depending on the substrate can drive molecular activation or degradation. Additionally, it is thought that controlled MMP activity may be important in scar-less wound healing, and may protect against bone destruction (Bjornsson et al., 2004). (Page-McCaw et al., 2007)
The actions of MMPs in inflammation are more complex than originally thought, and are unlikely to be entirely deleterious. Further investigations into the mechanisms that regulate excessive MMP production and activity in periodontitis and their functional relevance are required to improve our understanding of the role of these powerful enzymes in periodontitis, and how we might best target them to modulate the pathogenesis of periodontitis.

1.10 Mechanisms of MMP regulation during inflammation
MMPs are regulated at multiple levels (Page-McCaw et al., 2007; Murphy and Nagase, 2008). Regulatory mechanisms exist at the level of the input stimuli and cell signalling, transcription, translation and secretion, activation, localisation and substrate specificity, inhibition and clearance (Page-McCaw et al., 2007; Murphy and Nagase, 2008). Disruption of any of these regulatory mechanisms could alter MMP-mediated tissue remodelling, and therefore many studies have investigated the regulation of MMPs in destructive chronic inflammatory diseases, such as periodontitis.

1.10.1 Stimuli that regulate MMP expression
Basal expression levels of most MMPs are low, but are greatly enhanced by particular stimuli (Murphy and Nagase, 2008). The lipid mediator prostaglandin (PG)E_2 (Ruwanpura et al., 2004), LPS (Bodet et al., 2007), immune cell microparticles (vesicles that are increasingly produced by immune cells during activation and apoptosis) (Distler et al., 2005), ECM fragments (Fichter et al., 2006) and pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, oncostatin M (OSM), IL-17) (Catterall et al., 2001; Morgan et al., 2006; Cortez et al., 2007; Gosset et al., 2010) all increase the production of MMPs in *in vitro* cell-based studies. For example, expression levels of MMP-3 by human synovial fibroblasts can be increased to 50-fold that of basal by IL-1 (MacNaul et al., 1990; Birkedal-Hansen, 1993). Interestingly, *P. gingivalis* increases the production of gelatinases in an engineered human oral mucosa (EHOM) (Andrian et al., 2004). Andrian et al. (2004) found that this activity was reduced in strains with mutations in proteases (called gingipains), suggesting that the production of gelatinases in this model required the presence of gingipains. Additionally, purified arginine gingipains increase MMP-1 production, although it is possible this was due to LPS contamination (Matsushita et al., 2006). In contrast, other mediators such as transforming growth factor (TGF)-β and IFN-γ, suppress the production of MMPs (Birkedal-Hansen, 1993). The adipokine adiponectin, which is thought to have immunoregulatory potential, also regulates MMP production in synovial fibroblasts.
(Kim et al., 2010b) and human oral epithelial cells (Kraus et al., 2012), although both increases and decreases have been described, and may be due to the different cell types studied. Trombone et al. (2009) found that periodontal levels of MMP-2 and MMP-13 (and osteoclastogenic mediators) are increased in mice that are prone to a strong acute inflammatory reaction (AIR) compared to controls in the A. actinomycetemcomican-induced periodontitis model. In contrast, no differences in the management of the bacterial load between AIR and control mice were detected (Trombone et al., 2009). This suggests that the regulation of ECM remodelling in periodontitis depends on the strength of the inflammatory response, and is regulated differently to the mechanisms that control infection. Overall, these studies illustrate that host and microbial-derived stimuli can increase the production of MMPs by cells found in the periodontium during inflammation.

1.10.2 Intracellular signalling pathways and transcription factors regulate MMP expression

The expression of most MMPs requires the transduction of extracellular stimuli via intracellular signalling pathways (Murphy and Nagase, 2008). The upregulation of MMP gene expression requires the immediate early synthesis of transcription factors (such as c-Fos and c-Jun which are activated by MAPK activity), which then bind to MMP promoters to enhance gene expression (Sorsa et al., 2006). As such, increases in MMP production can take several hours to detect, but can remain upregulated for days (Birkedal-Hansen, 1993). This is in contrast to the expression of many inflammatory mediators which are induced within minutes and switched off again within hours (Barksby et al., 2009). Binding sites for the transcription factor activator protein-1 (AP-1) (which is composed of c-Fos and c-Jun) are found in the promoters of many MMPs (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13); STAT and NF-κB binding sites are also found in many MMP promoters (Vincenti and Brinckerhoff, 2002). A combination of multiple transcription factors and enhancers on an MMP promoter helps to drive maximal gene expression (Cortez et al., 2007). Indeed, combinations of stimuli synergistically increase the production and activity of MMPs by chondrocytes in cartilage (Catterall et al., 2001; Koshy et al., 2002; Morgan et al., 2006), and human synovial fibroblasts (MacNaul et al., 1990). Other combinations have an antagonistic relationship (Sukkar et al., 2007). During inflammation it is likely that multiple signals are integrated via intracellular signalling pathways by resident and recruited cells to regulate MMP production in the connective tissue microenvironment.
A polymorphism in the promoter region of MMP-1 is associated with both aggressive and chronic periodontitis (de Souza et al., 2003; Cao et al., 2005). Bioinformatics analysis identified that this polymorphism introduces an additional binding site for the transcriptional enhancer Ets and may therefore promote MMP-1 expression (Sorsa et al., 2006). Similar polymorphisms in MMP-3 and MMP-9 genes have been identified but they are less strongly associated with periodontitis (Loo et al., 2011). Individuals that have these polymorphisms could be more likely to progress from gingivitis to periodontitis due to the potential for increased MMP expression.

1.10.3 Regulated secretion of MMPs

MMP secretion can be constitutive or regulated (Page-McCaw et al., 2007). Of relevance to periodontitis, neutrophil MMP-8 is expressed and translated prior to cell activation and stored in intracellular granules; upon activation, MMP-8 containing granules are secreted extracellularly (Birkedal-Hansen, 1993). This makes sense as the innate immune response is required to activate within minutes; something that de novo expression of MMP-8 would hamper. Another level of MMP regulation relevant to neutrophils is that the cells have a short half-life (Scott and Krauss, 2012). Therefore, continued secretion of neutrophil-derived MMPs requires the infiltration of new neutrophils which will depend on local chemotactic gradients (Van Dyke and Serhan, 2003). Inflammatory stimuli may enhance MMP expression and secretion suggesting that MMPs drive inflammatory tissue remodelling, however the mere presence of MMPs does not result in proteolysis until the enzymes are activated (Sorsa et al., 2006).

1.10.4 Activation of pro-MMPs

MMPs are synthesised as zymogens and require proteolytic cleavage of the prodomain to become active (Nagase et al., 2006). Numerous host proteases activate MMPs, including MMPs themselves (Murphy and Nagase, 2008). For example, membrane bound MMP-14 activates MMP-2, which might help to localise MMP-2 activity in the pericellular region (Hannas et al., 2007). Another MMP-activating protease, extracellular MMP inducer (EMPRIN) has been identified in the gingiva and may be produced by PDL cells, but whether levels of EMPRIN increase during periodontitis is not clear (Dong et al., 2009; Xiang et al., 2009). Perhaps the levels of MMP activators are less important than the concentrations of secreted MMP, although clearly once some MMP activity is established, there is the potential for a cascade of MMP-induced MMP activity. Interestingly, a P. gingivalis proteinase (presumably a gingipain) activates MMPs (DeCarlo et al., 1997). DeCarlo et al. (1997) found that the in vitro incubation of
this *P. gingivalis* proteinase with MMP-3 and MMP-1 resulted in the production of superactive MMP-1 in only 1 minute; in contrast, MMP-3 activated MMP-1 but no superactive form was identified. Additionally, the *P. gingivalis* proteinase cleaved at a location distinct to the usual activatory or auto-catalytic sites (DeCarlo et al., 1997). The extent to which this *in vitro* finding translates to MMP activation in the gingiva is unknown. However, it is possible that enhanced MMP activation by microbial-derived proteinases during periodontal inflammation plays a critical role in the tissue destruction observed in periodontitis.

### 1.10.5 Inhibitors of MMPs

Four TIMPs have been described in humans, and these MMP inhibitors non-covalently bind to both inactive and active forms of MMPs in a 1:1 ratio (Nagase et al., 2006; Hannas et al., 2007). All four TIMPs have been identified in periodontal tissues (Soell et al., 2002; Pozo et al., 2005; Andrian et al., 2007; Nakasone et al., 2009). TIMP-1 and TIMP-2 levels show some variation between periodontitis and health; however, the relationship between TIMP levels and periodontitis is unclear (Soell et al., 2002; Pozo et al., 2005). One of the key determinants for ECM degradation by MMPs is the ratio of MMPs to TIMPs or other inhibitors (Murphy and Nagase, 2008). Overall, current observations of MMPs and TIMPs in periodontitis indicate that the levels of MMP are enhanced while the levels of TIMPs stay fairly stable (Soell et al., 2002; Pozo et al., 2005; Andrian et al., 2007), suggesting the MMP:TIMP ratio is increased in periodontitis and that MMPs play a role in periodontal tissue destruction. The protein α2-macroglobulin (α2M) is found in body fluids, including GCF and saliva, and covalently binds and inhibits MMPs (Birkedal-Hansen, 1993). Other MMP inhibitors have been described (Murphy and Nagase, 2008), and it is likely more will be identified in the future. MMP activity may also be controlled by the sequestration of MMPs in areas of ECM that do not contain their preferred substrates and by MMP degradation (Page-McCaw et al., 2007).

Almost each level of MMP regulation is susceptible to alteration by molecules found in the inflamed periodontium. Increased concentrations and activities of MMPs are found in the periodontium during periodontitis (Hannas et al., 2007), and MMPs are part of a select group of enzymes that can degrade demineralised periodontal ECMs (Nagase et al., 2006). This suggests that excessive MMP activity is involved in the pathogenesis of periodontitis however, little direct evidence in support of this hypothesis is available. The inflammatory mechanisms of gingivitis and periodontitis are essentially the same.
Therefore the stimuli that promote MMP production are also present during non-destructive inflammation of the periodontium. What are the mechanistic cues that promote excessive MMP activity in periodontitis? Could a stronger inflammatory response be initiated in the periodontium of particular populations which enhances MMP production and activity? Leptin enhances MMP production, alone and in combination with inflammatory mediators (Section 1.4). Leptin is found in the periodontium, and both resident and recruited cells express the leptin receptor (Section 1.8). Circulating levels of leptin are elevated in obesity and T2DM (Section 1.3), and both of these conditions are positively associated with periodontitis (Section 1.7). In this thesis I hypothesise that leptin acts as a molecular link between obesity, T2DM and periodontitis by regulating the production of MMPs.

1.11 The role of gingival fibroblasts in inflammation and immunity

Cells with fibroblast morphology are the most numerous cell type in gingival connective tissue (Section 1.5) (Wikesjö and Selvig, 1999; Bartold and Narayanan, 2006). Gingival fibroblasts are mesenchymally-derived cells characterised by their ability to synthesise and remodel ECM components, their spindle-shaped morphology in vitro, and their lack of a consistent cell surface marker (McCulloch and Bordin, 1991; Lekic et al., 1997; Kalluri and Zeisberg, 2006; Pilling et al., 2009). Gingival fibroblasts recognise inflammatory changes in the gingival microenvironment, such as the presence of bacterial or white blood cells, or damage to the gingival ECM (Bartold et al., 1992; Scragg et al., 1999; Weinberg et al., 2009). These cells, which are positioned in the centre of the complex gingival microenvironment, respond to inflammatory cues by regulating tissue remodelling, inflammatory and immune responses (Bartold and Narayanan, 2006). Due to these functions, and their predominance in gingival connective tissue, fibroblasts have been implicated in the pathogenesis of periodontitis.

Most studies that have investigated the functions of gingival fibroblasts have performed in vitro experiments on primary hGFs isolated in house, although primary hGFs are also available commercially (ATTC). The gingiva is a fairly accessible tissue, and several minimally invasive surgical procedures are routinely employed to acquire gingival or oral mucosal tissue for fibroblast isolation. Out of the studies that do report the procedure used to acquire gingival tissue, the most common source is waste tissue derived from tooth (particularly third molar) extractions. Gingival tissue is also acquired from tooth exposure surgery and gingivectomy procedures. One surgical approach in
the management of periodontitis is flap surgery. Gingival tissue acquired by flap surgery is also used to study fibroblasts derived from a site of periodontitis.

1.11.1 Regulation of ECM remodelling

Gingival fibroblasts are implicated in tissue remodelling during inflammation as they can synthesise ECM. The expression of ECM components by gingival fibroblasts, including type I and type III collagens and fibronectin, are regulated by growth factors and cytokines (Murakami et al., 2001; Leivonen et al., 2002; Palmqvist et al., 2008; Ren et al., 2009; Sume et al., 2010; Arancibia et al., 2012). For example, gingival fibroblasts stimulated with TGF-β or connective tissue growth factor increase collagen deposition in an integrin α6β1-dependent manner (Heng et al., 2006). Interestingly, older fibroblasts produce less collagen (Varani, 2010) which could be mechanistically relevant to the increased risk of periodontitis with age (Huttner et al., 2009).

Gingival fibroblasts may be able to remodel tissue by degrading ECM components during inflammation. During homeostasis, fibroblasts in the periodontium are thought to remodel ECM components (in particular type I collagen) by a highly controlled, pericellular and phagocytic pathway (Beertsen et al., 1997). In gingival fibroblasts, this intracellular collagen-degrading activity is increased after exposure to LPS in a TLR4-dependent manner (Bhide et al., 2005; Takahashi et al., 2008), and when compromised, is implicated in the development of gingival fibrosis (McKleroy et al., 2013). These studies suggest that homeostatic collagen turnover by gingival fibroblasts is modulated by inflammatory mediators. Interestingly, proinflammatory cytokines, bacterial TLR agonists, growth factors and PGE2 regulate the production and secretion of ECM-degrading enzymes by gingival fibroblasts (Tervahartiala et al., 2000; Yamada et al., 2000; Dahan et al., 2001; Domeij et al., 2002; Leivonen et al., 2002; Ruwanpura et al., 2004; Beklen et al., 2006; Cox et al., 2006; Zhou and Windsor, 2006; Bodet et al., 2007; Cury et al., 2007; Zhou et al., 2007; Smith et al., 2009; Zhang et al., 2011; Ujii et al., 2012). The increased production of collagenolytic enzymes (MMP-1/MMP-8/MMP-13) by stimulated gingival fibroblasts has been extensively studied (Tervahartiala et al., 2000; Yamada et al., 2000; Domeij et al., 2002; Leivonen et al., 2002; Beklen et al., 2006; Cox et al., 2006; Zhou and Windsor, 2006; Cury et al., 2007; Zhou et al., 2007). In several studies this increase in collagenase production correlates to an enhanced ability of stimulated gingival fibroblasts to degrade type I collagen gels (Yamada et al., 2000; Domeij et al., 2002; Leivonen et al., 2002; Cox et al., 2006; Cury et al., 2007; Zhou et al., 2007), suggesting that the pro-MMPs secreted by gingival fibroblasts can
be activated. Gingival fibroblasts stimulated with proinflammatory mediators increase the production of other proteases (MMP-3/MMP-14/MMP-16/cathepsin B/cathepsin L/plasminogen activator) (Tervahartiala et al., 2000; Dahan et al., 2001; Domeij et al., 2002; Ruwanpura et al., 2004; Beklen et al., 2006; Cox et al., 2006; Zhou and Windsor, 2006; Bodet et al., 2007; Cury et al., 2007; Smith et al., 2009; Ebisawa et al., 2011)

These other proteases degrade ECM components and activate pro-MMPs, thereby both directly and indirectly regulating ECM degradation. This MMP-activating function may be mechanistically relevant to the increased activity of gingival fibroblast-derived collagenases observed during inflammatory conditions. In addition, the activation of gingival fibroblast-derived proteases in vivo may be performed by enzymes derived from other sources, including neutrophils or P. gingivalis (Grayson et al., 2003; Andrian et al., 2004; Beklen et al., 2006). Increased activities of the gelatinolytic MMPs (MMP-2/MMP-9) have also been detected in the supernatants of gingival fibroblasts stimulated with proinflammatory mediators (Dahan et al., 2001; Arris et al., 2003; Beklen et al., 2006; Cox et al., 2006; Zhou and Windsor, 2006; Bodet et al., 2007; Cury et al., 2007; Zhou et al., 2007; Zhang et al., 2011). Increased levels of MMPs and other proteases have been observed in inflamed gingiva by IHC, and in fibroblasts isolated from inflamed gingival tissue (Tervahartiala et al., 2000; Cox et al., 2006; Cury et al., 2007). Overall, these studies suggest that during inflammatory conditions gingival fibroblasts increase the secretion and activity of MMPs and other proteases, which implicates gingival fibroblasts in tissue degradation during the pathogenesis of periodontitis.

Gingival fibroblasts express several protease inhibitors (TIMP1-4/α2M/plasminogen activator inhibitor-1(PAI-1)) supporting the notion that gingival fibroblasts inhibit local tissue remodelling (Condacci et al., 1988; Yamada et al., 2000; Leivonen et al., 2002; Arris et al., 2003; Zhou and Windsor, 2006; Bodet et al., 2007; Cury et al., 2007; Nakasone et al., 2009). For example, LPS and TGF-β increase the production of TIMP-1 and PAI-1 by gingival fibroblasts respectively. Additionally, gingival fibroblasts stimulated with LPS or PGE₂ increase the production of OPG at levels that inhibit the development of osteoclasts from monocytes (Nagasawa et al., 2002; Kiji et al., 2007). However, gingival fibroblasts are also stimulated by growth factors to produce RANKL which promotes osteoclastogenesis. These studies suggest that gingival fibroblasts regulate alveolar bone remodelling, as well as tissue remodelling in the gingiva. While
gingival fibroblasts clearly have the potential to remodel gingival ECM, whether this is beneficial or deleterious in the context of gingivitis and periodontitis is unknown.

1.11.2 Innate recognition of microbes
Gingival fibroblasts recognise and respond to TLR agonists. Gingival fibroblasts express up to nine TLRs, including TLR2 and TLR4, which suggests that these cells recognise microbe-associated molecular patterns in the gingiva (Kiji et al., 2007; Mahanonda et al., 2007; Uehara and Takada, 2007). Interestingly, TNF-α, TLR2 agonists and bradykinin upregulate the surface levels of TLR2 on gingival fibroblasts (Gutiérrez-Venegas and Arreguín-Cano, 2011; Davanian et al., 2012; Faria Morandini et al., 2012). This function is antagonised by anti-inflammatory prostaglandins (Davanian et al., 2012), and suggests that the ability of gingival fibroblasts to recognise TLR2 agonists depends on which inflammatory mediators are present in the gingiva. Gingival fibroblasts stimulated with LPS also increase the secretion of high mobility group box-1 (HMGB1), independently of cell death (Feghali et al., 2009). HMGB1 can act as an endogenous TLR4 agonist, suggesting that gingival fibroblasts may initiate TLR4 signalling in TLR4-expressing cells. In response to stimulation with TLR agonists, gingival fibroblasts increase the production of several CSFs (Costa-Rodrigues and Fernandes, 2011; Khaled et al., 2013), which may drive increased production of leukocytes in the bone marrow. Levels of granulocyte-macrophage CSF in the gingival crevice are associated with generalised aggressive periodontitis (de Lima Oliveira et al., 2012), although this was unlikely to be derived entirely from fibroblasts.

1.11.3 Regulation of leukocyte recruitment
Fibroblasts may be a source of chemokines in the gingiva. Aside from a potential role in increasing the number of circulating lymphocytes, gingival fibroblasts secrete numerous chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL17, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12) that together are responsible for the recruitment of most types of circulating leukocytes (Imatani et al., 2001; Ozawa et al., 2003; Brunius et al., 2005; Mahanonda et al., 2007; Minami et al., 2007; Uehara and Takada, 2007; Hosokawa et al., 2008; Mahanonda et al., 2008; Hosokawa et al., 2009; Morandini et al., 2009; Dzierzewicz et al., 2010; Hosokawa et al., 2010a; Hosokawa et al., 2010b; Montreekachon et al., 2011; Morandini et al., 2012; Khaled et al., 2013). For example, increased production of CXCL8 by stimulated gingival fibroblasts may assist in the recruitment of neutrophils into the gingiva (Imatani et al., 2001; Ozawa et al., 2003; Brunius et al., 2005; Mahanonda et al., 2007; Minami et al., 2007; Uehara and Takada,
The production of chemokines by gingival fibroblasts is regulated by bacterially derived TLR agonists and inflammatory/immune response mediators; some of these stimuli synergise to further enhance chemokine secretion (Imatani et al., 2001; Ozawa et al., 2003; Brunius et al., 2005; Mahanonda et al., 2007; Minami et al., 2007; Uehara and Takada, 2007; Hosokawa et al., 2008; Mahanonda et al., 2008; Hosokawa et al., 2009; Morandini et al., 2009; Dzierzewicz et al., 2010; Hosokawa et al., 2010a; Hosokawa et al., 2010b; Montreekachon et al., 2011; Morandini et al., 2012; Khaled et al., 2013). However, other resident or recruited cell types may also affect chemotaxis in the gingiva, so the importance of gingival fibroblasts in leukocyte recruitment in vivo has not yet been confirmed. Interestingly, cytokine stimulation of gingival fibroblasts synergistically increases surface intercellular adhesion molecule-1 expression, which increases neutrophil binding in vitro (Ozawa et al., 2003; Mahanonda et al., 2008; Hosokawa et al., 2010a). This suggests that as well as recruiting lymphocytes, gingival fibroblasts are also able to promote retention of these cells within the gingiva. Dermal and synovial fibroblasts regulate the ability of endothelial cells to bind lymphocytes (McGettrick et al., 2009), but whether gingival fibroblasts perform the same function has not been established.

1.11.4 Regulation of inflammation and immune responses
Gingival fibroblasts may be a source of proinflammatory cytokines and PGE2 in the gingiva (Imatani et al., 2001; Coletta et al., 2002; Noguchi et al., 2002; Ohshima et al., 2002; Uehara et al., 2005; Kiji et al., 2007; Mahanonda et al., 2007; Uehara and Takada, 2007; Botero et al., 2008; Mahanonda et al., 2008; Palmqvist et al., 2008; Ara et al., 2009; Feghali et al., 2009; Chotjumlong et al., 2010; Dzierzewicz et al., 2010; Souza et al., 2010; Bäge et al., 2011; Belibasakis and Guggenheim, 2011; Bostanci et al., 2011; Zhu et al., 2011; Belibasakis et al., 2013; Khaled et al., 2013). Although highly responsive to proinflammatory cytokines within the IL-1 and TNF families gingival fibroblasts secrete these cytokines only at low levels, even though increased mRNA expression is induced by microbial stimuli (Botero et al., 2008; Bostanci et al., 2011; Belibasakis et al., 2013; Khaled et al., 2013). However, gingival fibroblasts are a source of the proinflammatory cytokine IL-6, and production is often synergistically regulated by IL-1, TNF-α, lipid mediators and bacterial stimuli (Imatani et al., 2001; Coletta et al., 2002; Noguchi et al., 2002; Uehara and Takada, 2007; Palmqvist et al., 2008; Ara et al., 2009; Dzierzewicz et al., 2010; Belibasakis and Guggenheim, 2011;
Khaled et al., 2013). Other IL-6 family cytokines, LIF and IL-11, are regulated in a similar manner (Palmqvist et al., 2008; Souza et al., 2010). Given the well-described functionality of the IL-6 family cytokines (Taga and Kishimoto, 1997b), these studies suggest that gingival fibroblasts drive wide-ranging inflammatory responses both locally and systemically. Additionally, gingival fibroblasts are a source of PGE₂, which is increased after stimulation with bacterial TLR agonists, IL-1β/TNF-α or bradykinin (Noguchi et al., 2002; Kiji et al., 2007; Chotjumlong et al., 2010; Båge et al., 2011; Belibasakis and Guggenheim, 2011; Gutiérrez-Venegas and Arreguín-Cano, 2011). Increased levels of PGE₂ are detected in fibroblasts from patients with periodontitis compared to healthy controls (Båge et al., 2011), although whether gingival fibroblast-derived PGE₂ drives gingival inflammation or is a cause of it remains to be determined. However, studies have suggested that PGE₂ has an autocrine effect on gingival fibroblasts by regulating their productions of IL-6 and OPG (Ko et al., 1977; Noguchi et al., 2002; Takashiba et al., 2003; Kiji et al., 2007).

Fibroblasts may regulate and suppress inflammatory and immune responses in the gingiva. Stimulation of gingival fibroblasts with TLR agonists or immunoregulatory cytokines (IFN-γ/IL-17/TNF-α) increases their production of the anti-inflammatory mediators indoleamine 2, 3-dioxygenase (IDO) and IL-10 (Mahanonda et al., 2007; Mahanonda et al., 2008; Khaled et al., 2013). Furthermore, gingival fibroblast-derived IDO reduces T lymphocyte proliferation in co-culture models (Mahanonda et al., 2007), supporting a role for gingival fibroblasts in suppressing inflammatory and immune responses in the gingiva. The adipokine adiponectin, which tends to be decreased in the circulation of obese individuals or those with T2DM, promotes anti-inflammatory gingival fibroblast responses (Iwayama et al., 2012). This suggests that gingival fibroblasts may enhance or drive inflammatory responses in individuals who have T2DM or are obese. Yet gingival fibroblasts may also be a source of cytokines that stimulate proliferation and activity of leukocytes (Khaled et al., 2013). Additionally, gingival fibroblasts stimulated with IFN-γ display increased surface MHC class II which supports the theory that fibroblasts promote adaptive immune responses in their local microenvironment (Mahanonda et al., 2008; Seguin-Estevez et al., 2009). Interestingly, IFN-γ also stimulates gingival fibroblasts to increase the surface expression of CD40 (Mahanonda et al., 2008), and ligation of CD40 enhances the inflammatory response of fibroblasts (Dongari-Bagtzoglou et al., 1997; Sempowski et al., 1997; Wassenaar et al., 1999). Overall, this suggests a bidirectional regulatory
relationship exists between gingival fibroblasts and lymphocytes which may be relevant to the pathogenesis of gingivitis and periodontitis.

1.11.5 Gingival fibroblasts as a unique cell type
Gingival fibroblasts have an ability to integrate their responses to environmental stimuli, which may determine how these cells regulate tissue remodelling, immune responses and inflammation. Distinct stimuli, or combinations of stimuli, can prompt the same outcome by activating similar intracellular pathways (e.g. NF-κB/MAPK/PI3K) (Kida et al., 2005; Bodet et al., 2007; Uehara and Takada, 2007; Hosokawa et al., 2008). Conversely, a single stimulus can promote different fibroblast responses, possibly in a concentration or time-dependent manner (Ruwanpura et al., 2004). Alternatively, variation in gingival fibroblast responses could be due to intrinsic heterogeneity in cell populations. There is good evidence to suggest that fibroblasts have a sense of positional identity. Dermal and oral fibroblasts possess distinct phenotypes which correlate to the structure and functions of the surrounding tissues (Irwin et al., 1994; Lekic et al., 1997; Chang et al., 2002a). For example, gingival fibroblasts from healthy human donors have a reduced ability to adhere and spread on ECMs after TGF-β stimulation compared to dermal fibroblasts (Guo et al., 2011), which could be relevant to why wounded gingival tissue is less likely to scar than dermal tissue (Szpaderska et al., 2003). However, phenotypic differences between gingival fibroblasts and other oral fibroblasts, and even between gingival fibroblasts (isolated from papillary and reticular layers) have been reported (Irwin et al., 1994; Han and Amar, 2002), which suggests that intra-site heterogeneity also exists. This may also support the hypothesis that fibroblasts and mesenchymal stem cells in the gingiva are essentially the same cell type, although perhaps at different states of differentiation and/or activation (Phipps et al., 1997; Haniffa et al., 2009). Distinct phenotypes between fibroblasts from healthy and inflamed periodontal sites have been identified (Kent et al., 1999), however in this study healthy and periodontal disease fibroblasts from different donors were compared so the differences observed could have been due to genetic heterogeneity between individuals. Overall, gingival fibroblasts certainly have the potential to regulate inflammatory and immune responses in the gingiva. However, the factors that determine the potentially beneficial or deleterious outcomes of gingival fibroblast stimulation during inflammation are still not fully understood.

It has been difficult to identify whether gingival fibroblasts have a protective or pathogenic role during inflammation in vivo. This is partly due to the redundancy of the
cellular sources of inflammatory molecules, such as proteases, cytokines and their regulatory counterparts in the gingiva coupled with the lack of a gingival fibroblast-specific marker that could facilitate genetic manipulation studies. Nonetheless, while there may be redundancy in the gingiva between the products of fibroblasts and other resident and recruited cells, it appears that fibroblasts generate these products in a manner that is quite unique (Black 2007). For example, profiling of intracellular signalling has shown that LPS-stimulated gingival fibroblasts upregulate IL-6 in a manner distinct to monocytes, which may result in different responses between these cells during inflammation (Jin et al., 2012). Additionally, unlike monocytes, gingival fibroblasts do not develop tolerance to E. coli or P. gingivalis LPS (Ara et al., 2009), which supports the theory that fibroblasts can drive the persistence of chronic inflammation, or lack of resolution of acute inflammation (Buckley, 2011; Naylor et al., 2012; Owens and Simmons, 2013). Are gingival fibroblasts a causal factor in the initiation of gingival inflammation and do they play a role in the pathogenesis of periodontitis? Could certain populations at risk of developing periodontitis promote deleterious gingival fibroblast responses during inflammation?

Gingival fibroblasts maintain the structure and function of the gingiva. This homeostatic function is evidenced by their ability to produce ECM components and to promote gingival tissue remodelling during wound repair. However, gingival fibroblasts are immunocompetent cells responsive to a wide range of inflammatory and immune stimuli. These stimuli cause gingival fibroblasts to alter their phenotype in a manner both similar and distinct to other fibroblasts and immunocompetent cells. While the biologically active mediators produced by gingival fibroblasts are not in themselves unique, the way inflammatory stimuli cause gingival fibroblasts to regulate their phenotype does differ from other cell types. This places gingival fibroblasts in a unique position to regulate inflammatory processes, and may be relevant to the pathogenesis of periodontitis. Studies that pinpoint the mechanisms and temporal relationship behind gingival fibroblast responses during inflammation, and in vivo studies that put these responses into context, will help to further our understanding of this important and unique cell during inflammation and immunity and may help to elucidate why periodontitis is currently so difficult to cure.

1.11.6 Gingival fibroblasts and leptin

Only one very recent study has investigated the ability of leptin to regulate gingival fibroblast responses. Park et al. (2013) showed that primary hGFs express both long and
short leptin receptor isoforms, but not leptin, at the mRNA level. The authors showed that recombinant leptin dose-dependently increases IL-6 and CXCL8 secretion by hGFs. This suggests that gingival fibroblasts respond to leptin in a proinflammatory manner, and may be mechanistically relevant to the pathogenesis of periodontitis in the context of elevated levels of leptin.

Circulating levels of leptin are elevated in obesity and T2DM, and both of these conditions are positively associated with periodontitis. Leptin promotes inflammation and ECM remodelling, processes implicated in the development of periodontitis. Fibroblasts are the predominant cell type in the gingiva and can sense and respond to numerous stimuli in their microenvironment. Inflammatory stimuli (including leptin) drive gingival fibroblasts towards a proinflammatory phenotype. Periodontitis is characterised by destruction of the periodontal tissues and gingival fibroblasts, stimulated by inflammatory mediators, enhance the degradation of ECM components. Leptin enhances the matrix-degrading potential of several cell types, however no-one has assessed whether leptin has a similar effect on gingival fibroblasts. In this thesis it is hypothesised that leptin acts as a mechanistic link between obesity, T2DM and periodontitis by promoting inflammatory responses in gingival fibroblasts. In particular, it is hypothesised that leptin (either alone or in synergy with other inflammatory mediators) stimulates the production of the ECM-degrading MMPs by gingival fibroblasts, and that this will increase gingival tissue degradation.
1.12 Aims

1. To determine whether leptin increases MMP expression in hGFs.
   Both semi-quantitative and real-time RT-PCR were used to assess mRNA expression levels of MMPs and TIMPs after leptin stimulation in hGFs. ELISA was used to determine the levels of MMPs in the supernatants of leptin-stimulated hGFs.

2. To determine whether leptin synergises with proinflammatory stimuli to increase the production of MMPs identified in (1) in hGFs.
   hGFs were stimulated with leptin in combination with proinflammatory mediators (IL-1, OSM) and TLR agonists (E. coli LPS, pam2CSK4). Both semi-quantitative and real-time RT-PCR were used to assess mRNA expression levels of MMPs and TIMPs in stimulated hGFs. ELISA was used to determine the levels of MMPs in the supernatants of stimulated hGFs. Surface expression levels of TLR2 and TLR4 on hGFs were measured by flow cytometry.

3. To investigate which signalling pathways are involved in leptin-mediated MMP-1 expression in hGFs.
   Semi-quantitative RT-PCR and flow cytometry were used to determine the expression of leptin receptor isoforms by hGFs. The phosphorylation statuses of intracellular signalling proteins were measured by Western blot in hGFs after stimulation with leptin, alone and in combination with IL-1 or pam2CSK4. Intracellular signalling pathways of interest identified by Western blot were pharmacologically inhibited and MMP gene expression was measured in hGFs after stimulation with leptin, alone and in combination with IL-1 or pam2CSK4.

4. To assess genome-wide RNA expression patterns in hGFs stimulated with leptin±IL-1.
   hGFs were stimulated with leptin±IL-1β and MMP-1 mRNA expression was assessed using real-time RT-PCR to determine whether the stimulation was successful. High quality RNA was used for genome-wide expression analysis. Individual and related groups of genes that were regulated by leptin±IL-1 were identified using bioinformatics analysis. Real-time RT-PCR was used to confirm the differential expression changes of 8 genes.

5. To investigate the role of leptin on tissue integrity in a gingival tissue explant model system.
   Gingival tissue was assessed for utility as a model system of tissue integrity by measuring explant viability and the ability of a potent cytokine stimulus (IL-
1+OSM) to promote gingival ECM degradation. Gingival tissue explants were stimulated with leptin+IL-1 and gingival tissue integrity was determined by measuring the proportion of collagen released from the gingival tissue, and by measuring MMP activity in culture supernatants.
Figure 1.1: The functions of leptin.

Leptin is produced predominantly from white adipose tissue. Much is known regarding leptin’s hormonal functions in the neuroendocrine system. Specifically, leptin drives physiological and psychological mechanisms that regulate food intake, energy homeostasis and metabolism. More recently, leptin has been found to regulate a wide range of other biological processes (in italics).
Figure 1.2: Aspects of the leptin signalling pathway.

Leptin (L) binds to the leptin receptor (LEPR) in a 2:2 ratio. This promotes the activation of constitutively-bound JAK2 and SFKs by transphosphorylation. The leptin receptor has no integral kinase activity. Activated JAK2 and SFKs phosphorylate three tyrosine residues in the membrane distal region of the intracellular domain of the leptin receptor (Y985, Y1077 and Y1138). These phosphorylated tyrosine residues facilitate the binding of SH2 domain-containing proteins, which are then phosphorylated (and usually activated) by JAK2 and SFKs. The phosphatase Shp2 binds to phosphorylated Y985, which in turn facilitates the binding of GRB2 and the guanine nucleotide exchange factor SOS. SOS activates membrane-bound Ras which activates the MAPK signalling pathway. The MAPK signalling pathway is a kinase cascade that results in the activation and phosphorylation of the MAPKs ERK, JNK and p38. These MAPKs activate transcription factors that relocalise to the nucleus to promote gene expression. STATs bind to phosphorylated Y1077 and Y1138 of the leptin receptor. JAK2 phosphorylates and activates STATs. Activated STATs form homodimers which function as transcription factors, and relocate to the nucleus to promote gene expression. MAPK signalling can regulate the activity of the NF-κB and STAT signalling pathways. Interestingly, leptin:leptin receptor binding also activates the PI3K signalling pathway; a function that may be independent of the phospho-tyrosine residues on the leptin receptor. Dashed arrows indicate possible interactions between different signalling pathways. Leptin affects the expression of a diverse range of genes including cytokines, chemokines and MMPs. L – leptin, JAK2 – Janus kinase 2, SFK – Src family kinase, P – phosphorylated molecule, Y – tyrosine residue, LEPR – leptin receptor, GRB2 – growth factor receptor-bound protein 2, SOS – Son of Sevenless, MAPK – mitogen-activated protein kinase, ERK – extracellular signal-related kinase, JNK – c-Jun N-terminal kinase, STAT – signal transducer and activator of transcription.
Dentine (D) which is found in the root of the tooth is covered by cementum. Cementum interacts directly with the periodontal ligament within the gingival connective tissue (CT). The gingival connective tissue is rich in collagen fibres. The epithelium (E) which covers the external surface of the gingiva consists of three distinct types. The keratinised gingival epithelium covers the external surfaces of the gingiva, from the muco-gingival margin to the free gingival margin (M). The sulcular epithelium covers the gingiva from the free gingival margin (M) into the gingival sulcus, essentially facing the enamel crown of the tooth. In this image there is a space where the enamel would have been (enamel space - ES); this is due to the processing required to section teeth for histological analysis. Junctional epithelium is found at the base of the gingival sulcus between the sulcular epithelium and the tooth surface at the cemento-enamel junction (CEJ). Image adapted from Preshaw (2014).
Table 1.1: The mammalian MMPs.

There are 26 mammalian MMPs which are split into 6 groups based on substrate specificity and structural characteristics. Example cellular sources of MMPs are displayed (Birkedal-Hansen, 1993; Kessenbrock et al., 2010). MMP – matrix metalloproteinase, DCs – dendritic cells.

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2 Chapter 2. Methods

2.1 Materials

Cell culture plastic-ware was supplied by Greiner Bio-one (Stonehouse, UK). All reagents were supplied by Sigma-Aldrich (Gillingham, UK) except where indicated. Molecular biology grade water was used where required; otherwise deionized, purified water was used. Chemical pathway inhibitors SP600125, UO126, Bay11-7085 and PD98059 were from Tocris (R&D systems, Abingdon, UK), while S3I-201, SB203580, Akt inhibitor VIII and IkB kinase (IKK) inhibitors VI and VIII were from Merck Millipore (Watford, UK). All chemical pathway inhibitors were reconstituted in dimethyl sulphoxide (DMSO). The MMP inhibitor GM6001 (Tocris) was prepared at 20 mM in DMSO. Pam2CSK4 (Invivogen, Source Bioscience, Nottingham, UK) was prepared as a 100 µg/ml solution in Limulus amebocyte lysate (LAL) reagent grade water (<0.005 EU/ml endotoxin). LPS from Escherichia coli (E. coli) strain 0111:B4 (Invivogen) was prepared as a 5 mg/ml solution in sterile water. Aliquots of inhibitors, pam2CSK4 and LPS were stored at -20 °C.

2.1.1 Recombinant proteins

Lyophilised human recombinant leptin (R&D Systems) was prepared as a 1 mg/ml solution in 20 mM tris(hydroxymethyl)aminomethane (TRIS)-HCl, pH 8. Human recombinant IL-1α (670 ng/ml) was a gift from Dr Keith Ray (GlaxoSmithKline). Recombinant OSM (10 µg/ml) was prepared by researchers in the musculoskeletal research group (Newcastle University) according to the method of Staunton et al (1998). Human recombinant IL-1β (R&D Systems) was prepared at 25 µg/ml in 0.1 % w/v endotoxin-free bovine serum albumin (BSA) in Dulbecco’s phosphate buffered saline (PBS). Aliquots of reconstituted proteins were stored at -80 °C. Further dilutions were prepared in serum-free Dulbecco’s modified eagle medium (DMEM). Recombinant human MMP-1 (R&D Systems) was stored at -80°C at 0.458 mg/ml.

Leptin reconstituted in the Translational Oral Biosciences lab was tested for endotoxin contamination by Dr Katrin Jaedicke using the LAL assay (Jaedicke, 2010). Recombinant human leptin at 100 µg/ml contained a concentration of 53 pg/ml LPS. In this study the highest concentration of leptin used was 25 µg/ml, which would have contained 13.3 pg/ml LPS. Previous studies demonstrated that leptin at this concentration failed to stimulate TNF-α production by monocytes, further confirming the lack of biologically active LPS in this preparation (Jaedicke et al., 2013). Several
studies have utilised *E. coli* LPS to stimulate gingival fibroblasts (Kent *et al.*, 1999; Xiao *et al.*, 2001; Nagasawa *et al.*, 2002; Tardif *et al.*, 2004; Mahanonda *et al.*, 2007; Minami *et al.*, 2007; Ara *et al.*, 2009; Feghali *et al.*, 2009; Jin *et al.*, 2012). Concentrations in the ng – μg/ml range were required to generate responses. Additionally, leptin strongly activates the JAK/STAT signalling pathway in hGFs, and has little effect on NF-κB signalling which is activated downstream of TLR ligation (Section 5.3.4).

### 2.2 Tissue culture techniques

All tissue culture was performed using aseptic technique in a class II laminar flow unit (BioAir, Biological Instrumentation Services, Manchester, UK). Where available reagents purchased were sterile and endotoxin-free. Subsequent sterilisation was performed by using a high temperature, high pressure autoclave and/or a 0.22 μm filter. Tissue and cells were incubated in a 5 % CO₂ atmosphere at 37 °C unless otherwise indicated. Ca²⁺/Mg²⁺-free PBS was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and nystatin (40 U/ml). DMEM containing 4.5 g/L glucose was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). DMEM was either used as a serum-free medium (SFM) or supplemented with 10% v/v foetal bovine serum (FBS) (DMEM+). Nutrient mixture F-12 Ham medium was supplemented with penicillin (200 U/ml), streptomycin (200 μg/ml) and nystatin (80 U/ml).

#### 2.2.1 Primary hGF isolation

Gingival tissue was acquired anonymously from patients undergoing canine tooth exposure surgery (performed by Dr John Meech, Newcastle Dental Hospital). Ethical approval was obtained from the national research ethics service committee North East (County Durham and Tees Valley). The tissue was placed in F-12 Ham medium on ice until processed. The tissue was orientated using a stereo microscope. Gingival tissue has a distinct, smooth keratinised epithelium, and a more vascular, less dense connective tissue layer. After three washes in PBS the epithelium was removed by scalpel and the remaining tissue was dissected into 2 mm³ sections. Tissue sections were squashed onto the surface of a tissue culture plate and just covered in DMEM+ to promote hGF adherence and outgrowth. The medium was replaced as required and the tissue was incubated for approximately 2 weeks to allow for hGF migration, forming a monolayer on the plate surface. The outgrowth protocol for hGFs has been published previously.
Consequently, the remaining tissue was removed and the hGFs subcultured.

2.2.2 Primary hGF subculture

hGFs are adherent cells that grow in monolayer to confluence on tissue culture plasticware in DMEM+. These cells demonstrate the spindle-shaped morphology characteristic of fibroblasts (Figure 2.1), and towards confluency the cells align in the same orientation (Figure 2.2), similar to that described previously (Kanda-Nakamura et al., 1996; Beertsen et al., 1997; Chang et al., 2002b). hGFs were cultured in monolayer until 80% confluent, as determined by visual inspection with a light microscope (DM IL, Leica Microsystems, Milton Keynes, UK). To passage, cells were washed in PBS then covered with 0.25 % w/v trypsin-ethylenediaminetetraacetic acid (EDTA) solution and incubated until detached (< 3 minutes). An excess of DMEM+ was added to the cell suspension to quench the trypsin. The cells were pelleted by centrifugation (168 x g, 5 minutes, 20°C, CR3i multifunction centrifuge (ThermoFisher Scientific, Loughborough, UK)) and then resuspended in DMEM+. At this point hGFs were routinely > 99 % viable by trypan blue exclusion (Section 2.2.4). Cells were subsequently seeded either into culture flasks to maintain cultures, seeded into plates for stimulation experiments, or cryopreserved (Section 2.2.5) for future use. hGFs remained viable but were found to stop dividing at passage 13, indicative of cellular senescence. hGFs from one donor were tested for mycoplasma by Dr Helen Bosomworth and no trace of contamination was detected.

2.2.3 hGF characterisation

Vimentin is an intermediate filament protein which is expressed in mesenchymally-derived cells, such as fibroblasts. Therefore, vimentin expression was determined by immunocytochemistry (Section 2.3) in hGF cultures to investigate whether the cells isolated from the gingival tissue were indeed fibroblasts. Cells from all donors tested which were incubated with mouse monoclonal anti-human vimentin antibody stained positive strongly compared to the no antibody control (NAC), which suggests that the cells isolated from gingival tissue using the technique in Section 2.2.1 are mesenchymally-derived (Figure 2.2). The staining observed was limited to the cytoplasm but extended throughout the cell in the manner expected of an intermediate filament. In dividing cells staining was particularly intense at the periphery of the cell (Figure 2.2B). Vimentin has an altered distribution during mitosis (Aubin et al., 1980),
so this staining pattern is evidence in support of the antibody binding vimentin and not other cellular targets in a non-specific manner.

2.2.4 Cell counting and viability assessment by haemocytometer

Equal volumes of cell suspension and trypan blue (0.4 %) were incubated at room temperature (RT) for 30 seconds, after which a sample of the mixture was loaded onto a haemocytometer. The cells were counted and assessed for viability, by trypan blue exclusion, under a light microscope. The Bright-Line Haemocytometer (Hausser Scientific, VWR, Lutterworth, UK) used has a Neubauer ruling pattern and therefore the number of cells/ml was determined using the formula:

\[
\text{cells/ml = number of cells counted in } 1 \text{ mm}^2 \times 2(\text{dilution factor}) \times 10^4
\]

Cell morphology and adherence to culture surfaces were also noted. Cells which were <90% viable or poorly adherent were not used for stimulation experiments.

2.2.5 Cryopreservation and recovery of hGFs

hGFs were pelleted by centrifugation (168 x g, 5 minutes, 20°C, CR3i multifunction centrifuge). Pellets of hGFs were resuspended in DMEM+ supplemented with 10% v/v glycerol to give a cell density of > 1x10^6 cells/ml. Aliquots were dispensed into cryovials and placed in a propan-2-ol-containing freezing container in a -80°C freezer. Once cooled to -80°C the cryovials were transferred to liquid nitrogen for long term storage.

hGFs were revived from cryopreservation by quickly warming to 37°C. hGFs were seeded into a cell culture flask and DMEM+ was slowly added to the flask. hGFs were then incubated and after the cells were adherent the medium was changed to remove any residual cryopreservant and non-viable cells. hGFs were subcultured at least once before being used in an experiment.

2.2.6 In vitro stimulation of hGFs

hGFs were seeded at various densities in DMEM+ depending on plastic-ware format (Table 2.1). In the 96-well format wells at the edge were filled with PBS to stop evaporation of medium from hGF-containing wells. Seeded hGFs were incubated until 80-90 % confluent at which point the cells were either processed for downstream applications, or serum-starved (washed in PBS and media replaced with SFM) for 18 hours and then stimulated for ≤ 48 hours. hGFs were used in stimulation experiments between passages 5 and 9.
2.2.6.1 Chemical pathway inhibitors

The ability to inhibit specific signalling molecules, and therefore, particular signalling pathways is a useful tool for understanding the mechanisms of cell signalling events. Small molecule, chemical pathway inhibitors have been used extensively for this purpose, including with hGFs (Ravenhall et al., 2000; Yucel-Lindberg and Brunius, 2006; Tong et al., 2008). It is important to test that these inhibitors are specifically inhibiting their target, and that at the concentrations required to do this there is no alteration in cell viability.

Chemical pathway inhibitors (Section 2.1) were tested for their potential to affect cell viability. For each inhibitor, a 6-point, 3-fold dilution series (n=2 per concentration) was performed using concentrations which included those suggested to effectively inhibit the pathway of interest according to the manufacturer. Inhibitors were diluted in SFM. DMSO controls of 1, 0.4, 0.1 and 0.03 % v/v which were equivalent to the highest concentration of DMSO in inhibitor treatments were performed to investigate any effect of DMSO on cell viability. hGFs were stimulated for 24 h at which point viability was assessed using a colourimetric cell proliferation assay (Section 2.5.1).

Morphological changes indicative of toxicity (rounding/shrinking/poor adherence) were observed at 24 h by light microscope for hGFs treated with the highest concentrations of PD98059 and Akt inhibitor VIII. Indeed, Akt inhibitor VIII caused a reduction in hGF proliferation in a dose-dependent manner (Figure A.1B). In contrast, a slight increase in proliferation was observed in hGFs treated with PD98059 although not as much as the DMSO-treated control (Figure A.1D). It is likely however, that the increased proliferation and morphological changes observed by the highest concentrations of PD98059 were due to DMSO and not PD98059 itself. The highest concentration of PD98059 tested contained 1 % DMSO and this concentration of DMSO alone caused an 18 % increase in proliferation compared to non-treated cells. A dose-dependent decrease in hGF proliferation was demonstrated by Bay11-7085 treatment, while hGFs treated with the highest concentrations of S3I-201 and SB203580 had reduced proliferation compared to the untreated and DMSO controls (Figure A.1). No dose-dependent changes in proliferation were observed for hGFs treated with U0126, SP600125 or IKK inhibitors VI and VIII, and proliferative responses correlated to the untreated and DMSO controls (Figure A.1).
Concentrations of chemical pathway inhibitors that had no negative effect on cell proliferation were used to test for efficacy in inhibiting the phosphorylation of cell signalling intermediates. hGFs were pre-treated with inhibitors or a 0.1 % DMSO control for 30 min. hGFs were then stimulated with 0.05 ng/ml IL-1α and 5 ng/ml OSM for 20 minutes. At this time point hGFs were lysed in preparation for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting (Section 2.11).

The MAPK/Erk kinase (MEK) inhibitor U0126, but not the MEK inhibitor PD98059, greatly inhibited the phosphorylation of ERK1/2 in hGFs after IL-1+OSM stimulation compared to the DMSO-pretreated control (Figure B.1A). As such, U0126 was chosen as the inhibitor used in this work to study ERK signalling. The p38 inhibitor SB203580 affects p38 activity independently of its phosphorylation by binding to the ATP-binding pocket. However, SB203580 has been reported to modulate ERK activity (Henklova et al., 2008). In hGFs, SB203580 had no effect on ERK phosphorylation induced by IL-1+OSM (Figure B.1A). The JNK inhibitor SP600125 inhibited both JNK and ERK phosphorylation in a dose-dependent manner in hGFs after IL-1+OSM stimulation (Figure B.1B). The higher concentration of SP600125 (10 μM) was chosen to study JNK activity in this thesis; however the concomitant inhibition of ERK by this inhibitor was a limitation that was taken into account. The NF-κB inhibitors IKK VIII and Bay11-7085, but not the NF-κB inhibitor IKK VI, inhibited NF-κB p65 phosphorylation in hGFs after IL-1+OSM stimulation (Figure B.1C). Bay11-7085 was selected to study NF-κB activity in this study. Akt inhibitor VIII greatly reduced the phosphorylation of both Ser473 and Thr308 in hGFs after IL-1+OSM stimulation (Figure B.1D), and was selected for use in this thesis. The STAT3 inhibitor S3I-201 slightly reduced the phosphorylation of Tyr705, but had little effect on Ser727 phosphorylation, in hGFs after IL-1+OSM stimulation (Figure B.1E).

2.2.7 Preparation of hGFs for flow cytometry
Adherent hGFs were washed with PBS then covered with 0.25 % Trypsin-EDTA and incubated until detached (< 3 minutes). An excess of DMEM+ was added to the cell suspension to quench the trypsin. hGFs were pelleted by centrifugation (168 x g, 5 minutes, 20 °C, CR3i multifunction centrifuge (ThermoFisher Scientific)) and then resuspended in 0.5 % w/v BSA in PBS. hGFs were counted and assessed for viability by trypsin blue exclusion using a haemocytometer (Section 2.2.4). The cells were
centrifuged (168 x g, 5 minutes, 20 °C) and the pellet resuspended in 0.5 % w/v BSA in PBS to give a density of 4x10⁶ cells/ml for flow cytometry analysis (Section 2.10).

2.2.8 Staining gingival tissue for triglycerides
Gingival tissue was acquired and processed according to Section 2.2.1. Dissected gingival tissue pieces were placed into 24-well plates and washed in PBS. Tissue pieces were resuspended in 1 ml PBS to which 30 μl AdipoRed (Lonza, Slough, UK) was added. Tissue pieces were then incubated for 10 minutes at RT. AdipoRed contains the stain Nile Red and allows for the vital visualisation of lipids by assessing the fluorescence which occurs when Nile Red is partitioned in fat droplets. However, a pink-coloured staining can also be observed with AdipoRed using a light microscope (CKX41 model, Olympus, Southend-on-Sea, UK); this crude approach allows for quick generation of images that show tissue morphology and fat droplet localisation.

2.2.9 Gingival tissue stimulation
Gingival tissue was acquired and processed according to Section 2.2.1. One 2 mm³ piece of tissue was placed per well (96-well format), washed with PBS then suspended in 200 μl DMEM+ overnight. The following day the DMEM+ was aspirated, and the tissue was washed with PBS. Tissue pieces were resuspended in stimulation treatments diluted in SFM. Treatments were changed routinely every 3-4 days over the 3 week culture period and removed supernatants were stored at -20 °C until analysis. At the end of the experiment the tissue pieces were washed in PBS before storage at -20 °C for subsequent analysis.

Gingival tissue pieces used for ex vivo culture were susceptible to both fungal and bacterial contamination. Both yeast-like single cells and hyphae were observed by light microscopy in fungal contaminations indicative of Candida spp, while cells with cocci morphology were observed in bacterial infection. Microbial contamination only occurred in the first few days of ex vivo culture, and only in particular donors. Therefore, as the oral environment from which the tissue was isolated is microbe-rich it was thought that the most likely source of contamination was the tissue itself. If any gingival tissue pieces had visual signs of microbial contamination all tissue pieces from this donor were destroyed.

2.2.9.1 Papain-mediated digestion of gingival tissue
Papain is a cysteine protease and is effective at digesting numerous proteins, including collagen and other ECM components. Digestion of gingival tissue with papain thus
disrupts gingival tissue ECM allowing for downstream measurement of the hydroxyproline and glycosaminoglycan (GAG) content of gingival tissue. Stimulated gingival tissue pieces were digested in 200 μl of 4.5 mg/ml papain (Merck, Hoddesdon, UK) in a 0.1 M phosphate (NaH₂PO₄/Na₂HPO₄ (both VWR)) buffer, pH 6.5 containing 5 mM EDTA (disodium salt) and 5 mM cysteine-HCl. Tissue-containing wells were sealed to eliminate evaporation and incubated until digested (≤ 18 h). Once cooled, acid hydrolysis was performed on the digests (Section 2.13.1).

2.3 Immunocytochemistry
Glass slides (13 mm diameter) were washed (1x 100% ethanol, 3x PBS) and placed into 24 well plates. Cells were seeded into the plates (Table 2.1) and incubated until confluent. Next, cells were fixed in ice-cold methanol (Fisher Scientific) for 15 minutes, and then washed once in TRIS-buffered saline (TBS), pH 7.6. The slides were then incubated for 1 h at RT in either mouse monoclonal anti human vimentin primary antibody (IgG2b, Clone Vim 3B4) (Dako, Ely, UK) diluted 200-fold in diluent (3% v/v FBS in TBS, pH 7.6) or diluent alone as a NAC. The antibody was removed by washing (3x 5 min in TBS, pH 7.6) before the slides were incubated in Envision polymer (Dako) for 30 min at RT. The slides were washed again (3x 5 min in TBS, pH 7.6) before incubation in freshly prepared diaminobenzidine solution (Dako) for 5 min at RT. Excess diaminobenzidine was removed by washing (1x 5 min in water) before the slides were counterstained with haematoxylin for 1 min at RT. Excess haematoxylin was removed by washing (1x 5 min in water) before the slides were mounted onto microscope slides. Cells were visualised and images taken with a DM500 microscope (Leica microsystems).

2.4 Histological processing of gingival tissue
Gingival tissue was acquired according to Section 2.2.1. The tissue was placed in a 10 % formalin solution for 24 h at RT either as unprocessed tissue or after sectioning using precision cutting (Section 2.4.1). Next, the tissue was rinsed in water for 10 min and stored in 70 % v/v ethanol for approximately 72 h.

Whole tissue samples were sent to a Biobank facility (Newcastle University) for processing and embedding in wax. Sections of whole gingival tissue were cut using a microtome (Leica microsystems) and placed onto microscope slides. Sections were left to dry overnight by incubating at 37 °C, and then stored at RT prior to staining (Section 2.4.2). In contrast, precision-cut tissue pieces were sent to the Royal Victoria Infirmary
(Newcastle upon Tyne) for processing, embedding and staining with haematoxylin and eosin (H+E).

2.4.1 Precision slicing of gingival tissue
This procedure was performed by Dr Stephen Hill (Fibrosis Research Group, Newcastle University). Briefly, freshly acquired gingival tissue was glued onto the stage of a vibratome (Model VT1200S, Leica Microsystems). Precision cut sections of 250 μm were made using a blade speed of > 0.3 mm/s. Sections were suspended in Hank’s balanced salt solution supplemented with 10 % FBS before the tissue was fixed and processed.

2.4.2 H+E staining of gingival tissue sections
Gingival tissue sections (from whole tissue samples) were de-waxed in xylene (2 x 5 min washes). Next, the sections were rehydrated through graded concentrations of ethanol (100-95-75-50 %, 3 min at each concentration). The sections were washed in water, and then stained with Mayer’s haematoxylin for 90 sec. Next, the sections were washed in water for 1 min before being placed in Scott’s tap water substitute for 1 min to stain the nuclei blue. The sections were washed in water for 3 min, then stained with eosin for 2 min and finally washed in water for 30 sec. The sections were then dehydrated through graded concentrations of ethanol (50-75-97-100 %, 2 min at each concentration). The slides were then cleared in xylene (2 x 5 min washes). DPX was used to mount a cover slip over the tissue section and left to dry. Tissue sections were visualised and images taken with a DM500 microscope.

2.5 Colourimetric cell proliferation assays

2.5.1 Tetrazolium compound method
Proliferation was assessed in hGFs using the Celltiter 96® AQqeous one solution cell proliferation assay kit (Promega, Southampton, UK). The solution provided in this kit contains a tetrazolium compound which is reduced to form an insoluble coloured formazan product which can be detected by spectrophotometry. This conversion is presumed to be carried out depending on the levels of NAD(P)H, and can therefore provide an indication of the metabolic activity of a cell. However, metabolic activity may not always correlate to cell proliferation and this limitation must be taken into account.
SFM was added to two empty wells to allow for the assessment of background absorbance. After 22-23 hours post-stimulation Celltiter reagent (20 µl/100 µl supernatant) was added and mixed. hGFs were incubated until 24 hours post-stimulation, when all wells were mixed by pipette and the absorbance was measured at 490 nm using a Synergy HT plate reader (BioTek, Potton, UK). Results were calculated as % unstimulated or untreated control after the subtraction of the mean background absorbance. In the preliminary experiment assessing dose-dependent toxicity of chemical pathway inhibitors a four-parameter logistic (4PL) curve was fitted to the data in SigmaPlot (Systat Software, London, UK) (Section 2.2.6.1). In stimulation experiments hGFs exposed to DMEM supplemented with 5/10 % FBS were used as a positive control for proliferation (Figure 2.3).

2.5.2 Lactate dehydrogenase assay
Lactate dehydrogenase (LDH) is an enzyme found constitutively within all cells. When cell death occurs and plasma membrane integrity is lost, the contents of the cytosol are released into the supernatant. LDH activity in cell supernatants is therefore used to indicate the level of cell death in a sample.

In this study, LDH activity was measured using a LDH cytotoxicity assay kit (Thermofisher Scientific). Substrate solution was reconstituted in 11.4 ml dH2O. A reaction mix was prepared by adding assay buffer (0.6 ml) to the substrate solution and was stored at -20 °C until use. The positive control was prepared by diluting 1 µl LDH positive control with 10 ml 1 % w/v BSA in PBS (assay diluent). Gingival tissue supernatants, the positive control, the diluent (blank control) and a SFM control were dispensed into a 96-well plate in duplicate (25 µl/well). Reaction mix (25 µl) was added to each well, mixed and incubated at RT for 30 min in the dark. Stop solution (25 µl) was added to each well, mixed and absorbance was measured at 490 nm and 680 nm using a plate reader. The readings at 680 nm were subtracted from the readings at 490 nm to correct for plate interference. Results were calculated as % unstimulated control after the subtraction of the mean blank absorbance. A lower limit of quantification was determined as blank absorbance + 10SD as suggested by Armbruster et al. (1994). LDH activity was detected in the positive control supplied with the assay kit.
2.6 DNA and RNA extraction techniques

2.6.1 Total RNA purification
For conventional RT-PCR or where an assessment of the yield and quality of RNA was required, hGFs were lysed and RNA extracted from the lysate using the GenElute mammalian total RNA purification kit or RNeasy kit (Qiagen, Manchester, UK) following the manufacturer’s instructions. Briefly (and for both kits), the culture medium was removed and cells were covered with ice-cold lysis buffer (containing guanidine thiocyanate and supplemented with 1 % v/v β-mercaptoethanol) for 1-2 minutes. The plates were rocked and tapped to promote total cell lysis and RNase inactivation before the lysate was transferred to a 1.5 ml tube and stored at – 80 °C if necessary. An equal volume of 70 % v/v ethanol was added to defrosted cell lysates and mixed by vortexing. This mixture was added to a total RNA binding silica column, centrifuged at 13000 rpm for 15 sec (Microcentaur centrifuge, MSE, London, UK), and the flow through was discarded. The RNA-bound column was washed three times to improve RNA purity. Finally, elution solution was added to the column and RNA eluted into a clean collection tube by centrifugation (13000 rpm, Microcentaur centrifuge, 1 min). RNA yield and quality were assessed by measuring absorbance at 260 and 280 nm on a spectrophotometer (Nanodrop, Thermofisher scientific). A 260/280 ratio of approximately 2 indicated pure RNA. RNA was kept on ice short-term or stored at -80 °C.

2.6.2 Nucleic acid stabilisation
hGFs stimulated in a 96-well format were lysed and nucleic acids stabilised using the Sidestep lysis and stabilisation buffer (Stratagene, Agilent Technologies, Wokingham, UK). Briefly, the supernatant was removed and the cells were washed once with ice-cold PBS. To each well, 20 μl of lysis buffer was added, and cells were fully disturbed using a plate shaker (2 minutes, 900 rpm). Lysates were kept on ice short-term or stored at -80 °C.

2.7 RT-PCR

2.7.1 Reverse transcription reaction
Reverse transcription was performed on RNA/lysates using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Paisley, UK) according to the manufacturer’s instructions using a T100 thermal cycler (Bio-Rad, Hemel Hempstead, UK). Samples derived from total RNA purification were adjusted so
that they all contained the same RNA concentration. Samples derived from nucleic acid stabilisation were diluted 20-fold for reverse transcription reactions (1 μl per reaction as recommended by lysis buffer manufacturer). Controls containing nucleic acids but no reverse transcriptase were performed and were included in all downstream RT-PCR experiments. RNA (10 μl diluted in molecular grade H₂O as necessary) was mixed with 10 μl reverse transcription mastermix containing 8 mM dNTPs, 5 U/μl MuLV reverse transcriptase, and random primers and a buffer mix diluted according to the manufacturer’s protocol. Samples were incubated at 25 °C for 10 min to promote primer annealing, then 37 °C for 120 min to promote reverse transcriptase-mediated extension, and finally at 85 °C for 5 min to inhibit enzyme activity. cDNA was stored at 4°C. cDNA derived from stabilised nucleic acid lysates were diluted 2.5-fold in water prior to use.

2.7.2 Conventional RT-PCR
RT-PCR was performed on cDNA (2.5 μl) by incubation with specific primers (2 μM final concentration), 1x BioMix Red reaction mix containing Taq DNA polymerase (Bioline, London, UK) and water for a 25 μl reaction. Controls containing water instead of cDNA were performed to control for contamination. Thermal cycling was performed in a T100 thermal cycler under the following conditions:

Step 1 (denaturation): 94°C, 5 min

Step 2: (60°C, 1 min for annealing, 72°C, 1 min for extension, 94°C, 1 min for denaturation) x 35 cycles

Step 3 (final extension): 72°C, 10 min

Table 2.2 displays the primers used and the expected product size. PCR products were confirmed by GelRed (Biotium, Cambridge Bioscience, Cambridge, UK)-containing agarose gel electrophoresis and images were captured under ultraviolet transillumination using Genesnap software on a G:Box imaging system (Syngene, Cambridge, UK). Hyperladders IV or V (Bioline) were run on all gels to confirm product size.

2.7.3 Real-time RT-PCR
cDNA (diluted in molecular grade H₂O as necessary) was mixed with 600 nM forward and reverse primers, 1X TaqMan universal mastermix (Applied Biosystems) and either 150 nM 5’carboxyfluorescein/3’tetramethylrhodamine PrimeTime qPCR probes
(Integrated DNA Technologies, Glasgow, UK) or 1X TaqMan assay (POLR2A Hs00172187_m1, IL24 Hs01114274_m1, COL6A3 Hs00915125_m1) (Applied Biosystems) to give a final reaction volume of 10 µl per well in a 96-well PCR plate (Applied Biosystems). Controls containing water instead of cDNA were performed to control for contamination. PCR was performed on a 7900 HT real-time PCR system (Applied Biosystems). Table 2.2 lists the primer/probe sequences used. The cycling conditions used were as follows:

Step 1 (denaturation): 95°C, 10 min

Step 2 (annealing, extension, denaturation): (95°C for 15 s, 60°C for 1 min) x 40 cycles

Threshold cycle (Ct) values were acquired and the 2^(-ΔΔCt) analysis method was performed (Livak and Schmittgen, 2001) (Figure C.1). Relative quantitation was performed by normalisation to the reference gene RNA polymerase II (RNAP). The amount of cDNA used was determined by the ability of samples to generate sigmoidal amplification curves with a distinct exponential phase which generated a C_t between 15 and 35.

18S rRNA was also assessed for use as a reference gene in preliminary experiments. However, the C_t values for RNAP had a lower coefficient of variance (CV) than 18S rRNA both within and between treatment conditions suggesting that 18S rRNA was a less appropriate reference gene for hGFs (Figure C.2). Similar findings have been reported elsewhere (Schmittgen and Zakrajsek, 2000). As such, RNAP was chosen as the reference gene for real-time data in this study. Replicate PCR reactions were performed to establish the extent of technical variation. The CV was < 4 % and therefore subsequent PCR reactions for each sample were performed in single for each sample.

2.8 Microarray analysis

Two array formats were used to probe leptin stimulated hGFs for mRNA expression at a time point of 24 h. Ideally, gene expression would have been measured at multiple time points but only limited sample numbers could be analysed in this study. Microarray analysis requires a large quantity of good quality RNA. Therefore, samples for microarray analysis were generated using hGFs in a 6-well format.

2.8.1 TaqMan Low Density Array

The human immune panel TaqMan Low Density Array (TLDA) (Applied Biosystems) was used to explore gene expression in hGFs. For each sample, this array quantified the
expression of 96 genes. The genes on the array were pre-selected because their gene products function in immunity (Table D.1). Gene expression was determined by real-time RT-PCR but instead of preparing a reaction mix which contains primers and probes, the primers and probes were already distributed onto the microfluidic card.

Total RNA was purified from stimulated hGFs and quantified using the GenElute kit (Section 2.6.1). Equal amounts of RNA were used to generate cDNA by reverse transcription (Section 2.7.1). The amount of input RNA was calculated to generate cDNA (assuming 100 % conversion) in the range of 100-500 ng per reaction well to assess moderate levels of gene expression (as recommended in the manufacturer’s protocol). cDNA (diluted in H₂O) and 1X TaqMan universal mastermix were mixed and then loaded by pipette into the sample reservoirs on the microfluidic card. The cards were then centrifuged (1200 rpm, 2 x 1 min, Heraeus multifuge 3L, Thermofisher Scientific) to distribute the sample into the reaction wells. Finally, the reaction wells were sealed using a card sealer (Applied Biosystems) and the sample reservoirs were removed.

Prior to RT-PCR of the TLDA a background run was performed to control for the microarray card format. Real-time PCR of the 384-well TLDA microarray card was performed on a 7900 HT real-time PCR system. The cycling conditions for real-time RT-PCR analysis were used (Section 2.7.3). Cₜ values were acquired and relative quantitation performed by normalisation to the reference genes 18S rRNA or β-actin (Livak and Schmittgen, 2001).

2.8.2 Genome-wide expression microarray

The Human HT-12 v4 expression beadchip (Illumina, Little Chesterford, UK) was used to explore genome-wide transcriptional profiles in hGFs. This array contains over 47000 probes from the National Center for Biotechnology Information RefSeq database release 38 that detects well-characterised genes as well as candidate genes and splice variants. This exploratory approach has the potential to identify novel genes regulated by leptin in hGFs. Each array has the capacity for 12 samples. The 12 samples chosen for analysis in this study included 4 treatment conditions (unstimulated, leptin, IL-1β, leptin+IL-1β) in three donors to allow for statistical analysis to be performed on the data.

hGFs from donors which were responsive to leptin and IL-1 were used in the stimulations for microarray analysis. hGFs were stimulated in duplicate to allow for
either pooling of samples or for choosing one sample for downstream analysis if poor quality RNA was generated. Total RNA was purified from stimulated hGFs using the RNeasy kit (2x10^5 cells/well) and quantified using the Nanodrop spectrophotometer (Section 2.6.1).

RNA (500 ng per sample) was sent to Cambridge Genomic Services (CGS, Cambridge, UK) for expression analysis using the Illumina beadchip. This service involved an additional quality control (QC) analysis. The RNA samples were analysed using an electrophoresis-based approach (2100 Bioanalyzer, Agilent Technologies) to generate a RNA integrity number (which should be > 8). Then, RNA amplification was performed using the TotalPrep 96-RNA amplification kit (Ambion, Life Technologies) to create biotin-labelled cRNA. This cRNA was hybridised onto the beadchip and assessed according to the workflow in Figure E.1A. Essentially, cRNA was hybridised to a complementary oligonucleotide probe. Each probe is linked via an address sequence to a bead which sits in a specific location on the array. Streptavidin conjugated to the fluorophore Cy3 is bound to biotin-conjugated cRNA and fluorescence is quantitatively detected local to each bead. The fluorescent signal intensity generated for each probe was reported by CGS and was used for bioinformatics analysis (Stekel, 2003).

Analysis of the microarray dataset was performed with the assistance of Mr Andrew Skelton (Musculoskeletal Research Group and Bioinformatics Support Unit, Newcastle University) using R Studio software (Boston, USA). The dataset acquired from CGS was normalised using the robust spline method. The quality of the dataset was confirmed using MA plots, mean v SD plots, box and whisker plots and density plots (Figure E.2). Principle component analysis identified that the datasets for the samples from one donor were different to the others in one dimension (Figure E.2E). Therefore, the dataset was corrected using the “ComBat/surrogate variable analysis” add-on to the R studio software to eliminate this variation, and to maintain the statistical power of the experiment (Johnson et al., 2007; Leek and Storey, 2007).

After correction, the datasets for different treatment conditions were compared (unstimulated v leptin, unstimulated v IL-1, unstimulated v leptin+IL-1, leptin v leptin+IL-1, IL1 v leptin+IL-1) to generate lists of differentially expressed genes. Only genes which had a raw (not log-transformed) fold change of > 2 and a corrected p value of < 0.01 were considered differentially expressed. Corrected p values were generated
using the Benjamini-Hochberg method for multiple comparisons to reduce the likelihood of false positives.

Two enrichment analyses were performed on the differentially expressed gene lists to identify biological processes and pathways significantly over-represented in the selected comparisons (unstimulated v leptin, unstimulated v IL-1, unstimulated v leptin+IL-1). Gene ontology (GO) analysis (biological processes only) was performed using the “GOstats” add-on to the R Studio software. GO terms are a consistently labelled group of biological processes, arranged hierarchically, that are affiliated to genes based on existing biological knowledge regarding gene product function. Therefore, numerous genes are affiliated to the same GO term. Kyoto encyclopaedia of genes and genomes (KEGG) analysis was performed using the “pathview” add-on to the R Studio software. The KEGG database contains biological pathways in which the link between different gene products can be visualised. Therefore KEGG analysis can generate information relevant to the overall biological pathways that may be active under the conditions used to generate a dataset (e.g. pathways upregulated in leptin-stimulated hGFs compared to unstimulated hGFs).

Significant GO terms and KEGG pathways were deduced by performing a hypergeometric test. Essentially, this statistical test determines whether the observed number of genes associated with a GO term or KEGG pathway in a list of genes is significantly different to the number expected in a universal list of genes. In this study, only GO terms over-represented with a corrected p-value of <0.001 were considered significant. Only KEGG pathways over-represented with a corrected p-value of <0.01 were considered significant.

Only selected genes, GO terms and KEGG pathways relevant to inflammation or other processes in which leptin or IL-1 are thought to function (based on the existing literature) are presented in this study.

2.9 ELISA

hGF supernatants were assessed for human total MMP-1, total MMP-3, IL-6 and IL-24 by ELISA (R&D systems) according to the manufacturer’s protocol. The MMP ELISAs detected active and inactive MMP. Supernatants were stored at – 80 °C prior to analysis. The plates for the IL-6 ELISA were pre-coated and samples/standards could therefore be directly dispensed into the wells. For the other ELISAs (MMP-1, MMP-3 and IL-24), 96-well plates (R&D Systems) were coated with capture antibody diluted in
PBS at RT overnight. Next, the plates were aspirated and washed with 0.05 % Tween 20 in PBS (wash buffer) three times using a plate washer (BioTek). The plates were then blocked by filling each well with 1 % BSA in PBS (reagent diluent) for 1 h at RT. Meanwhile, 2-fold serial dilutions were performed with the standard in reagent diluent to give a seven-point standard curve (MMP-1 top standard: 10 ng/ml, MMP-3 top standard: 2 ng/ml, IL-6 top standard: 300 pg/ml, IL-24 top standard: 4 ng/ml). Similarly, cell culture supernatants were diluted in reagent diluent as necessary. The aspiration/wash step was repeated as above, and then 100 µl standards, samples or blank (reagent diluent) in duplicate were dispensed in the wells and incubated for 2 h at RT. Next, the aspiration/wash step was repeated as above, before a biotinylated detection antibody (diluted in reagent diluent) was added to the wells and incubated for 2 h at RT. Plates were aspirated/washed as above and then a streptavidin-horseradish peroxidase (HRP) solution (diluted in reagent diluent) was added to the wells for 20 min at RT in the dark. A final aspiration/wash step was performed as above before a 1:1 mixture of H₂O₂ and tetramethylbenzidine was added to the wells for 20 min at RT in the dark. Colour development was stopped by adding 2 N H₂SO₄ to the wells. The optical density of each well was determined at 450 nm and 550 nm using a Synergy HT plate reader. The readings at 550 nm were subtracted from the readings at 450 nm to correct for plate imperfections.

The mean readings for each standard concentration and the blank were used to generate a 4PL curve fit (Appendix K). Sample concentrations were determined from the standard curve and multiplied by any dilution factor. In some of the results obtained in this study there was substantial variation in the protein concentrations detected between donors; however the pattern of response remained the same. In this circumstance, protein levels were expressed in relation to the unstimulated control (either as a percentage or fold change).

2.10 Flow cytometry

hGFs isolated according to the procedures described previously (Section 2.2.7), were split into fluorescence-activated cell sorting (FACS) tubes and washed in 0.5 % v/v FBS in PBS (FACS buffer) before centrifugation at 1500 rpm for 5 min (Heraeus Megafuge 16R Thermofisher Scientific). The pellet was resuspended in residual FACS buffer and then either left unstained or stained with an isotype control antibody or target antibody for 30 min at 4 °C (Table 2.3). The cells were washed in FACS buffer and centrifuged (1500 rpm, Heraeus Megafuge 16R, 5 min). The resulting cell pellets were resuspended
in 300 μl FACS buffer then assessed for antibody staining using a FACScaliber flow cytometer (BD, Oxford, UK) by Professor Steve Todryk (Northumbria University). ≥ 10000 gated events were acquired for each sample and data was collected using CellQuest software (BD). Data analysis was performed using winMDI software. hGFs were gated as shown in Figure 2.4 to remove likely cell debris from further analysis.

2.11 SDS-PAGE and immunoblotting

SDS-PAGE uses an electric current to separate proteins in a polyacrylamide gel according to mass. Once separated the proteins are transferred to a membrane and probed with specific antibodies conjugated to HRP. The membrane is covered with a substrate that when oxidised by HRP results in the emission of light. Proteins of interest are then detected by chemiluminescence at the area of the membrane that corresponds to the proteins known molecular mass.

2.11.1 Cell preparation for SDS-PAGE

hGFs were stimulated in 6 well plates or 25 cm² culture flasks to generate enough protein for detection by immunoblotting. At selected time-points post stimulation (0 - 4 h) hGFs were washed in ice-cold PBS and the culture surface aspirated thoroughly to ensure that the lysis buffer was not diluted. Next, 100 μl (6-well plate) or 130 μl (T25) lysis buffer (50 mM TRIS/HCl (Melford, Ipswich, UK) pH 7.5, 1 mM ethylene glycol tetraacetic acid, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 50 mM sodium fluoride, 10 % v/v glycerol, 0.3 % v/v Triton X100, 5 mM sodium pyrophosphate, 1 μM microcystin LR, 1 x complete cocktail inhibitor (Roche, Burgess Hill, UK), 0.1 % v/v β-mercaptoethanol) was added per flask while on ice. This lysis buffer was based on that used by Hui et al (2012) except the amount of Triton X100 was reduced from 1 % to 0.3 % to eliminate interference with the Bradford assay (Table 2.4). Cells were scraped from the flask into a 1.5 ml centrifuge tube and then incubated on ice for 20 min. Next, the lysates were centrifuged at 15493 x g for 3 min at 4 °C (3K10 centrifuge). The resulting supernatant was transferred to a new ice-cold tube and stored at -80 °C.

2.11.2 Bradford assay

The Bradford assay was used to assess protein concentration in cell lysates. BSA standards (0.4 – 4 μg/well) (Thermofisher Scientific) and a water blank were added to 96-well plates in duplicate and used to generate a standard curve. For sample analysis, cell lysates or lysis buffer as a control, were diluted in water (final volume 10 μl) and
added to 96-well plates in duplicate. Bradford assay reagent (Pierce, Thermo Scientific) (150 μl/well) was added, mixed, and the plates were incubated for 5 min at RT. Absorbance at 595 nm was assessed using a Synergy HT plate reader.

The blank absorbance was subtracted from all samples and standards. The mean readings for each standard concentration were used to generate a 4PL curve fit (Figure 2.5). Samples with a CV > 10 % were repeated. Protein concentrations in samples were determined from the standard curve and used to normalise the concentration between lysates. Subsequently, samples were diluted in sample buffer (0.52 M TRIS, 0.16 M sodium dodecyl sulphate (SDS), 33 % v/v glycerol, 0.03 % w/v bromophenol blue, 17 % v/v β-mercaptoethanol) at a ratio of 4:1. Next, diluted samples were boiled for 5 min to promote protein denaturation and reduction, and then stored at -20 °C.

2.11.3 SDS-PAGE and immunoblotting
Polyacrylamide gels (10 %) were cast (Separating gel: 350mM Tris-base pH 8.8, 0.1 % w/v SDS, 10 % v/v bis/acrylamide (NBS Biologicals, Huntingdon, UK), 0.2 % w/v ammonium persulphate (APS), 3 % v/v tetramethylethylenediamine (TEMED)). Stacking gel (125 mM Tris-base pH 6.8, 0.1 % w/v SDS, 4.5 % v/v bis/acrylamide, 0.1 % APS, 2 % v/v TEMED) was cast on top of the separating gel and combs were inserted. Once set, samples (≤ 20 μg and equal volume for each sample per gel) or Pageruler prestained protein ladder (5 μl) (Thermo Scientific) were loaded onto the gel. Gels were placed in a tank (Mini-PROTEAN Tetra Cell system, Bio-Rad) and submerged in electrophoresis running buffer (25 mM Tris-base, 200 mM glycine, 1 % w/v SDS). Electrophoresis was performed at 100 V while samples moved through the separating gel, and then increased to 150 V until sufficient separation was achieved. Next, the proteins were transferred from the gel to nitrocellulose membranes using a 7 min pre-programmed blotting protocol using the iBlot gel transfer device (Invitrogen, Life Technologies) according to the manufacturer’s instructions.

After transfer membranes were washed in methanol (Thermo Fisher Scientific) and rinsed in water. The membranes were then blocked (5 % w/v non-fat dry milk powder (Marvel, Premier Foods, St. Albans, UK) in TBS/Tween-20 (TBS/T) (150 mM NaCl, 10 mM Tris/HCl pH 7.5, 0.1 % v/v Tween-20)) for 1 h at RT. Blocking buffer was removed by washing in TBS/T (3 x 5 min) before membranes were incubated with primary antibody (diluted in 5 % w/v BSA, 0.02 % w/v sodium azide in TBS/T) for 18 h at 4 °C (Table 2.5). Primary antibody solution was washed from the membranes using
TBS/T (3 x 5 min) and then membranes were incubated for 1 h at RT with HRP-conjugated secondary antibody (diluted in 5% w/v non-fat dry milk powder, 0.02% w/v sodium azide in TBS/T) with specificity for the species the primary antibody was raised in (Table 2.5). Excess secondary antibody was removed by washing the membranes in TBS/T (3 x 5 min) before the western blots were developed using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare, Hatfield, UK), Immobilon Western chemiluminescent HRP substrate (EMD Millipore, Watford, UK) or ECL Select Western blotting detection reagent (GE Healthcare). Band detection and image acquisition were performed using a Chemigenius II Bioimager (Syngene). Where required, antibodies were stripped from the membranes by washing for 30 min (3x) in 100 mM glycine, pH 2 heated to boiling point.

2.12 GAG assay
This assay compares the amount of GAG in tissue pieces to that of the tissue supernatant which gives an indication of ECM GAG degradation. This simple assay is based on that of Farndale et al. (1986) using the dye dimethyl-methylene blue (DMB). Cationic DMB binds highly negatively charged sulphated GAGs. DMB is metachromatic and this binding causes a colour change that can be measured by spectrophotometry.

2.12.1 GAG assay procedure
Chondroitin 4-sulphate standards were prepared at concentrations between 0 - 40 μg/ml in 0.1 M phosphate buffer (Section 2.2.9.1 for buffer details). DMB reagent was prepared by adding 16 mg DMB to a solution of 40 mM glycine, 40 mM NaCl in 10 mM HCl, pH3. DMB reagent was stored at RT away from direct light. The absorbance of the DMB reagent at 525 nm should be approximately 0.3 (Farndale et al., 1986). To confirm this, DMB (250 μl) was dispensed into 4 wells of a 96-well plate and the mean absorbance at 525 nm was measured to be 0.25 using a plate reader.

Gingival tissue digests (Section 2.2.9.1) or tissue supernatants (diluted in 0.1 M phosphate buffer as required) and standards were dispensed into a 96-well plate in duplicate (40 μl/well). DMB reagent (250 μl) was dispensed into each well and the absorbance at 525 nm was read immediately. Background absorbance was subtracted from each well. A linear standard plot was generated from the standards which was subsequently used to calculate the concentrations of GAG in samples (taking account of
any dilutions) (Figure 2.6). These concentrations were used to determine the amount of GAG in the original supernatant or tissue digest. Results are reported as % GAG release calculated using the formula:

\[
\left( \frac{\text{GAG in supernatant (\(\mu g\))}}{\text{total GAG (\(\mu g\)) (supernatant + tissue)}} \right) \times 100
\]

2.13 Hydroxyproline assay

Hydroxyproline is an unusual amino acid formed by the hydroxylation of proline, is found almost exclusively in collagen, and therefore, hydroxyproline residues released from tissue indicate that collagen fibrils have been proteolytically cleaved (Gorres and Raines, 2010). Hydroxyproline levels are therefore used as a way of assessing relative collagen concentrations. This assay compares the amount of hydroxyproline in tissue pieces to that of the tissue supernatant, which gives an indication of collagen release from the tissue, most likely caused by collagenase activity. To analyse hydroxyproline, proteins must first undergo hydrolysis to release individual amino acids into solution. Hydroxyproline is then oxidised to form a pyrrole. This pyrrole interacts with the organic molecule 4-(dimethylamino)benzaldehyde (DAB) to form a coloured product that can be measured by spectrophotometry. This assay is based on the method of Bergman and Loxley (1963).

2.13.1 Acid hydrolysis of gingival tissue digests and supernatants

Digested gingival tissue and tissue supernatants (120-260 \(\mu l\)) were mixed with an equal volume of concentrated HCl in a deep-well 96 well plate. The sample/HCl mixtures were sealed thoroughly and incubated for 4 – 18 hours at 105 \(^\circ\)C in an oven (LTE Scientific, Oldham, UK). HCl donates a proton which promotes peptide bond cleavage. Over time, this results in a mixture containing single amino acids.

Once hydrolysis was complete, the samples were briefly cooled and then the liquid was removed using an aqueous solvent protocol on the EZ-2 centrifugal evaporator (Genevac, Ipswich, UK) for \(\geq 7\) hours at a temperature of 55 \(^\circ\)C. Dried samples were resuspended in dH\(_2\)O and the samples were centrifuged (CR3i multifunction centrifuge) briefly to pellet any insoluble material.

2.13.2 Hydroxyproline assay

L-hydroxyproline standards were prepared at concentrations between 0 - 30 \(\mu g/ml\). Standards or hydrolysed samples (40 \(\mu l\) diluted in dH\(_2\)O if required) were dispensed in duplicate into a 96-well plate. Chloramine T (250 mM, prepared fresh daily) was diluted
5-fold in acetate-citrate buffer (420 mM sodium citrate, 128 mM trisodium citrate, 26 mM citric acid and 38.5 % v/v propan-2-ol), and then 25 μl was added per well. Reaction mixtures were incubated at RT for 4 minutes. DAB (4.5 M in 70 % v/v perchloric acid) was diluted 4-fold in propan-2-ol and then 150 μl was added per well. The plate was sealed well and incubated at 65 °C for 35 min in an oven. The plate was then cooled briefly, before absorbance at 560 nm was detected using a plate reader.

Background absorbance (dH₂O) was subtracted from each well. A linear plot was generated from the standards and was subsequently used to calculate the unknown concentrations of hydroxyproline in samples (taking account of any dilutions) (Figure 2.7). These concentrations were used to determine the amount of hydroxyproline in the acid hydrolysis reaction. This value was used to calculate the amount of hydroxyproline in the original supernatant or tissue digest. Results are reported as % hydroxyproline release calculated using the formula:

\[
\left( \frac{\text{OHP in supernatant (ng)}}{\text{total OHP (ng) (supernatant + tissue)}} \right) \times 100
\]

2.14 MMP activity assay

This assay for MMP activity is based on the method of Neumann et al (2004). The FS-6 substrate is a 9 amino acid peptide which contains a site that is cleaved by multiple MMPs. The fluorophore (7-methoxycoumarin-4yl)acetyl is bound to the C-terminal of the substrate with an internal dipicolinic acid quencher on the N-terminal side of the cleavage site. Therefore, before cleavage, fluorescence is quenched due to FRET. After substrate cleavage the fluorophore is no longer quenched and fluorescence can be detected and quantified.

The MMP substrate was dissolved in dH₂O at a concentration of 5 mM and stored in aliquots at -20 °C. The fluorophore 7-amino-4-methylcoumarin (AMC) was dissolved in methanol at a concentration of 5 mM and stored at -20 °C. When needed, this MMP substrate stock was diluted to 250 μM in assay buffer (100 mM TRIS, 100 mM NaCl, 10 mM CaCl₂, 0.1% (w/v) PEG6000, 0.05% (w/v) Brij 35). Similarly, AMC was diluted to a working concentration of 25 μM in assay buffer.

The total assay volume was 100 μl in 96-well format and 30 μl in 384-well format opaque black microtitre plates. In the 384-well format assay 1.5 – 6 μl of neat test sample (hGF or gingival tissue supernatant) was dispensed per well. The test sample was diluted in assay buffer to a volume of 24 μl/well. Then 6 μl of 250 μM MMP
substrate was added per well (final concentration: 50 μM). This mixture was incubated at 37 °C to promote MMP activity and substrate cleavage. In this study incubation times of between 0 – 4 h were assessed. Fluorescence was measured using a plate reader (Excitation 310 nm, Emission 420 nm). In the 96-well format assay the volumes of sample and MMP substrate were increased by 3.33-fold to account for the increased total assay volume per well.

Several controls were performed in each assay; a blank (assay buffer only), a control for background substrate fluorescence (50 μM substrate, no sample), and a positive control (5 μM AMC). 5 μM AMC gives a fluorescence reading that would be expected for 10% degradation of the substrate as the substrate is used at a final concentration of 50 μM. The endpoint of the assay should be timed so that substrate fluorescence does not exceed that of the AMC control to ensure an excess of substrate for MMPs to target.

This assay can be adapted to measure total MMP activity by incubating samples in the presence of 4-aminophenylmercuric acetate (APMA). Where used, APMA was dissolved at a concentration of 100 mM in DMSO, and spiked into the sample/substrate mixture to give a final concentration of 1 mM.

In preliminary experiments, fluorescence was measured at different sensitivities. Assay buffer (blank control), 50 μM substrate or 5 μM AMC were dispensed in duplicate into a 96-well plate (100 μl/well) or 384-well plate (30 μl/well). Plates were incubated for 30 minutes at 37 °C and fluorescence was measured using a plate reader (BioTek). The 384-well format was adopted at a sensitivity of 120 (Table 2.6). Using this protocol, the assay had a low % CV, the greatest range between the background substrate fluorescence and the AMC control, and required less reagent/sample volume.

Recombinant human MMP-1 was spiked (100 pg) into assay buffer and tissue culture medium in the presence of APMA. After a 2 h incubation significant MMP activity could be detected in both spiked assay buffer and tissue culture medium confirming that this assay can measure MMP activity (Figure 2.8).

2.15 Statistical analysis

Experiments were performed at least in triplicate unless indicated otherwise, and each experiment was repeated in at least three different hGF donors. SPSS 15.0 (IBM, Portsmouth, UK) was used for statistical analysis. Statistical analysis of real-time RT-PCR data was performed on δCt values as previously described (Yuan et al., 2006).
Means + SD, or medians ± interquartile range are presented depending on whether the datasets had parametric or non-parametric distributions respectively. This was assessed by using the Shapiro-Wilk test for normality and the Levene test for homogeneity of variance. Parametric data were analysed using the Student’s t test, while non-parametric data were analysed using the Mann Whitney U test as indicated in the results. Both tests were performed with the Bonferroni-Holm correction for multiple comparisons. Differences were considered significant if p < 0.05 for all experiments except for the Illumina microarray data analysis where p < 0.01 was required for significance (Section 2.8.2). Only selected comparisons are shown for clarity.
Primary hGFs were isolated from gingival tissue by outgrowth. hGFs were maintained in DMEM+ in plastic culture flasks. Once confluent, hGFs were subcultured and seeded into new flasks at a lower cell density. This image was taken using a CKX41 light microscope and displays viable hGFs at a low confluency at passage 9. At low confluency the characteristic spindle-shaped morphology of fibroblasts is evident. 100x magnification.
Figure 2.2: hGFs are vimentin positive.

hGFs were cultured on glass slides until confluent. Cells were fixed and stained for vimentin by immunocytochemistry (Section 2.3). Stained samples were mounted onto microscope slides and visualised by light microscopy [A] No antibody control [B] anti-vimentin antibody. Data presented is from one donor and representative of the three donors tested independently. Immunocytochemistry was performed in duplicate for each donor. Images are at x200 magnification.
Table 2.1: A summary of the plastic-ware formats, seeding densities and endpoints used throughout this study.

Seeding densities were chosen to ensure sufficient cell numbers to prevent apoptosis, but with enough space on the growing surface to allow for cell attachment and growth. RT-PCR – reverse transcription PCR, ELISA – enzyme-linked immunosorbant assay.

<table>
<thead>
<tr>
<th>Plastic-ware format</th>
<th>Seeding density (cells/well or flask)</th>
<th>Maximum volume/surface</th>
<th>Endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well plate</td>
<td>0.3-1.4x10^4</td>
<td>200 µl</td>
<td>real-time/conventional RT-PCR proliferation assay ELISA</td>
</tr>
<tr>
<td>6 well plate</td>
<td>1-2x10^5</td>
<td>2 ml</td>
<td>conventional RT-PCR ELISA immunoblotting microarray</td>
</tr>
<tr>
<td>24 well plate</td>
<td>4x10^4</td>
<td>800 µl</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>25 cm² cell culture flask</td>
<td>2-4x10^5</td>
<td>2.8 ml</td>
<td>immunoblotting</td>
</tr>
</tbody>
</table>
Figure 2.3: The effect of serum on hGF proliferation.

Seeded hGFs incubated in DMEM+ were washed in PBS. PBS was replaced with either SFM (serum starved) or DMEM+ and hGFs were incubated overnight (18 h). hGFs were then either maintained in SFM or DMEM+, or SFM was replaced with DMEM supplemented with 5 or 10 % FBS. hGFs were incubated for a further 24 h at which time proliferation was assessed by a colourimetric proliferation assay. Data (median±IQR, n=10-41 from 11 independent experiments in 5 different donors) are presented as % SFM-treated control. Statistics: Mann Whitney U test for independent samples (Bonferroni-corrected). ***=p<0.001 compared to SFM-treated control. SFM – serum-free medium.
Table 2.2: The forward and reverse primer and probe sequences used for RT-PCR analysis of gene expression.

Primers and probes were aliquoted and stored at -20 °C. β2m – β2-microglobulin, LEPR – leptin receptor (long isoform).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>5’-AAGATGAAAGTGGAACAAAATT-3</td>
<td>5’-CCAAGAGAATGGCGGAGTTCC-3’</td>
<td>5’-FAM-CAGAGAGTCAACTACATCGTGTTCGGGCTC-3’-TAMRA-3’</td>
<td>150 bp</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5’-TGCCGATGGATACCCCCTTT-3’</td>
<td>5’-TTCCTCCCAAGGTCCATAGCTCAT-3’</td>
<td>5’-FAM-CTCCTGGCTCATGCCCTTGCCC-TAMRA-3’</td>
<td>425 bp</td>
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<tr>
<td>MMP-3</td>
<td>5’-TCCGCTTGTTCAAGATGATAT-3’</td>
<td>5’-AAAGGACAAAGCAGGATACAGT T-3’</td>
<td>5’-FAM-TGATCCCTTGACACCTCCCCCTGAC-TAMRA-3’</td>
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<tr>
<td>MMP-7</td>
<td>5’-CTTTGCGGAGGAGCTCA-3’</td>
<td>5’-CAGCCGAAAGGATGA-3’</td>
<td>5’-FAM-CCATTGATGGGAGAGAAGACG-TAMRA-3’</td>
<td>428 bp</td>
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<tr>
<td>MMP-8</td>
<td>5’-CAGGCCCTCAAGATGACATCGA-3’</td>
<td>5’-ACGGAGTGTTGCTATGACATCA-3’</td>
<td>5’-FAM-CAAGCACCCCTATCCACCTACTGGACCAA-TAMRA-3’</td>
<td>425 bp</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5’-CCTGGGCAGATCACAACCT-3’</td>
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<td>MMP-10</td>
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<td>MMP-13</td>
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<tr>
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<td>5’-AGGAGATGTAGCACGGGATCA-3’</td>
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<td>5’-ATTAGCTCAGAATACGTTATG TATCC-3’</td>
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<tr>
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<tr>
<td>β2m</td>
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<td>5’-CTTATGCAAGCTTAACTATC-3’</td>
<td>5’-FAM-CTCGGAGTGAAGATACGCGC-TAMRA-3’</td>
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</table>
Table 2.3: Details of the antibodies used for flow cytometry.

PE – phycoerythrin, APC - allophycocyanin, TLR – Toll-like receptor. EBioscience, Hatfield, UK; AbD Serotec, Kidlington, UK

<table>
<thead>
<tr>
<th>Target</th>
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<th>Clone</th>
<th>Isotype</th>
<th>Species</th>
<th>Supplier</th>
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<td>R&amp;D systems</td>
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</tbody>
</table>


Figure 2.4: Forward and side scatter properties of hGFs.

hGFs were prepared for flow cytometry (Section 2.2.7). Forward and side scatter were assessed on unstained cells according to the methods, and this data is presented in dot plots. The region highlighted in this dot plot was used to gate cells and omit cellular debris.
Table 2.4: Absorbance values derived from the Bradford assay.

Cell lysis buffer with two different concentrations of Triton X100 or Triton X100 alone (at concentrations of 5 – 0.1 %) were used as samples for the Bradford assay (Section 2.11.2). Data (n=2-4) shows absorbance values measured at 595 nm which had the background absorbance (from a water blank) subtracted.

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<tr>
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</tr>
<tr>
<td>5</td>
<td>&gt; top standard</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt; top standard</td>
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</table>
Figure 2.5: Representative standard curve for the Bradford assay.

Blank-subtracted absorbance (blank 595 nm) was plotted against standard protein (BSA) concentrations (0 – 4 µg/well, n=2 per concentration). A 4-parameter logarithmic curve was fitted and used to determine the unknown concentration of proteins in samples.
Table 2.5: Targets, species of origin, dilutions and suppliers for antibodies used in this study.

The site/s of phosphorylation in the phospho-epitopes detected by the antibodies used in this study are displayed in brackets using the single letter amino acid code to indicate the amino acid type, and a number which indicates how far downstream of the translation initiating methionine this amino acid residue is found in the protein (e.g. for ERK, T202 represents the amino acid threonine at position 202 in this protein). GAPDH – Glyceraldehyde-3’-phosphate dehydrogenase, JNK – c-Jun N-terminal kinase, SAPK – stress-activated protein kinase. Cell Signaling Technology, New England Biolabs, Hitchin, UK

<table>
<thead>
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<th>Target (phosphorylation site/s)</th>
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<th>Dilution</th>
<th>Manufacturer</th>
</tr>
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<td>Polyclonal anti-mouse</td>
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<td>Dako</td>
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</table>
Figure 2.6: A representative standard plot for the GAG assay.

The mean blank-subtracted absorbance at 525 nm was plotted against chondroitin sulphate standard concentrations (0 – 40 μg/ml, n=2 per concentration). A linear trend line was fitted. The equation of the trend line and the R$^2$ value are also displayed.
Figure 2.7: Representative standard plot for the hydroxyproline assay.

Blank-corrected absorbance (595 nm: mean±SD n=2) was plotted against standard hydroxyproline (OHP) concentrations (0 – 30 µg/ml). A linear trend line was fitted and used to determine the unknown concentration of hydroxyproline in samples. The equation of the trend line and the R² value are also displayed.
Table 2.6: Comparison of FS6 assay controls at different sensitivities.

Relative fluorescence units (RFU) measured in the blank, substrate and AMC controls are displayed in this table at sensitivities between 35-120 (excitation 310 nm, emission 420 nm). The mean RFU (n=2) and % CV are displayed for both 96- and 384-well plate assay formats. The fold difference between the substrate and AMC control is presented as an indication of the dynamic range of the assay. ND – not done.

<table>
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<tr>
<td></td>
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</table>
Figure 2.8: MMP activity in spiked assay buffer and tissue culture medium.

Recombinant human MMP-1 was spiked into MMP activity assay buffer or serum-free medium to result in 100 µg MMP-1/well. APMA was added to MMP-1-containing wells to activate the recombinant MMP-1 and samples were then incubated with the MMP substrate FS-6 for 2 h at 37 °C. Fluorescence was measured (excitation 310 nm, emission 420 nm). Data (RFU) are presented as mean+SD (n=2-4). Statistics: T tests for independent samples (Bonferroni-corrected) ***=p<0.001, **=p<0.01 as indicated.
Chapter 3. Regulation of MMP and TIMP production by gingival fibroblasts stimulated with leptin

3.1 Introduction
Leptin has a broad range of functions, including regulating energy expenditure, reproductive capability and immunocompetence (Myers et al., 2008). Leptin appears to regulate these functions both by signalling to the CNS, and by signalling directly to peripheral cells (Mantzoros et al., 2011). One of the peripheral outcomes of leptin signalling is a change in cellular phenotype relevant to tissue remodelling (Schram et al., 2010). Leptin decreases the production of the collagenase MMP-1 in HSCs (Cao et al., 2007), but increases MMP-1 production in cartilage and chondrocytes (Koskinen et al., 2011; Hui et al., 2012). Similarly, leptin increases the expression of the MMP inhibitor TIMP-1 in HUVECs (Park et al., 2001), but decreases TIMP-1 expression in a murine cardiomyocyte cell line (Schram et al., 2010). These conflicting results are likely due to the different cells and tissues assessed, and the concentrations of leptin used. Several studies report that leptin increases the production of the collagenase MMP-13 in glioma cells, cartilage and chondrocytes (Yeh et al., 2009; Koskinen et al., 2011; Hui et al., 2012). Only one study has attempted to investigate whether leptin regulates the production of the collagenase MMP-8 (Koskinen et al., 2011), but no MMP-8 was detected in any of the human osteoarthritis cartilage samples used, suggesting that leptin cannot induce MMP-8 in this tissue. Several studies have reported that leptin increases the expression, secretion and activity of the gelatinases (MMP-2/9) in HUVECs (Park et al., 2001), a breast cancer cell line (McMurtry et al., 2009), a cardiomyocyte cell line (Schram et al., 2010), and cardiac fibroblasts (Schram et al., 2011). In contrast, leptin has no effect on MMP-2 and MMP-9 expression and activity in a glioma cell line (Yeh et al., 2009). Increased production of the stromelysin MMP-3 and the membrane-type MMP-14 is also stimulated by leptin (Koskinen et al., 2011; Schram et al., 2011). Overall, these studies support a role for leptin in regulating ECM remodelling. However, only one of these studies was performed in fibroblasts, and this study used fibroblasts derived from rats (Schram et al., 2011). There are no reports on the ability of leptin to regulate MMP and TIMP production by human fibroblasts.

Fibroblasts sense and respond to stimuli in the gingival microenvironment (Bartold and Narayanan, 2006). Gingival fibroblasts regulate gingival tissue integrity, particularly by altering the production of MMPs and TIMPs (Ebisawa et al., 2011). Dysregulated tissue
remodelling occurs in periodontitis (Preshaw, 2008), and therefore, gingival fibroblasts are implicated in the disease pathogenesis. *In vitro* experiments have shown that primary hGFs produce MMP-1 constitutively (Yamada *et al.*, 2000; Domeij *et al.*, 2002). *In situ* hybridisation and IHC approaches showed that MMP-13 localises to fibroblasts (among other cells) within the gingiva of individuals with periodontitis (Tervahartiala *et al.*, 2000). However, in this study no healthy gingival tissue was used as a control, so it is unclear whether MMP-13 is also present in healthy gingiva.

Similarly, MMP-8 protein is produced by gingival fibroblasts isolated from individuals with chronic periodontitis (Cox *et al.*, 2006); again no healthy control fibroblasts were used for comparison. The production of MMP-1 and MMP-13 by hGFs is increased by a variety of cytokines and growth factors (Domeij *et al.*, 2002; Leivonen *et al.*, 2002; Cox *et al.*, 2006; Cury *et al.*, 2007; Ujii *et al.*, 2012). Interestingly, Domeij *et al.* (2002) identified that increased MMP-1 gene expression is initially observed at a time point of 8 h, and is maintained up to 48 h (the longest time point tested). This suggests that gingival fibroblasts, unlike neutrophils which store pre-synthesised MMP protein in intracellular granules (Birkedal-Hansen, 1993), increase the production of MMPs in a manner that requires the immediate early synthesis of transcription factors which bind to MMP promoters to increase gene expression (Sorsa *et al.*, 2006).

Gingival fibroblasts isolated from individuals with periodontitis produce MMP-2 mRNA and protein (Dahan *et al.*, 2001; Cox *et al.*, 2006). Stimulation of hGF with bacteria, bacterial supernatants and LPS mildly increases the production of MMP-2 (Dahan *et al.*, 2001; Zhou and Windsor, 2006; Bodet *et al.*, 2007; Zhang *et al.*, 2011), while growth factors and nicotine have no similar effect (Cury *et al.*, 2007; Zhou *et al.*, 2007; Zhang *et al.*, 2011; Ujii *et al.*, 2012). MMP-2 is not highly regulated at the level of gene expression, and these studies suggest that this is true for gingival fibroblasts (Birkedal-Hansen, 1993). Interestingly, *P. gingivalis* supernatant and TNF-α increase the amount of active MMP-2 produced by hGFs. Also, TNF-α induces the expression of MMP-9 in rat gingival fibroblasts (Kim *et al.*, 2009).

The stromelysins MMP-3 and MMP-10 are expressed in unstimulated hGFs (Domeij *et al.*, 2002; Mah *et al.*, 2014). Using IHC, MMP-3 was found to localise to fibroblasts in the gingiva of individuals with periodontitis (Beklen *et al.*, 2006). Cytokines, PGE₂, and LPS regulate the production of MMP-3 by hGFs, generally enhancing MMP-3 production (Domeij *et al.*, 2002; Ruwanpura *et al.*, 2004; Bodet *et al.*, 2007; Cury *et al.*, 2007). Beklen *et al.* (2006) found that TNF-α-stimulated hGFs increase the production
of inactive or pro-MMP-3, and suggested that the activation of MMP-3 requires proteases from exogenous sources. In support of this, *P. gingivalis* culture supernatant (which contains numerous potent proteases) has no effect on MMP-3 gene expression in hGFs, but increases the amount of active MMP-3 (Zhou and Windsor, 2006).

The matrilysin MMP-7 is localised to fibroblasts in the gingiva of individuals with periodontitis (Tervahartiala et al., 2000). Increased gene expression and protein levels of MMP-7 are detected in hGFs stimulated with epidermal growth factor (EGF) (Cury et al., 2007). Similarly, EGF increases the expression of the elastase MMP-12 by hGFs (Zhou and Windsor, 2006).

MMP-14 is produced by gingival fibroblasts isolated from individuals with periodontitis (Dahan et al., 2001; Cox et al., 2006). Depending on the stimuli tested, both increases and decreases in the production of MMP-14 in gingival fibroblasts have been observed (Zhou and Windsor, 2006; Smith et al., 2009; Zhang et al., 2011); other stimuli have no effect on MMP-14 production (Zhou et al., 2007; Zhang et al., 2011). Both *P. gingivalis* supernatant and nicotine increase the amount of active MMP-14 in hGFs, although the mechanism underlying this effect was not explored (Zhou and Windsor, 2006; Zhou et al., 2007).

The MMP inhibitors TIMP-1, TIMP-2, TIMP-3 and TIMP-4 are produced constitutively by gingival fibroblasts (Yamada et al., 2000; Bodet et al., 2007; Nakasone et al., 2009; Leivonen et al., 2013). TIMP-1 gene expression by hGFs is increased by bacterially-derived molecules (Zhou and Windsor, 2006; Bodet et al., 2007). However, in these studies there are inconsistencies regarding whether the changes in TIMP-1 gene expression translate to protein. Both LPS and nicotine have no effect on the protein levels of TIMP-2 in hGFs (Bodet et al., 2007; Zhou et al., 2007; Zhang et al., 2011), however increased TIMP-2 gene expression is described in hGFs stimulated with *P. gingivalis* supernatant (Zhou and Windsor, 2006). TIMP-2 forms complexes with MMP-14 and MMP-2, and is thought to be important in regulating the activity of MMP-2 at the plasma membrane (Nagase et al., 2006). In support of this, there is some evidence that components of cigarette smoke increase the localisation of TIMP-2 to the surface of hGFs (Zhou et al., 2007; Zhang et al., 2011).

Overall, there is good evidence to suggest that gingival fibroblasts express and secrete a variety of MMPs and TIMPs. Numerous and diverse inflammatory stimuli increase the production of MMPs by gingival fibroblasts; this may be relevant to the ECM
destruction that occurs in periodontitis. Stimuli that bear structural and functional similarities to leptin (e.g. cytokines and growth factors) regulate the production of MMPs in hGFs. Leptin enhances the matrix-degrading potential of several cell types, however no-one has assessed whether leptin has a similar effect on gingival fibroblasts; there is a lack of data on this phenomenon in human fibroblasts and mesenchymally-derived cells in general. Therefore, the aim of the experiments presented in this chapter was to determine whether leptin increases MMP and TIMP expression in hGFs.

3.2 Basal expression of MMPs and TIMPs in gingival fibroblasts

Unstimulated hGFs from up to seven individual donors were lysed and cDNA prepared by reverse transcription. RT-PCR was used to determine the basal mRNA expression levels of MMPs and TIMPs by hGFs (Table 3.1). Regarding the collagenases, MMP-1 and MMP-8 mRNAs were detected in unstimulated hGFs from all donors tested. However, the third collagenase, MMP-13, was not detected in unstimulated hGFs from any donor.

The gelatinase MMP-2 was expressed in unstimulated hGFs derived from 3 donors, while no basal expression of MMP-9 was detected in the donors tested (Table 3.1). The stromelysin MMP-3 was not consistently expressed at a basal level in hGFs; the expression of MMP-3 varied between donors (n=4) and between replicates (n=4 different RNA samples for each donor). Another stromelysin, MMP-10, was expressed basally in the one donor tested.

No basal expression of the matrilysin MMP-7 was detected in hGFs from both donors tested (Table 3.1). In contrast, both the membrane type-MMP MMP-14 and MMP-12 were detected in unstimulated hGFs in all donors tested (n=3). Additionally, the MMP inhibitors TIMP-1, TIMP-2 and TIMP-3 were all expressed in unstimulated hGFs from all donors tested.

3.3 Regulation of MMPs and TIMPs in gingival fibroblasts by leptin

hGFs were stimulated with leptin (0.1 – 25 µg/ml) for 6 – 48 h. hGFs were lysed and cDNA prepared by reverse transcription while the supernatants were collected. RT-PCR was used to determine the relative MMP and TIMP gene expression levels after leptin stimulation. MMP concentrations in the supernatants were assessed by ELISA. Unstimulated hGFs at each time point were used as controls.
3.3.1 MMP-1

Leptin significantly increased MMP-1 mRNA levels in hGFs at 24 h in a dose-dependent manner (Figure 3.1). A 4.4-fold increase in MMP-1 expression was observed in hGFs treated with 10 µg/ml leptin (p<0.01), while an 8.6-fold increase was detected for the 25 µg/ml leptin concentration (p<0.001) compared to the unstimulated control. To determine whether leptin had a similar effect on MMP-1 secretion, MMP-1 concentrations in the supernatants of leptin-stimulated hGFs were assessed at a 24 h time point. In agreement with the MMP-1 gene expression data, secreted MMP-1 levels were also significantly increased by leptin in a dose-dependent manner (Table 3.2). However, the concentrations of MMP-1 detected varied between donors. In one of the donors, MMP-1 was only detected in the supernatants of hGFs that had been stimulated with 10 and 25 µg/ml leptin (Table 3.2). In the other donors, MMP-1 protein was detected in the supernatants of hGFs from all treatment conditions (0 – 25 µg/ml leptin). In these donors the concentrations of MMP-1 detected were significantly higher in supernatants from hGFs stimulated with 10 or 25 µg/ml leptin compared to the unstimulated control, and the lower concentrations of leptin (0.1, 0.5, 1 µg/ml) (all p<0.001). In two of the three donors, the concentrations of MMP-1 detected were significantly higher in supernatants from hGFs stimulated with 25 µg/ml leptin compared to those stimulated with 10 µg/ml leptin (Table 3.2).

Leptin-simulated MMP-1 gene expression was assessed at two time points (6 and 24 h) to determine how MMP-1 expression in hGFs related to time (Figure 3.2). The levels of MMP-1 gene expression after leptin stimulation (10 µg/ml) in hGFs were significantly higher at 24 and 48 h respectively (both p<0.001) compared to the 6 h time point in the one donor tested. No significant difference between the levels of MMP-1 at 24 h and 48 h after leptin stimulation were detected (Figure 3.2). Additionally, the level of MMP-1 expression after leptin stimulation for 6 h was not significantly different to the unstimulated 6 h control. As MMP-1 gene expression correlated with secreted MMP-1 in cell supernatants previously (as shown in Figure 3.1 and Table 3.2), MMP-1 concentrations were assessed in hGF supernatants at 24 and 48 h time points (Figure 3.3). Secreted levels of MMP-1 were significantly higher for leptin-stimulated hGFs (10 µg/ml) at 24 and 48 h (both p<0.001) compared to the unstimulated control at the same time point. The concentration of MMP-1 detected in 10 µg/ml leptin-stimulated hGF supernatants was significantly higher (p<0.001) at 48 h compared to 24 h (Figure 3.3).
3.3.2 Other collagenases
MMP-8 and MMP-13 gene expression were assessed by real-time RT-PCR (Table 3.1). Leptin (10 µg/ml) significantly increased MMP-8 mRNA expression by 2.6-fold compared to the unstimulated control at 24 h (p<0.05). In contrast, MMP-13 mRNA was not detected at any leptin concentration tested (0, 0.1, 0.5, 1, 10, 25 µg/ml) at a 24 h time point in the two donors assessed (Table 3.1).

3.3.3 Gelatinases
Although MMP-2 was expressed constitutively (Table 3.1), leptin (0.1 – 25 µg/ml) had no significant effect on MMP-2 mRNA levels in hGFs after a 24 h stimulation (Figure 3.4). Similarly, MMP-9 mRNA, which was not detected basally by hGFs (Table 3.1), was also not detected after stimulation with leptin at any concentration tested (0.1, 0.5, 1, 10, 25 µg/ml) at a 24 h time point in the two donors assessed (using real-time RT-PCR).

3.3.4 Stromelysins
MMP-3 mRNA was not consistently detected using real-time RT-PCR in unstimulated hGFs or those stimulated with leptin (0.1 – 25 µg/ml) (Table 3.1), possibly due to transcript levels below the level of detection of the RT-PCR method used in this study. Similar inconsistencies in MMP-3 gene expression have been described in the literature (Domeij et al., 2002; Zhou and Windsor, 2006). Therefore, semi-quantitative RT-PCR was used to assess MMP-3 gene expression (Figure 3.5). Very faint bands of the correct product size for MMP-3 were detected in leptin-stimulated (0.1 and 10 µg/ml) and unstimulated hGFs at a 24 h time point. No clear differences in the intensity of the bands between leptin-stimulated or unstimulated hGFs were identified.

Because of the inability to quantitatively detect MMP-3 mRNA levels, MMP-3 concentrations in hGF supernatants were assessed at a 24 h time point (Table 3.3A). In two of the three donors, MMP-3 was only detected in the supernatants of hGFs that had been stimulated with 25 µg/ml leptin. Interestingly, in a third donor, MMP-3 protein was detected in the supernatants of hGFs from all treatment conditions (0 – 25 µg/ml leptin). In this donor the concentrations of MMP-3 detected were significantly higher in supernatants from hGFs stimulated with 10 or 25 µg/ml leptin compared to the unstimulated control, and the lower concentrations of leptin (0.1, 0.5, 1 µg/ml) (all p<0.001).
MMP-3 concentrations were also assessed at a 48 h time point (Table 3.3B). This was done to identify whether or not MMP-3 secretion increased over time, similar to that observed for MMP-1 (Figure 3.3). In one donor, no MMP-3 was detected at either 24 or 48 h time points under both unstimulated and leptin-stimulated (10 µg/ml) treatment conditions. In the other two donors tested, MMP-3 concentrations were higher in supernatants from leptin-stimulated (10 µg/ml) hGFs compared to the unstimulated controls at 24 h (donor 2: p<0.001, donor 3: p<0.01) and 48 h (both donors p<0.001). Additionally, in both of these donors the levels of leptin-stimulated MMP-3 protein were increased at 48 h compared to 24 h (both donors p<0.001). In donor 2 the levels of MMP-3 detected in supernatants from unstimulated hGFs were significantly higher at 48 h compared to 24 h (p<0.05); no significant difference in unstimulated MMP-3 levels were detected between 24 and 48 h in donor 3.

Gene expression levels (assessed by semi-quantitative RT-PCR) of another stromelysin, MMP-10, appeared to be higher in hGFs after leptin stimulation (0.1 and 10 µg/ml) compared to the unstimulated control at 24 h (Figure 3.6). However, MMP-10 gene expression was only assessed in one donor.

3.3.5 Other MMPs
Gene expression levels of a matrilysin (MMP-7), a membrane-type MMP (MMP-14) and the metalloelastase (MMP-12) were assessed by real-time RT-PCR. MMP-7 mRNA was not detected at baseline nor after stimulation with any leptin concentration tested (0.1, 0.5, 1, 10, 25 µg/ml) at 24 h in the two donors assessed (Table 3.1). Similarly, leptin (10 µg/ml) had no significant effect on MMP-12 mRNA expression (n=6 from 3 donors stimulated in independent experiments) compared to the unstimulated control at 24 h (Table 3.1). In contrast, leptin significantly increased MMP-14 mRNA levels in hGFs from 3 donors at 24 h in a dose-dependent manner (Figure 3.7). A 2.6-fold increase in MMP-14 gene expression was detected in hGFs treated with 10 µg/ml leptin (p<0.05) compared to the unstimulated control; no significant difference in MMP-14 gene expression was observed after stimulation with 0.1 µg/ml leptin compared to unstimulated hGFs.

3.3.6 TIMPs
TIMP-2 gene expression was assessed by semi-quantitative RT-PCR (Figure 3.6). Bands of the correct product size for TIMP-2 were detected in leptin-stimulated (0.1 and 10 µg/ml) and unstimulated hGFs at 24 h. The intensity of the band observed for hGFs
stimulated with 10 μg/ml leptin appeared to be fainter than that observed for the unstimulated control or 0.1 μg/ml leptin treatment condition, suggesting that leptin dose-dependently decreases TIMP-2 gene expression. The caveat to this conclusion is that this analysis was only completed in hGFs from a single donor.

TIMP-1 and TIMP-3 gene expression were determined by real-time RT-PCR. Leptin (10 μg/ml) had no significant effect on TIMP-3 mRNA levels (n=12 from 3 donors stimulated in independent experiments) compared to the unstimulated control at 24 h (Table 3.1). Similarly, TIMP-1 mRNA levels in hGFs were not significantly altered by leptin stimulation (0.1 and 10 μg/ml) compared to the unstimulated control (Figure 3.8).

3.3.7 MMP activity
hGF culture supernatants were tested for MMP activity at two dilutions (1.5 or 3 μl sample in a total volume of 30 μl) (Section 2.14). These supernatants were derived from hGFs stimulated for 24 h with leptin (0.1, 0.5, 1 10 and 25 μg/ml) or unstimulated controls. Across all treatment conditions, no fluorescence was detected in hGF supernatants above that of the substrate control, suggesting that unstimulated or leptin-stimulated hGF supernatants contained no measurable MMP activity.

3.4 Proliferative response of gingival fibroblasts to leptin stimulation
Leptin promotes cell proliferation and survival (Farooqi et al., 2002; Saxena et al., 2007; Shan et al., 2008; Mattioli et al., 2009; Fernandez-Riejos et al., 2010; Uddin et al., 2010). Although the use of a reference gene should account for differences in cell proliferation and activity over the course of an experiment, gene expression levels of reference genes can change after stimulation with leptin (Section 2.7.3). This can result in data that does not truly represent the expression levels of the gene of interest. In an effort to confirm whether the changes in MMP production observed in hGFs after leptin stimulation (Section 3.3) were due to non-specific increases in metabolic or cell division rates, a cell proliferation assay was performed.

hGFs were stimulated with leptin (0.1 – 25 μg/ml) for 24 h. Relative levels of cell proliferation were assessed using a tetrazolium compound-based assay (Section 2.5.1). Unstimulated hGFs were used as a negative control. Leptin dose-dependently increased hGF proliferation (Figure 3.9). hGF proliferation was significantly increased 16 % after treatment with 10 μg/ml leptin (p<0.01) and by 20 % after treatment with 25 μg/ml leptin (p<0.001) compared to the unstimulated control. hGF proliferation was increased 45 % in the FBS-stimulated positive control compared to the unstimulated control.
Interestingly, after 24 h stimulation with leptin (10 and 25 μg/ml) or IL-1α (0.05 ng/ml, as a positive control for hGF proliferation) there was a notable change in hGF morphology with high concentration leptin-stimulated cells looking larger than unstimulated cells by visual inspection under light microscopy. This suggests that leptin increases the growth of hGFs, and supports the results of the proliferation assay.

3.5 Discussion
Gingival fibroblasts, stimulated by inflammatory mediators such as cytokines and growth factors, enhance the production of MMPs (Domeij et al., 2002). As such, gingival fibroblasts are implicated in gingival tissue ECM remodelling (Sorsa et al., 2006). However, gingival fibroblasts also produce TIMPs which function to regulate MMP activity (Zhou and Windsor, 2006). Leptin regulates MMP and TIMP production (Schram et al., 2010), but no studies have yet investigated whether leptin has a similar effect in gingival fibroblasts. In the results presented in this chapter the basal gene expression profile of a number of MMPs and TIMPs in hGFs was described. Furthermore, this study was the first to show that leptin stimulated hGFs to enhance the production of several MMPs, but had no similar regulatory effect on TIMP expression. These results suggest that leptin promotes an ECM remodelling in the gingiva by increasing the ratio of MMPs to TIMPs expressed by gingival fibroblasts.

In agreement with the existing literature, gingival fibroblasts from all donors tested constitutively expressed MMP-1 (Yamada et al., 2000; Domeij et al., 2002), MMP-2 (Zhou et al., 2007; Zhang et al., 2011), MMP-10 (Mah et al., 2014), MMP-14 (Zhou and Windsor, 2006; Zhou et al., 2007; Zhang et al., 2011), TIMP-1 (Yamada et al., 2000; Zhou and Windsor, 2006; Cury et al., 2007), TIMP-2 (Zhou and Windsor, 2006; Cury et al., 2007; Zhang et al., 2011) and TIMP-3 (Arris et al., 2003). Furthermore, the lack of detectable basal gene expression of MMP-9 (Zhou and Windsor, 2006; Kim et al., 2009) and MMP-13 (Leivonen et al., 2002) in gingival fibroblasts observed in this study has also been described previously. No MMP-7 gene expression was detected basally in hGFs which is in agreement with Zhou and Windsor (2006), and similar to Mah et al. (2014) who could only detect very low basal expression levels of MMP-7 mRNA in hGFs. Only one other study has reported assessing MMP-8 and MMP-12 gene expression in unstimulated hGFs (from an undisclosed number of donors), and the authors found that neither MMPs were expressed in contrast to the results of the current study (Zhou and Windsor, 2006). It is likely that this discrepancy is due to different RT-PCR methodologies as Zhou and Windsor (2006) performed 30 cycles of semi-
quantitative RT-PCR, whereas in this study 40 cycles of real-time RT-PCR were performed. Overall, the basal profile of MMPs expressed by hGFs in this study tends to agree with the existing literature, and supports the hypothesis that ECM remodelling is a key role for fibroblasts in the gingiva. Further work to characterise the basal protein levels and activities of gingival fibroblast-derived MMPs and TIMPs may help to identify the functional role of fibroblast-derived MMPs in homeostatic ECM remodelling in the gingiva.

This is the first study to report leptin-stimulated MMP and TIMP production in any human fibroblasts. Leptin dose-dependently stimulated the expression of two collagenases, namely MMP-1 and MMP-8, by hGFs. MMPs are the only enzymes which degrade collagen (Burrage et al., 2006), and therefore this finding suggests that leptin could promote gingival fibroblast-mediated collagen degradation via the action of collagenases.

MMP-1 expression in other mesenchymal cells is regulated by leptin, although both upregulation (Hui et al., 2012), and downregulation (Cao et al., 2007) have been described. This discrepancy could be due to the different cell types studied previously (chondrocytes and HSCs) in comparison to hGFs in the current study, but could also be a dose-dependent effect of leptin. Cao et al. (2007) used lower concentrations of leptin (25 – 100 ng/ml) than in this study (0.1 – 25 μg/ml) and in the study by Hui et al. (2012). However, a 100 ng/ml leptin concentration was tested in the current study and did not alter MMP-1 gene expression in hGFs, in contrast to the decrease in MMP-1 expression at this concentration detected by Cao et al. (2007). The JAK/STAT and MAPK signalling pathways have a well-established positive regulatory effect on MMP expression (Vincenti and Brinckerhoff, 2002), however Cao et al. (2007) presented evidence that leptin suppressed MMP-1 expression via these same pathways and provided no explanation for this inconsistency.

MMP-8 is the main target of SDD, and SDD is approved as an adjunct therapy for periodontitis as it reduces the destruction of the periodontal tissues (Preshaw, 2008). It is suggested that SDD is an effective adjunct treatment for periodontitis because it is more effective at inhibiting ‘neutrophil-derived’ MMP-8 than ‘fibroblast-derived’ MMP-1 (Preshaw, 2008). This has resulted in the hypothesis that neutrophils are the mediators of periodontal tissue destruction in periodontitis, while fibroblast-derived MMPs are required for beneficial ECM remodelling. However, neutrophils are not the
only source of MMP-8; a tissue cell-derived MMP-8 (which is processed slightly differently to its neutrophil-derived counterpart) has been described (Kiili et al., 2002). Additionally, MMP-8 protein is produced by gingival fibroblasts derived from individuals with chronic periodontitis (Cox et al., 2006). Therefore, the implications of leptin increasing MMP-8 expression in hGFs are important, and suggest that gingival fibroblasts could be a source of MMP-8 in conditions of hyperleptinaemia, and that via gingival fibroblast derived MMP-8, leptin may promote deleterious ECM remodelling in the gingiva. Further study of MMP-8 protein secretion by leptin-stimulated hGFs should be carried out by Western blotting to determine which molecular weight forms are secreted; this should identify whether leptin drives MMP-8 secretion by hGFs, and whether any secreted MMP-8 is active.

In a previous study, leptin significantly increased the expression of the third collagenase MMP-13 in primary chondrocytes (Hui et al., 2012), while leptin did not induce MMP-13 expression in hGFs in this study. This discrepancy could be due to the different cell types studied and the different anatomical locations that they were isolated from. In support of this, the cartilage specific transcription factor RUNX2 is required to promote MMP-13 expression in chondrocytes (Burrage et al., 2006). This suggests that the responses that leptin generates may vary depending on cell-type and anatomical location due to inherent differences between cells/tissues.

In this study, leptin dose-dependently increased the secretion of the stromelysin MMP-3 by hGFs, which is in agreement with a previous study investigating leptin-stimulated MMP-3 production in cartilage (Koskinen et al., 2011). Similarly, leptin appeared to enhance the expression of another stromelysin, MMP-10, in hGFs; no other studies have reported an ability of leptin to regulate MMP-10. However, MMP-10 expression was only assessed in one donor by semi-quantitative RT-PCR. For increased confidence in this finding, future studies should assess leptin-mediated MMP-10 expression quantitatively at the RNA and protein level, and in more donors. The stromelysins have a broad ECM substrate specificity including non fibrillar collagens, fibronectin, elastin, aggrecan and laminin, but also cleave (and therefore activate) other MMPs including the collagenases (Burrage et al., 2006). Consequently, increases in, and accumulation of, fibroblast-derived collagenases and stromelysins in the gingiva at the same time may enhance widespread ECM remodelling including fibrillar collagen degradation, via the activation of collagenases by stromelysins.
The gelatinase MMP-2 is the only MMP which has no TATA box and no Ets transcription factor binding sites within its promoter (Vincenti and Brinckerhoff, 2002). Therefore, unlike the other MMPs, MMP-2 is not highly regulated at the level of gene expression. In agreement with this, leptin has no ability to regulate MMP-2 expression in a glioma cell line (Yeh et al., 2009), and had no ability to regulate MMP-2 expression in hGFs in this study, although MMP-2 mRNA was detected constitutively. In contrast, leptin dose-dependently increased MMP-2 expression as assessed by semi-quantitative RT-PCR in HUVECs (Park et al., 2001). However, the time point at which gene expression was assessed was not reported in this study, and may explain this inconsistency. Several studies have reported that leptin increases MMP-2 activity (McMurtry et al., 2009; Schram et al., 2010; Schram et al., 2011). As well as targeting ECM components, membrane-type MMP-14 (in complex with TIMP-2) is thought to promote pericellular MMP-2 activity (Nagase et al., 2006; Henderson et al., 2007).

Leptin increases MMP-14 protein production and surface expression in primary rat cardiac fibroblasts (Schram et al., 2011), and similarly, leptin increased MMP-14 expression in hGFs in this study. Presuming the MMP-2 and MMP-14 gene expression levels identified in this study translate to protein levels, it is possible that leptin may enhance MMP-2 activity by increasing MMP-14 expression in hGFs. Therefore, pericellular ECM remodelling by hGFs could be increased during hyperleptinaemia.

However, as leptin appeared to reduce TIMP-2 expression in hGFs in this study, further work must be done to determine if leptin affects the activity of MMP-2 via the MMP-14:TIMP-2 axis in hGFs. One approach would be to use gelatin zymography to assess the levels of active MMP secreted by hGFs. However, TIMP-2 expression was only assessed in one donor and by using semi-quantitative RT-PCR. For increased confidence in this result, leptin-mediated TIMP-2 expression should also be assessed in future studies quantitatively at the RNA and protein level, and in more donors.

Two other studies have investigated the effect of leptin on expression of the gelatinase MMP-9, both using semi-quantitative RT-PCR (Park et al., 2001; Yeh et al., 2009). Yeh et al. (2009) found that leptin had no effect on MMP-9 expression in a glioma cell line, while Park et al. (2001) found that leptin dose-dependently increased MMP-9 expression in HUVECs, although the time point at which gene expression was assessed was not reported in this study. However, MMP-9 was constitutively expressed in both these cell types, in contrast to the findings in gingival fibroblasts in this study. This suggests that gingival fibroblasts lack cell-specific transcription factors like RUNX2.
that are required for MMP-9 expression (or at least expression at a detectable level), similar to that described for MMP-13 earlier (Pratap et al., 2005).

MMP inhibitors, such as TIMPs, have a well established functional ability to reduce ECM degradation (Ellis et al., 1994). Leptin had no ability to increase the expression of TIMP-1, TIMP-2 and TIMP-3 in hGFs. While no other studies have reported an ability of leptin to regulate TIMP-2 and TIMP-3, the finding regarding TIMP-1 in this study is inconsistent with previous studies (Park et al., 2001; Schram et al., 2010), although neither of these previous studies assessed TIMP-1 expression in human fibroblasts. For example, Park et al. (2001) found that leptin increased TIMP-1 expression, as assessed by semi-quantitative RT-PCR, in HUVECs. In contrast, Schram et al. (2010) found that leptin reduced TIMP-1 expression in a murine cardiac cell line. These inconsistencies suggest that TIMP-1 expression is regulated in a cell type-specific manner. Together, the results in this study suggest that leptin does not increase TIMP-mediated MMP inhibition by gingival fibroblasts. However, TIMPs also appear to have functions outside of regulating MMP activity. For example, TIMP-3 inhibits angiogenesis by blocking VEGF binding to its receptor (Qi et al., 2003). The lack of increased TIMP expression by leptin-stimulated hGFs suggests that leptin may not affect other functions of fibroblast-derived TIMPs in the gingiva, at least in respect to functions affected by TIMP-1-3 expression levels.

The dose-dependent effect of leptin on MMP-1 and MMP-3 production was consistent between donors in this study, while the absolute concentrations of MMP-1 and MMP-3 produced by leptin-stimulated hGFs were donor-dependent. Similar differences in responses between hGF donors have been described previously (Faria Morandini et al., 2012). The implications of this finding are important and suggest that the concentrations of fibroblast derived MMP-1 and MMP-3 in the gingiva, and therefore the potential for gingival ECM degradation, may be inherently different between individuals. The analysis of leptin-mediated MMP-1 and MMP-3 production in a larger number of hGF donors could identify the extent of donor-dependent variation in leptin responses. If this is confirmed then future studies could aim to identify if these donor-dependent differences in leptin responses were due to differences in the characteristics of individual donors (e.g. gender, age, BMI) or perhaps the result of genetic polymorphism.
Overall, the results presented in this chapter demonstrate that leptin-stimulated gingival fibroblasts regulate a specific group of MMPs (MMP-1, MMP-3, MMP-8, MMP-10 and MMP-14). Other MMPs are regulated by leptin in other cell types (Section 1.4.3), indicating that gingival fibroblasts have an innate ability to determine which MMPs are regulated by leptin. Nonetheless, leptin regulated (or failed to regulate) a particular selection of MMPs in hGFs which was different to the MMPs that other stimuli such as cytokines and bacterial supernatants regulate in gingival fibroblasts (Section 1.11.1). The increased hGF proliferation stimulated by leptin in this study was unlikely to account for the changes in MMP and TIMP expression observed because the extent of expression change differed between individual MMPs and TIMPs. Together, this indicates that leptin regulates MMP and TIMP expression in gingival fibroblasts in a specific manner. Several mechanisms may determine how leptin regulates the MMPs in gingival fibroblasts that can be expressed such as increasing MMP gene transcription, mRNA stability or secretion rate.

All of the promoters of the MMPs upregulated by leptin contain binding sites for the transcription factor Ets. Ets is activated downstream of the MAPK signalling pathway (Yordy and Muise-Helmericks, 2000), and this signalling pathway is activated by leptin (Section 1.2.2). Therefore, leptin could regulate MMP expression in hGFs via Ets. However, leptin activates a number of intracellular signalling pathways that are implicated in regulating MMP production (Section 1.2.2), and MMP gene expression is widely thought to be regulated by the coordinate action of multiple cis-acting factors (Fanjul-Fernandez et al., 2010). Further studies investigating the intracellular signalling pathways and the transcription factors by which leptin regulates MMP synthesis in hGFs are warranted; subsequently an investigation into which signalling pathways are involved was performed in this study (Chapter 5).

The results presented in this chapter also suggest that leptin-stimulated gingival fibroblasts promote MMP activity by increasing the MMP:TIMP ratio. However, the results of the MMP activity assay described in this chapter suggest that either MMP activity in hGF supernatants was below the sensitivity of the assay, or that leptin only increases the production, and not activity, of MMPs by hGFs. If the latter is true, these results could still be relevant to ECM degradation in the gingiva during hyperleptinaemia because proteases derived from non-gingival fibroblasts and microbial proteases are implicated in activating MMPs (Beklen et al., 2006; Cox et al., 2006). Therefore, gingival fibroblasts could be a source of pro-MMPs, but these pro-MMPs
may only be activated in the presence of existing protease activity in the gingiva, which is not present in \textit{in vitro} single cell type studies.

The results presented in this chapter suggest that leptin promotes gingival ECM remodelling. ECMs are not fixed structures; for example, altered ECM remodelling is a feature of fibrosis and tissue destruction (Lu \textit{et al.}, 2011), however ECM remodelling (and MMP activity) also serves physiological purposes (e.g. in development and wound healing) (Page-McCaw \textit{et al.}, 2007). Therefore, while leptin may promote gingival ECM remodelling, this may not necessarily be involved in the deleterious tissue destruction of periodontitis. Indeed, while elevated levels of MMPs are found in numerous pathologies related to ECM degradation, evidence is lacking to show that MMPs play a causal role (Fanjul-Fernandez \textit{et al.}, 2010). For example, many clinical studies have identified increased MMP levels in individuals with chronic periodontitis compared to health (Section 1.9.2). It would be more informative to measure MMP levels across a range of clinical categories (e.g. gingivitis and mild to severe periodontitis). This kind of approach has been performed in our laboratory and it was found that total salivary MMP-8 (i.e. active and inactive) is highly predictive for both gingivitis and periodontitis (Preshaw \textit{et al.}, 2013). Therefore, if MMP-8 is involved in ECM destruction in periodontitis, the MMP-8 measured in gingivitis must be predominantly inactive. Alternatively, MMP-8 may not be as important in the deleterious ECM destruction observed in periodontitis as first thought.

Numerous physiological and biochemical alterations have been described as part of the starvation response (Friedman and Halaas, 1998). Perhaps in periods of starvation (and consequently hypoleptinaemia) the optimal level of gingival ECM remodelling would cost too much energy. Leptin could serve to inform gingival fibroblasts of the metabolic status of an organism, thereby ensuring that the functions of gingival fibroblasts (e.g. ECM remodelling) use an available amount of energy. A similar link between leptin, energy status and peripheral cell function has been described previously regarding the immunocompetence of T cells (Saucillo \textit{et al.}, 2014).

Further lines of work could be pursued in relation to the results presented in this chapter. It would be interesting to identify the full profile of MMPs, TIMPs, other proteases/inhibitors and ECM components produced by hGFs and how the production of these molecules are affected by leptin in an effort to gain an holistic understanding of the ECM remodelling potential of leptin-stimulated gingival fibroblasts. It would be
useful to use alternative methods to assess whether the MMPs produced by leptin-stimulated hGFs are active; gelatin zymography could be used to assess MMP-2 activity, while an immunoblotting approach could be used to assess whether the inactive or active forms of other MMPs are produced. As the gingival microenvironment is complex, it is highly improbable that leptin would be the only stimulus affecting gingival fibroblasts. Therefore, studies investigating how leptin regulates the ECM-degrading phenotype of gingival fibroblasts in combination with other stimuli relevant to gingivitis and periodontitis are warranted; this is presented in Chapter 4. Further, it would be valuable to determine whether the ability of leptin to regulate the ECM-degrading phenotype of gingival fibroblasts translates to ECM degradation in the gingiva, and whether this is beneficial or deleterious; in vivo or 3-dimensional tissue models may be suitable tools for this (see Chapter 7).

In conclusion, the results presented in this chapter provide the first evidence that leptin selectively regulates MMP and TIMP expression in hGFs. These results imply that leptin promotes an ECM-degrading gingival fibroblast phenotype by increasing the MMP:TIMP ratio, and by increasing the expression of MMPs that can activate other MMP family members. These results add valuable information regarding the potential for gingival fibroblast-mediated ECM remodelling during hyperleptinaemia.
Table 3.1: MMP and TIMP gene expression in hGFs under basal conditions and after stimulation with leptin.

cDNA was prepared from unstimulated hGFs or hGFs after stimulation with leptin (0.1, 0.5, 1, 10, 25 μg/ml) for 24 h and gene expression was determined by RT-PCR. For each MMP/TIMP the number of donors assessed is indicated. + indicates basal gene expression in all donors, +/- indicates basal gene expression in some donors, - indicates no basal gene expression in all donors assessed. The value for MMP-8 mRNA expression (fold unstimulated control) is presented as mean±SD (n=6 from three donors stimulated in independent experiments), and was tested for statistical significance using the t test for independent samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>MMP</th>
<th>Basally expressed</th>
<th>Regulated by leptin (10μg/ml)</th>
<th>Number of donors tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>MMP-1</td>
<td>+</td>
<td>Yes - upregulated</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>+</td>
<td>Yes – upregulated</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>MMP-13</td>
<td>-</td>
<td>2.6±1.0-fold control</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Gelatinase</td>
<td>MMP-2</td>
<td>+</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>-</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>Stromelysin</td>
<td>MMP-3</td>
<td>+/-</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MMP-10</td>
<td>+</td>
<td>Yes - upregulated</td>
<td>1</td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>-</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>Membrane-type MMP</td>
<td>MMP-14</td>
<td>+</td>
<td>Yes - upregulated</td>
<td>3</td>
</tr>
<tr>
<td>Other MMPs</td>
<td>MMP-12</td>
<td>+</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>TIMPs</td>
<td>TIMP-1</td>
<td>+</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td>+</td>
<td>No - downregulated</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TIMP-3</td>
<td>+</td>
<td>No</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 3.1: MMP-1 mRNA expression in hGFs after leptin stimulation.

hGFs were stimulated with leptin (0 – 25 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the $2^{-\Delta\Delta C_t}$ method by using RNAP as the reference gene. Data are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) **=p<0.01, ***=p<0.001 compared to unstimulated control.
Table 3.2: MMP-1 protein secretion by hGFs after leptin stimulation.

hGFs were stimulated with leptin (0 – 25 µg/ml) for 24 h. Supernatants were collected and MMP-1 concentrations were assessed by ELISA. Table displays the mean±SD (n=4) MMP-1 concentration (ng/ml) for each donor tested at each concentration of leptin. Statistics: T tests for independent samples (Bonferroni-corrected) ***=p<0.001 compared to 0, 0.1, 0.5 and 1 µg/ml leptin, ###=p<0.001 compared to 10 µg/ml leptin. ND – not detected.

<table>
<thead>
<tr>
<th>Leptin (µg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.32±0.08</td>
<td>1.07±0.14###</td>
</tr>
<tr>
<td>Donor 2</td>
<td>0.34±0.05</td>
<td>0.34±0.10</td>
<td>0.36±0.03</td>
<td>0.42±0.02</td>
<td>2.74±0.29***</td>
<td>7.64±1.07****</td>
</tr>
<tr>
<td>Donor 3</td>
<td>0.72±0.14</td>
<td>0.85±0.26</td>
<td>0.66±0.02</td>
<td>0.79±0.10</td>
<td>2.48±0.52***</td>
<td>3.06±0.67***</td>
</tr>
</tbody>
</table>
Figure 3.2: MMP-1 mRNA expression in hGFs after different durations of leptin stimulation.

hGFs were stimulated with leptin (10 µg/ml) for 6 - 48 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene and the 6 h unstimulated control as the reference treatment condition. Data are shown as mean+SD (n=4) from one donor as fold MMP-1 gene expression compared to the 6 h unstimulated control (not shown). Statistics: T tests for independent samples (Bonferroni-corrected) *** = p<0.001 compared to 6 h time point.
Figure 3.3: MMP-1 protein secretion by hGFs after different durations of leptin stimulation.

hGFs were stimulated with leptin (10 µg/ml) for 24 or 48 h. Supernatants were collected and MMP-1 concentrations were assessed by ELISA. Data (median +IQR, n=12) are presented as fold unstimulated control at 24 h from three donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control at the same time point unless indicated otherwise.
Figure 3.4: MMP-2 mRNA expression in hGFs after leptin stimulation.

hGFs were stimulated with leptin (0 – 25 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-2 and RNAP gene expression. Relative MMP-2 mRNA expression was determined using the $2^{-\Delta\Delta C_t}$ method by using RNAP as the reference gene. Data are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected).
Figure 3.5: MMP-3 mRNA expression in hGFs after leptin stimulation.

hGFs were stimulated with leptin (0 – 10 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Semi-quantitative RT-PCR was used to assess MMP-3 and 18S rRNA gene expression. The expected product size for MMP-3 was 150 bp, and for 18S rRNA was 85 bp. For clarity several other lanes from these gels have been omitted; however the entire gel is displayed in Figure 4.3. The MMP-3 mRNA expression levels detected in this donor are representative of those observed in two other donors stimulated with leptin in independent experiments.
Table 3.3: MMP-3 protein secretion by hGFs stimulated with leptin.

hGFs were stimulated with 10 µg/ml leptin (unless otherwise indicated for [A] 24 h or [B] 24-48 h. Supernatants were collected and analysed for MMP-3 by ELISA. Tables displays the mean±SD (n=4) MMP-3 concentration (pg/ml) for each donor tested. Statistics: T tests for independent samples (Bonferroni-corrected) [A] ***=p<0.001 compared to 0, 0.1, 0.5 and 1 µg/ml leptin. [B] **=p<0.01, ***=p<0.001 compared unstimulated control at the same time point; # =p<0.05, ###=p<0.001 compared to 24 h time point for the same treatment conditions. ND – not detected.

[A]

<table>
<thead>
<tr>
<th>Leptin (µg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>123 ± 13</td>
</tr>
<tr>
<td>Donor 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>302 ± 24</td>
</tr>
<tr>
<td>Donor 3</td>
<td>236 ± 66</td>
<td>235 ± 15</td>
<td>222 ± 23</td>
<td>226 ± 49</td>
<td>667 ± 56***</td>
<td>838 ± 31***</td>
</tr>
</tbody>
</table>

[B]

<table>
<thead>
<tr>
<th></th>
<th>24 h unstimulated</th>
<th>24 h leptin</th>
<th>48 h unstimulated</th>
<th>48 h leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 2</td>
<td>95.1 ± 27</td>
<td>437 ± 62***</td>
<td>152 ± 34#</td>
<td>825 ± 12****###</td>
</tr>
<tr>
<td>Donor 3</td>
<td>432 ± 72</td>
<td>1760 ± 510**</td>
<td>930 ± 440</td>
<td>3350 ± 180****###</td>
</tr>
<tr>
<td>Donor 4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 3.6: TIMP-2 and MMP-10 gene expression in hGFs after leptin stimulation.

hGFs from one donor were stimulated with leptin (0 – 10 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Semi-quantitative RT-PCR was used to assess TIMP-2, MMP-10 and 18S rRNA gene expression. The expected product size for TIMP-2 was 77 bp, MMP-10 was 113 bp and for 18S rRNA was 85 bp. For clarity several other lanes from these gels have been omitted; however the entire gel is displayed in Figure 4.7. The faint band for 18S observed in the –RT control may be due to the nature of the cell lysis and cDNA generation protocol. No similar band was detected in the H2O control.
hGFs were stimulated with leptin (0 – 10 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-14 and RNAP gene expression. Relative MMP-14 mRNA expression was determined using the 2^(-ΔΔCt) method by using RNAP as the reference gene. Data are shown as median+IQR (n=12-26) from 3 donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) *p<0.05 compared to unstimulated control.
Figure 3.8: TIMP-1 gene expression in hGFs after leptin stimulation.

hGFs were stimulated with leptin (0 – 10 µg/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess TIMP-1 and RNAP gene expression. Relative TIMP-1 gene expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. Data are shown as mean±SD (n=12-26) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected).
Figure 3.9: hGF proliferation after stimulation with leptin

hGFs were stimulated with leptin for 24 h. Proliferation was assessed using a tetrazolium compound based assay. Data (mean+SD, n=12) are shown relative to the unstimulated control (% control) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected): **=p<0.01, ***=p<0.001 compared to unstimulated control.
Chapter 4. Synergistic regulation of MMP and TIMP production in gingival fibroblasts by leptin and pro-inflammatory mediators

4.1 Introduction

Leptin is one of a number of inflammatory mediators that regulate MMP and TIMP production in mesenchymal cells (Vincenti and Brinckerhoff, 2002). The pro-inflammatory cytokine IL-1β enhances the expression of the collagenases MMP-1, MMP-8 and MMP-13 in gingival fibroblasts (Abe et al., 2001). IL-1β also stimulates the secretion of MMP-1 and the stromelysin MMP-3 by gingival fibroblasts (Domeij et al., 2002; Kida et al., 2005). Increased levels of active or partially active MMP-1 and MMP-13 are observed in gingival fibroblasts isolated from individuals with chronic periodontitis after stimulation with IL-1β (Cox et al., 2006); whether or not IL-1β elicits the same response in fibroblasts isolated from periodontally healthy individuals was not tested. IL-1β also significantly increases the production of the gelatinase MMP-2 in PDL cells (Xiang et al., 2009). The pro-inflammatory cytokine TNF-α stimulates the secretion of MMP-1, MMP-3, MMP-8 and the gelatinase MMP-9 by gingival fibroblasts (Domeij et al., 2002; Beklen et al., 2006). The adipokine adiponectin, lipoxin A4, and the cytokines IL-17 and TGF-β also increase the production of MMPs and TIMPs in fibroblasts (Sodin-Semrl et al., 2000; Stuelten et al., 2005; Cortez et al., 2007; Kim et al., 2010b). Taken together, these studies support an ability for numerous, and distinct, endogenous inflammatory mediators to regulate the ECM-degrading phenotype of fibroblasts and cells found within the periodontium by controlling MMP and TIMP production.

Supernatants from P. gingivalis cultures enhance the proportions of active MMP-1, MMP-2 and MMP-3 in cultures of gingival fibroblasts; these supernatants also increase the collagen degrading ability of gingival fibroblasts (Zhou et al., 2007; Zhang et al., 2010). This suggests that microbial molecules can increase MMP activity. Additionally, LPS derived from numerous bacteria (including periodontal pathogens) regulates MMP and TIMP production by gingival fibroblasts; however, MMPs and TIMPs are differentially regulated by LPS from different bacteria (Bodet et al., 2007; Herath et al., 2013). The regulation of gingival fibroblast-derived MMPs and TIMPs by bacteria is potentially relevant to periodontitis as microbial products are implicated in disease pathogenesis (Darveau, 2010). It is possible that microbial stimuli regulate MMP and
TIMP production due to their ability to stimulate IL-1β and TNF-α (Wang and Ohura, 2002; Tardif et al., 2004).

IL-1α and OSM synergise to increase MMP-1 secretion by gingival and synovial fibroblasts (Cawston et al., 1998; Sukkar et al., 2007). Only one other study has investigated the interaction between pro-inflammatory stimuli in regulating the ECM-degrading phenotype of gingival fibroblasts (Ruwanpura et al., 2004). In this study, PGE$_2$ regulated the ability of IL-1β to promote MMP-3 secretion by gingival fibroblasts. However, the regulatory effect of PGE$_2$ was positive in fibroblasts derived from individuals with periodontitis, and negative in fibroblasts derived from healthy periodontium. These studies suggest that the extent of gingival fibroblast-mediated ECM degradation depends, in part, on the combination of inflammatory mediators present.

Leptin synergises with LPS derived from periodontal pathogens (*P. gingivalis* and *P. intermedia*) to increase the production of TNF-α by THP-1 monocytes or macrophages (Kim, 2010; Jaedicke et al., 2013). However, leptin and LPS have no synergistic ability to regulate IL-6 and CXCL8 production by PDL fibroblasts (Park et al., 2013). However, Park et al. (2013) did not report the type of LPS used, the number of donors tested and experimental repeats performed. As such, the ability of leptin to synergise with inflammatory mediators in models relevant to periodontitis is unclear. However, leptin synergises with IL-1α to increase MMP-1 and MMP-13 production in chondrocytes, and to increase bovine cartilage degradation (Hui et al., 2012). Similarly, leptin and IL-1 synergistically increase the production of MMP-1, MMP-3 and MMP-13 by human cartilage acquired from individuals with osteoarthritis (Koskinen et al., 2011). These studies suggest that leptin may enhance ECM degradation under inflammatory conditions by promoting the production of MMPs. No studies have reported whether leptin interacts with pro-inflammatory mediators to regulate MMP and TIMP production in cells of the periodontium.

Periodontitis is characterised by destruction of the periodontal tissues, and I have already demonstrated that leptin may enhance an ECM-degrading gingival fibroblast phenotype (Chapter 3); however, other inflammatory mediators have a similar effect. Leptin can synergise with inflammatory mediators to enhance the production of MMPs in chondrocytes and cartilage explants. No-one has yet assessed whether the production of MMPs by gingival fibroblasts is similarly synergistically regulated, and therefore
whether or not leptin might have a role in gingival fibroblast-regulated ECM homeostasis during inflammation. Therefore, the aim of the experiments presented in this chapter was to determine whether leptin synergises with pro-inflammatory stimuli to regulate the production of MMPs and TIMPs in hGFs.

4.2 MMP and TIMP production by gingival fibroblasts in response to stimulation with leptin and pro-inflammatory cytokines

hGFs were stimulated for 24 h with or without leptin (0.1 and 10 μg/ml) and:

1. IL-1α (0.05 ng/ml).
2. OSM (5 ng/ml).
3. IL-1α+OSM (IL-1α 0.05 ng/ml, OSM 5 ng/ml)

A 24 h stimulation was chosen because increases in MMP production by gingival fibroblasts have been detected at this time point previously (See sections 1.11.1 and 3.3). The pro-inflammatory stimuli were chosen because they synergistically regulate MMP production in chondrocytes and cartilage (Cawston et al., 1998; Koshy et al., 2002; Milner et al., 2006; Hui et al., 2012). hGFs were either lysed and cDNA prepared by reverse transcription or relative levels of cell proliferation were assessed using a tetrazolium compound-based assay. RT-PCR was used to determine the relative MMP and TIMP gene expression levels after leptin stimulation. MMP concentrations in the supernatants were assessed by ELISA. Unstimulated hGFs at each time point were used as a control, while hGFs treated with DMEM+ were used as a positive control for the proliferation assay. A synergistic relationship was inferred if MMP/TIMP expression in hGFs was significantly different after stimulation with multiple stimuli in comparison to the individual stimuli (e.g. leptin+IL-1 significantly different to leptin or IL-1).

Leptin (0.1 μg/ml) did not synergise with IL-1α, OSM or IL-1α+OSM to enhance MMP or TIMP synthesis (Table 4.1). Because of these results, and the finding that 0.1 μg/ml leptin had no effect on MMP or TIMP production as observed previously (Section 3.3), the remaining results in this section refer to leptin at a concentration of 10 μg/ml unless otherwise indicated.

hGF proliferation was significantly increased by IL-1α, OSM and IL-1α+OSM at 24 h (Figure F.1). Leptin (0.1 and 10 μg/ml) did not synergise with IL-1α, OSM and IL-1α+OSM to increase hGF proliferation at 24 h.
A summary of the MMP/TIMP gene expression levels in hGFs after stimulation with 10 µg/ml leptin, IL-1 and OSM as determined by real-time RT-PCR can be found in Table 4.2.

4.2.1 Synergistic regulation of MMP-1 production by gingival fibroblasts after stimulation with leptin, IL-1 and OSM

Leptin and IL-1α synergised to increase MMP-1 mRNA levels in hGFs at 24 h (Figure 4.1). MMP-1 expression in leptin+IL-1α-stimulated hGFs was 16-fold higher than leptin and 15-fold higher than IL-1α-stimulated hGFs (both p<0.001). To determine whether leptin and IL-1α had a similar effect on MMP-1 secretion, MMP-1 concentrations in leptin and IL-1α-stimulated hGF supernatants were assessed. Leptin and IL-1α synergistically increased secreted MMP-1 protein levels in hGFs (Figure 4.2). MMP-1 secretion in leptin+IL-1α-stimulated hGFs (15.9 ng/ml, p<0.001 compared to the unstimulated control) was 11-fold higher than leptin and 27-fold higher than IL-1α-stimulated hGFs (both p<0.001) (Figure 4.2).

OSM increased MMP-1 mRNA levels in hGFs at 24 h (p<0.001), whether leptin was present or not (Table 4.2). Similarly, MMP-1 protein in leptin+OSM-stimulated hGF supernatants was significantly higher than in the unstimulated control (p<0.001, Table 4.3); secreted levels of MMP-1 after leptin+OSM stimulation were not significantly different to those detected for hGFs stimulated with leptin alone.

Leptin+IL-1α+OSM-stimulated hGFs did not significantly increase MMP-1 mRNA or protein levels in hGFs at 24 h above that detected for leptin+IL-1-stimulated hGFs (Table 4.2 and Table 4.3).

4.2.2 Synergistic regulation of MMP-3 production by gingival fibroblasts after stimulation with leptin, IL-1 and OSM

Leptin and IL-1α synergised to increase MMP-3 mRNA levels in hGFs at 24 h in all three donors tested (Figure 4.3), as assessed by semi-quantitative RT-PCR. However, semi-quantitative differences in MMP-3 mRNA levels in response to leptin and IL-1 between the donors tested were apparent. For example, in hGFs from donors 2 and 3, but not donor 4, IL-1α stimulation resulted in detectable MMP-3 gene expression (Figure 4.3). Similarly, the extent to which leptin (0.1 µg/ml) synergised with IL-1α to regulate MMP-3 gene expression in hGFs appeared to be donor-dependent. hGFs isolated from donor 2 had increased MMP-3 mRNA after stimulation with 0.1 µg/ml leptin+IL-1α; no similar effect was observed in donors 3 and 4 (Figure 4.3).
In donors 2 and 4 no MMP-3 mRNA was detected in hGFs stimulated with OSM (± leptin) at 24 h (Figure 4.3). In donor 3, very faint bands of the correct product size for MMP-3 were detected in hGFs stimulated with OSM (± leptin) at 24 h, and were possibly reduced in hGFs treated with 0.1 μg/ml leptin+OSM compared to OSM and 10 μg/ml leptin+OSM.

MMP-3 protein was not detected in the supernatants of unstimulated hGFs or hGFs stimulated with OSM (Table 4.3). However, leptin and IL-1α synergised to increase MMP-3 protein secretion by hGFs (Figure 4.4). MMP-3 secretion in leptin+IL-1α-stimulated hGFs was 16-fold higher than leptin and 13-fold higher than IL-1α-stimulated hGFs (both p<0.001) (Figure 4.4). The levels of MMP-3 protein detected were not significantly different between leptin and leptin+OSM-stimulated hGFs (Table 4.3).

Leptin+IL-1α+OSM-stimulated hGFs did not significantly increase MMP-3 mRNA or protein levels in hGFs at 24 h above that detected for leptin+IL-1-stimulated hGFs (Figure 4.3 and Table 4.3).

4.2.3 Effect of leptin, IL-1 and OSM on the expression of other MMPs and TIMPs in gingival fibroblasts

MMP-2, MMP-7, MMP-9, MMP-13, MMP-14 and TIMP-1 gene expression were assessed by real-time RT-PCR. No synergistic regulation of MMP-2 gene expression was detected in any combination of leptin, IL-1 and OSM-stimulated hGFs (Table 4.2). However, MMP-2 mRNA levels were 3.8-fold reduced in leptin+OSM-stimulated hGFs compared to cells stimulated with leptin at 24 h (p<0.05) (Figure 4.5).

No synergistic regulation of MMP-14 gene expression was detected in any combination of leptin, IL-1 and OSM-stimulated hGFs (Table 4.2) but, MMP-14 mRNA levels in hGFs were significantly increased after leptin+IL-1α stimulation at 24 h compared to the unstimulated control (p<0.001) (Figure 4.6). MMP-14 gene expression in leptin+IL-1α-stimulated hGFs was not significantly different from leptin-stimulated hGFs, but was 3.6-fold higher than hGFs stimulated with IL-1α (p<0.01) (Figure 4.6).

No synergistic regulation of TIMP-1 gene expression was detected in any combination of leptin, IL-1 and OSM-stimulated hGFs (Table 4.2).

MMP-7 and MMP-9 mRNAs were not detected in hGFs basally or after stimulation with any combination of leptin, IL-1 and OSM (Table 4.2). In contrast, MMP-13
mRNA was detected in hGFs stimulated with leptin+IL-1α at 24 h, but was not detected in any other treatment conditions (Table 4.2).

MMP-10 and TIMP-2 gene expression were determined by semi-quantitative RT-PCR (Figure 4.7). IL-1α and/or OSM (with or without leptin) appeared to have no ability to increase MMP-10 gene expression in hGFs at 24 h compared to the unstimulated control. In contrast, TIMP-2 expression in leptin+IL-1α-stimulated hGFs appeared to be reduced compared to either leptin or IL-1α-stimulated hGFs (at the 10 μg/ml leptin concentration) (Figure 4.7). Similarly, TIMP-2 expression in OSM (± leptin) -stimulated hGFs appeared to be reduced compared to leptin-stimulated hGFs and the unstimulated control, particularly in hGFs stimulated with 0.1 μg/ml leptin+OSM. Also, TIMP-2 expression in leptin+IL-1α+OSM-stimulated hGFs appeared to be reduced compared to either leptin or IL-1α+OSM-stimulated hGFs (at the 10 μg/ml leptin concentration) (Figure 4.7).

4.3 MMP and TIMP production by gingival fibroblasts after stimulation with leptin and TLR agonists

hGFs were stimulated for 24 h with or without leptin (10 μg/ml) and:

1. Pam2CSK4 (50 and 100 ng/ml), a TLR2/6 agonist.
2. *E. coli* LPS (10 and 1000 ng/ml), a TLR4 agonist.

These stimuli were chosen as they signal via specific TLRs and because signalling via TLRs regulates MMP production by gingival fibroblasts (Bodet et al., 2007; Herath et al., 2013). Two concentrations of both pam2CSK4 and *E. coli* LPS were used to stimulate hGFs in this study, chosen based on the existing literature and manufacturer recommendations (Nagasawa et al., 2002; Minami et al., 2007; Souza et al., 2010; Morandini et al., 2012). hGFs were either lysed and cDNA prepared by reverse transcription or relative levels of cell proliferation were assessed using a tetrazolium compound-based assay. RT-PCR was used to determine the relative MMP and TIMP gene expression levels after leptin stimulation. MMP concentrations in the supernatants were assessed by ELISA. Unstimulated hGFs at each time point were used as a control, while hGFs treated with DMEM+ were used as a positive control for the proliferation assay.

MMP-1 and MMP-3 protein concentrations for hGFs stimulated with pam2CSK4 were not significantly different between 50 and 100 ng/ml concentrations, and for hGFs
stimulated with LPS were not significantly different between 10 and 1000 ng/ml concentrations (Table 4.4). Subsequently, unless otherwise indicated the results presented in this section correspond to 50 ng/ml pam2CSK4 and 10 ng/ml LPS. A summary of the MMP/TIMP gene expression levels in hGFs after stimulation with 10 µg/ml leptin, 50 ng/ml pam2CSK4 and 10 ng/ml *E. coli* LPS as determined by real-time RT-PCR can be found in Table 4.5.

A similar pattern of response was detected between MMP-3 mRNA and protein levels in hGFs stimulated with leptin and pro-inflammatory cytokines previously (Figures 4.3 and 4.4). Therefore, in this section MMP-3 was only assessed at the protein level by ELISA.

hGF proliferation was significantly increased by pam2CSK4 (50 and 100 ng/ml) (p<0.01), but no similar effect was observed in hGFs stimulated with LPS (10 and 1000 ng/ml) (Figure F.2). Leptin did not synergise with pam2CSK4 (50 and 100 ng/ml) to increase hGF proliferation.

In a separate experiment, hGFs under basal conditions were prepared for flow cytometry (Section 2.2.7). This was performed to assess whether the hGFs used in this study expressed TLR2 or TLR4 on the cell surface.

### 4.3.1 Leptin and pam2CSK4 synergistically regulate MMP production by gingival fibroblasts

#### 4.3.1.1 MMP-1

Leptin and pam2CSK4 synergised to increase MMP-1 mRNA levels in hGFs (Figure 4.8). MMP-1 expression in leptin+pam2CSK4-stimulated hGFs (33-fold control, p<0.001) was 3-fold higher than leptin and 4-fold higher than pam2CSK4-stimulated hGFs (both p<0.05). To determine whether leptin and pam2CSK4 had a similar effect on MMP-1 secretion, MMP-1 concentrations in leptin and pam2CSK4-stimulated hGF supernatants were assessed. Leptin and pam2CSK4 synergised to increase MMP-1 protein levels in hGFs (Figure 4.9). MMP-1 secretion in leptin+pam2CSK4-stimulated hGFs (23-fold control, p<0.001) was 4-fold higher than leptin and 3-fold higher than pam2CSK4-stimulated hGFs (both p<0.001).

#### 4.3.1.2 MMP-3

Leptin and pam2CSK4 synergised to increase MMP-3 protein levels in hGF supernatants (Table 4.6). In donor 4, MMP-3 was only detected in the supernatants of
hGFs that had been stimulated with leptin+pam2CSK4. In donors 2 and 3, MMP-3 secretion in leptin+pam2CSK4-stimulated hGFs was higher than leptin and pam2CSK4-stimulated hGFs (leptin+pam2CSK4 compared to leptin: donor 2 p<0.05, donor 3 p<0.001; leptin+pam2CSK4 compared to pam2CSK4: both donors p<0.001) (Table 4.6).

4.3.1.3 MMP-14
MMP-14 mRNA levels in hGFs were increased 3.2-fold after leptin+pam2CSK4 stimulation compared to the unstimulated control (p<0.01) (Figure 4.10). MMP-14 gene expression in leptin+pam2CSK4-stimulated hGFs was not significantly different from leptin-stimulated hGFs, but was 3.3-fold higher than hGFs stimulated with pam2CSK4 (p<0.05).

4.3.1.4 TIMP-1
TIMP-1 gene expression was assessed by real-time RT-PCR. In comparison to the unstimulated control no significant differences in TIMP-1 mRNA levels were observed in pam2CSK4-stimulated hGFs, both in the absence and presence of leptin (n=12 from 3 donors stimulated in independent experiments) (Table 4.5).

4.3.1.5 TLR2 surface expression
hGFs isolated from all donors tested expressed TLR2 on the cell surface (Figure 4.11); the median fluorescence intensity (MFI) increased 6.7±0.8-fold in samples stained for TLR2 compared to the isotype control.

4.3.2 Synergistic regulation of MMP and TIMP production by gingival fibroblasts after stimulation with leptin and E. coli LPS

4.3.2.1 MMP-1
Leptin and E. coli LPS synergised to increase MMP-1 mRNA levels in a donor-dependent manner (Table 4.7A). MMP-1 expression in leptin+LPS-stimulated hGFs was significantly higher than leptin or LPS-stimulated hGFs (both p<0.05) in donor 3; no similar synergy was observed for donors 2 and 4. Leptin and LPS also synergised to increase MMP-1 protein levels in hGFs in a donor-dependent manner (Table 4.7B). MMP-1 secretion in leptin+LPS-stimulated hGFs was significantly higher than leptin or LPS-stimulated hGFs (both p<0.01) in donor 3; no similar synergy was observed for donors 2 and 4.
4.3.2.2 MMP-3

Leptin and *E. coli* LPS synergised to increase MMP-3 protein levels in a donor-dependent manner in hGF supernatants (Table 4.8). In donor 3, MMP-3 secretion in leptin+*E. coli*-stimulated hGFs was significantly higher than leptin and *E. coli*-stimulated hGFs (both p<0.01); no significant difference in MMP-3 secretion between leptin and leptin+*E. coli* LPS-stimulated hGFs was observed in donor 2. No MMP-3 protein was detected in any of the supernatants of donor 4 (Table 4.8).

4.3.2.3 MMP-14 and TIMP-1

MMP-14 and TIMP-1 gene expression was assessed by real-time RT-PCR (Table 4.5). No synergistic regulation of MMP-14 expression was observed in hGFs stimulated with leptin+LPS. In comparison to the unstimulated control no significant differences in TIMP-1 mRNA levels were observed in *E. coli* LPS-stimulated hGFs at 24 h, both in the absence and presence of leptin (both n=12 from 3 donors stimulated in independent experiments).

4.3.2.4 TLR4 surface expression

TLR4 surface expression on hGFs was assessed to determine whether the donor-dependent responses to LPS were due to the absence or presence of surface TLR4. hGFs isolated from all donors tested expressed TLR4 on the cell surface (Figure 4.12); the median fluorescence intensity (MFI) increased 3.8±0.5-fold in samples stained for TLR4 compared to the isotype control.

4.4 Discussion

Periodontitis is characterised by destruction of the periodontal tissues (Darveau, 2010). Gingival fibroblasts enhance the production of ECM-degrading MMPs after stimulation with inflammatory mediators (Domeij *et al.*, 2002) or leptin (Chapter 3). Circulating levels of leptin are elevated in obesity and T2DM (Maffei *et al.*, 1995; Coppari and Bjorbaek, 2012); both of these conditions are positively associated with periodontitis (Pischon *et al.*, 2007; Preshaw *et al.*, 2012). Therefore, leptin could act as a molecular link between obesity, T2DM and periodontitis by stimulating the production of MMPs by gingival fibroblasts. Leptin synergises with IL-1α and TNF-α to enhance the production of MMPs in chondrocytes and cartilage (Koskinen *et al.*, 2011; Hui *et al.*, 2012), yet no studies have investigated whether the production of MMPs by gingival fibroblasts is similarly regulated. However, the cytokines IL-1α+OSM synergistically
increase MMP-1 production by gingival fibroblasts (Sukkar et al., 2007); a finding confirmed in the present study.

The results presented in this chapter are the first to show that leptin synergised with IL-1α and the TLR2/6 agonist pam2CSK4 to enhance the production of the collagenase MMP-1 and the stromelysin MMP-3 by gingival fibroblasts. This is the first report of such synergies in any human fibroblasts, and the first report of synergy between leptin and a TLR2 agonist in any primary human cells.

Gene expression of the collagenase MMP-13 increases in gingival fibroblasts after stimulation with IL-1β at concentrations above and below the 0.05 ng/ml IL-1α used in the current study (Abe et al., 2001). In contradiction to this, MMP-13 gene expression was only induced in hGFs stimulated with leptin+IL-1α in this study; no similar induction of MMP-13 gene expression in hGFs was detected after the presumably equally potent leptin+IL-1α+OSM stimulation. It is difficult to understand the inconsistency between these studies as it is unclear how many donors the results presented by Abe et al. (2001) represent. Additionally, in the present study MMP-13 gene expression was only assessed in one hGF donor. Therefore, further analysis of MMP-13 expression in hGFs from other donors may help to understand the regulation of this MMP during hyperleptinaemic and inflammatory conditions. Nonetheless, these studies suggest that under certain stimulatory conditions MMP-13 expression, which is thought to be induced only in RUNX2-expressing osteogenic cells (including PDL fibroblasts (Iwayama et al., 2012)) as described in Section 3.5, can be upregulated in non-osteogenic cell types. If MMP-13 expression was found to be increased in hGFs more consistently, it would be interesting to measure RUNX2 expression to help understand the mechanism behind MMP-13 regulation in these cells.

Increased production of the stromelysin MMP-10 has been observed after IL-1α+OSM stimulation of chondrocytes and synovial fibroblasts (Barksby et al., 2006), in contradiction to the results presented in this chapter. This disparity may be because MMP-10 gene expression was only determined in one donor using semi-quantitative RT-PCR in this study. Further study of MMP-10 production by quantitative methods and in more donors will be required to better understand the regulation of MMP-10 in gingival fibroblasts, and how this compares to other mesenchymal cells.

Together, these findings have similar implications to those discussed in Section 3.5; leptin may enhance fibrillar collagen degradation in the gingiva as the stromelysin
MMP-3 can activate the collagenase MMP-1 (Burrage et al., 2006). The synergistic increases in MMP-1 and MMP-3 production by gingival fibroblasts described in this chapter add to the results presented in Chapter 3, and suggest that under inflammatory conditions leptin substantially enhances gingival fibroblast-mediated collagen remodelling. Both MMP-1 and MMP-3 target other ECM components (Nagase et al., 2006), and therefore, under inflammatory conditions leptin may also substantially enhance the remodelling of a range of ECM components by gingival fibroblasts.

IL-1α and IL-1α+OSM appear to increase MMP-14 gene expression in chondrocytes (although no statistical analysis was performed) (Koshy et al., 2002), which is in contrast to the results of the current study. This inconsistency may be explained by the different concentrations of IL-1α used (Koshy et al. (2002) used 1 ng/ml IL-1α while 0.05 ng/ml IL-1α was used in this study) or by the different cell types studied. The lack of regulation of MMP-2 gene expression observed in the results presented in this chapter are in agreement with the evidence that MMP-2 is not highly regulated at the transcriptional level (Burrage et al., 2006). However, the expression of MMP-2 is significantly increased in PDL cells after stimulation with concentrations of IL-1 similar to those used in this study (Xiang et al., 2009); this discrepancy could illustrate a difference between PDL and gingival cells. In this study, TIMP-2 mRNA levels appeared to be lower in hGFs after stimulation with any combination of leptin, IL-1α and OSM compared to the individual stimuli or unstimulated control. However, TIMP-2 expression was only determined in one donor using semi-quantitative RT-PCR. Further analysis of TIMP-2 production by quantitative methods and in more donors are required to confirm whether leptin, IL-1 and OSM have an additive or synergistic effect on TIMP-2 expression in hGFs. MMP-14, MMP-2 and TIMP-2 are implicated in regulating pericellular ECM remodelling as described in Section 3.5. As combinations of leptin, IL-1 and OSM had no ability to synergistically increase the expression of MMP-14, MMP-2 and TIMP-2, it is possible that this axis of pericellular ECM remodelling by gingival fibroblasts is not enhanced during hyperleptinaemic and inflammatory conditions above that observed in hyperleptinaemia.

Lack of detectable gene expression for the other gelatinase MMP-9 and the matrilysin MMP-7 have been described previously in gingival fibroblasts after stimulation with P. gingivalis supernatant (Zhou and Windsor, 2006), which is in agreement with the MMP-9 and MMP-7 expression results in stimulated hGFs in this study. This finding supports the evidence that MMP-9 (as described in Section 3.5) and MMP-7 are
expressed in a cell type-specific manner (Loffek et al., 2011). TNF-α increases MMP-9 secretion from gingival fibroblasts (Beklen et al., 2006), suggesting that MMP-9 production by gingival fibroblasts may be regulated at a level downstream of gene expression.

None of the combinations of leptin, IL-1α, OSM, pam2CSK4 and LPS had any ability to significantly regulate TIMP-1 expression in hGFs adding to the results of leptin-stimulated TIMP-1 expression presented in Section 3.3.6. Interestingly, TIMP-1 gene expression is increased in IL-1α+OSM-stimulated bovine cartilage (Milner et al., 2006), and possibly by gingival fibroblasts after stimulation with LPS derived from periodontal pathogens (Bodet et al., 2007). In the study by Bodet et al. (2007) no loading control was presented for the immunoblotting used to assess TIMP concentrations therefore it is possible that the results are biased by non-specific changes in protein production after LPS stimulation. The inconsistency between the results in this chapter and the results of Milner et al. (2006) regarding TIMP expression are likely to be due to the different cell types/tissue studied. In another study, 1 µg/ml E. coli LPS (which had no effect on TIMP-1 expression in this study) increased TIMP-1 gene expression in gingival fibroblasts (Herath et al., 2013); however, it was not reported whether the results in their study were representative of multiple gingival fibroblast donors. The results in this study imply that under hyperleptinaemic and pro-inflammatory conditions TIMP-1 (and TIMP-2)-mediated inhibition of MMPs by gingival fibroblasts is not increased.

However, there are still some inconsistencies in the literature regarding the regulation of TIMPs (TIMP-1 in particular) by gingival fibroblasts and further studies of TIMP expression, secretion and biological activity in hGF cultures are therefore warranted.

IL-1 concentrations (usually IL-1β) in the gingiva, GCF and saliva are generally positively associated with gingivitis and periodontitis (Preshaw and Taylor, 2011); IL-1 is likely produced after activation of TLRs or other PRRs by microbes in the periodontal plaque biofilm. IL-1 is thought to play a role in the pathogenesis of periodontitis by promoting inflammation and tissue destruction (Preshaw and Taylor, 2011). Gingival fibroblasts express the IL-1 receptor on their surface (Kanda-Nakamura et al., 1996), and IL-1 stimulates a range of responses in gingival fibroblasts relevant to inflammation and tissue homeostasis (Section 1.11). Therefore, IL-1 was used in this study to model one of the signals that fibroblasts are likely exposed to during gingival inflammation.
Numerous bacterial species are positively associated with periodontitis; however no individual microbe has been identified as causal in the pathogenesis of periodontitis (Darveau, 2010). Indeed, current hypotheses suggest that particular polymicrobial communities may determine the pathogenesis of periodontitis (Darveau, 2010). Dental plaque contains both TLR2 and TLR4 agonists (Yoshioka et al., 2008). Some of the well-established periodontal pathogens stimulate inflammatory responses by activating TLR2 (de Aquino et al., 2014). For example, *P. gingivalis* strains express a LPS that stimulates TLR2, but commensal microbes can also activate TLR2 responses (Darveau, 2010). TLR2 (but not TLR4) was found to promote periodontal bone resorption in a model of mouse periodontitis (Burns et al., 2006), which suggests that TLR2 activation promotes deleterious inflammatory responses in the periodontium.

Gingival fibroblasts express multiple TLRs (Section 1.11.2), including TLR2 and TLR4 which were detected on the surface of gingival fibroblasts in this study. It is likely that gingival fibroblasts express surface TLRs to activate inflammatory responses that help kill and clear any microbes in the gingival connective tissue. The approach in this study to stimulate TLR2 and TLR4 responses in gingival fibroblasts was performed in an attempt to model two of the relevant microbial inflammatory signals that exist in the periodontium.

Alone, both IL-1 and pam2CSK4 increased MMP-1 and MMP-3 production by gingival fibroblasts and had no effect on TIMP-1 expression, in agreement with previous studies (see Section 4.1). Therefore, these proinflammatory stimuli (and leptin) may increase gingival fibroblast-derived MMP activity in the gingiva by increasing the MMP:TIMP ratio. A key finding presented in this chapter is the scale of the synergistic upregulation of MMP-1 and MMP-3 production by gingival fibroblasts stimulated with IL-1+leptin and pam2CSK4+leptin. For example, the fold change in MMP-1 expression in leptin+IL-1-stimulated hGFs in this study was approximately 10 times higher than that observed in a previous study in chondrocytes using the same concentrations of leptin and IL-1 (Hui et al., 2012; Hui et al. (2012) found that these concentrations of leptin and IL-1 synergistically enhanced collagen degradation in bovine cartilage. Although these results are not entirely comparable, it could be speculated that during simultaneous hyperleptinaemic and inflammatory conditions gingival fibroblasts may promote ECM remodelling at a rate higher than ECM components can be laid down, thereby disturbing ECM homeostasis and promoting deleterious ECM degradation. Therefore, individuals who have gingivitis and are hyperleptinaemic may be more likely
to develop periodontitis. However, ECM components are not the only targets of MMPs; MMPs can also degrade molecules that have pro-inflammatory functions, potentially dampening the inflammatory response (Page-McCaw et al., 2007). Three-dimensional ex vivo or in vivo models of ECM degradation in the gingiva during hyperleptinaemic and inflammatory conditions may help to provide information regarding the functional relevance of elevated gingival fibroblast-derived MMPs.

One previous study stimulated gingival fibroblasts with leptin and *E. coli* LPS, and found that these stimuli do not synergistically regulate IL-6 and CXCL8 production (Park et al., 2013); although the number of donors tested was not reported. Unlike the other inflammatory mediators tested in this study, the ability of *E. coli* LPS (alone and in combination with leptin) to regulate MMP production by gingival fibroblasts was donor-dependent. As such, this is the first report that leptin and *E. coli* LPS can synergistically regulate MMP production in gingival fibroblasts (albeit in a single donor).

Cell surface levels of TLR4 on immune cells and synovial fibroblasts are modulated by cytokines and LPS respectively (Mita et al., 2002; He et al., 2013). Therefore, *E. coli* LPS responses could vary between gingival fibroblasts from different donors because surface expression of TLR4 is altered. In this study, TLR4 was expressed on gingival fibroblasts from all donors tested, including those that did not regulate MMP production in response to LPS. This suggests that a lack of TLR4 surface expression was not the reason why gingival fibroblasts from two of the three donors tested did not respond to LPS.

Fibroblasts from different areas of the periodontium have different phenotypes (Irwin et al., 1994; Jonsson et al., 2011), and donor-donor variability has been described in other studies of gingival fibroblasts (Tipton et al., 1991; Sukkar et al., 2007; Uehara and Takada, 2007). Another possible reason that in this study the gingival fibroblast response to LPS varied could be because an anatomically and/or phenotypically distinct gingival fibroblast population was isolated and selected from the gingival tissue donated by the individual that responded to *E. coli* LPS. Future studies of LPS responses by gingival fibroblasts from more donors and an extensive phenotyping of the isolated gingival fibroblasts may help to identify how often varied responses to LPS occur, and whether any particular fibroblast populations are involved.
Sensitivity to LPS varies between individuals (Michel et al., 2001; Wurfel et al., 2005). Together with the results in this study, this suggests that the ability of leptin to determine ECM remodelling during microbial-derived inflammation in the gingiva may be dependent on individual LPS sensitivity. The identification of the genetic or environmental basis for this finding may help to elucidate individual risks in the hyperleptinaemic population for developing periodontitis.

Overall, the results presented in this chapter demonstrated that leptin synergises with IL-1 and pam2CSK4 to regulate specific MMPs (MMP-1 and MMP-3) in gingival fibroblasts. A different MMP (MMP-13) is synergistically regulated by leptin and pro-inflammatory cytokines in chondrocytes (Hui et al., 2012), which adds to the idea discussed in Section 3.5 that gingival fibroblasts have an innate ability to determine which MMPs are subject to regulation. Not all the MMPs (e.g. MMP-14) upregulated by leptin were synergistically regulated by leptin in combination with these proinflammatory mediators. Together, these findings indicate that the regulation of MMP (and TIMP) expression in gingival fibroblasts during inflammatory and hyperleptinaemic conditions requires the intracellular integration of multiple signals at the same time. Mechanisms that may determine how MMP production is synergistically regulated in gingival fibroblasts include activating multiple signalling pathways/transcription factors that converge on MMP promoters to maximise gene expression, or increasing multiple processes in the pathway of MMP production (e.g. increasing transcription, translation and secretion rates). For example, cytokines increase MMP mRNA stability (Loffek et al., 2011); perhaps this complements the regulatory action of leptin on MMP production.

One previous study investigating leptin and OSM in combination found that leptin promoted cartilage collagen degradation independent of whether OSM was present (Hui et al., 2012). Similarly, in the present study leptin and OSM did not synergistically increase MMP-1 or MMP-3 production by gingival fibroblasts; however OSM alone did significantly increase MMP-1 production. Leptin and OSM are both members of the IL-6 cytokine family and share several characteristics; for example, both leptin and OSM activate the JAK/STAT intracellular signalling pathway (Taga and Kishimoto, 1997b). The MMP-1 promoter requires binding of transcription factors derived from several intracellular signalling pathways to promote maximal gene expression (Vincenti and Brinckerhoff, 2002). Therefore, it is possible that leptin and OSM regulate MMP-1 production by activating the same signalling pathways resulting in the activation of the
same transcription factors. As such, leptin and OSM may regulate MMP production by
gingival fibroblasts in a redundant manner. OSM is present in GCF albeit at levels not
significantly different between health and periodontitis (Lin et al., 2005; Becerik et al.,
2012). This suggests that OSM is present in the gingiva, and therefore, that leptin may
have no effect on MMP production by gingival fibroblasts in vivo. In this study OSM
did not promote MMP-3 production by gingival fibroblasts, in contrast to leptin. This
suggests that leptin and OSM do not function redundantly regarding the regulation of
MMP-3 production by gingival fibroblasts, although only one OSM concentration was
tested. Further work assessing how a range of OSM concentrations affect MMP
production by gingival fibroblasts would help to address this possibility.

Leptin activates a number of intracellular signalling pathways that are implicated in
regulating MMP production (Section 1.10). Similarly, IL-1 and TLR2 agonists activate
multiple intracellular signalling pathways, including the NF-κB and MAPK pathways
(Bowie and O’Neill, 2000). The MMP-1 and MMP-3 promoters contain NF-κB binding
sites (Fanjul-Fernandez et al., 2010), suggesting that the synergistic upregulation of
MMP-1 and MMP-3 in gingival fibroblasts could be due to a NF-κB-mediated increase
in gene expression. However, the MMP-14 promoter also contains an NF-κB binding
site and was not synergistically regulated in this study (Fanjul-Fernandez et al., 2010),
suggesting that other critical factors not activated by leptin are required for MMP-14
upregulation, or that increased NF-κB activity is not sufficient for synergistic increases
in MMP-1/3 expression in gingival fibroblasts. The MMP-1 and MMP-3 promoters
have two binding sites for the transcription factor AP-1, while most other MMP
promoters only have one (Fanjul-Fernandez et al., 2010). The MMP-9 promoter is an
exception to this as it has three AP-1 binding sites, but as discussed previously MMP-9
appears to be regulated by cell-type specific transcription factors (e.g. RUNX2) (Fanjul-
Fernandez et al., 2010). Increased expression of the proteins which AP-1 comprises
(e.g. c-Fos, c-Jun), and increased AP-1 activity are promoted by the MAPK signalling
pathway (Hess et al., 2004); leptin, IL-1 and pam2CSK4 all activate the MAPK
signalling pathway (Section 1.2) (Toshchakov et al., 2005). Therefore, leptin and IL-1 or
pam2CSK4 could synergistically regulate MMP-1 and MMP-3 production in
gingival fibroblasts by amplifying AP-1-mediated MMP gene expression.

In this study leptin and OSM increased MMP-14 gene expression, while IL-1α and
pam2CSK4 had no similar effect. This suggests that members of the IL-6 cytokine
family regulate MMP-14 gene expression in a manner distinct to that of other
inflammatory stimuli. Mechanistically, this could be regulated via the activation of signalling pathways (e.g. JAK/STAT) which IL-6 family cytokines, but not IL-1 or TLR2 agonists, stimulate (Taga and Kishimoto, 1997b). However, the MMP-14 promoter does not contain a STAT binding site (Fanjul-Fernandez et al., 2010). Interestingly, the MMP-14 promoter also lacks binding sites for AP-1 and other transcription factors that are important for regulating the expression of MMPs (Lohi et al., 2000) (Fanjul-Fernandez et al., 2010). MMP-14 was not synergistically regulated in this study and together this supports the idea discussed above that AP-1 activity could be involved in determining synergistic regulation of MMPs in gingival fibroblasts.

IL-1, TLR2 and TLR4 all activate the same canonical signalling events e.g. the NF-κB signalling pathway in a MyD88-dependent manner (Bowie and O'Neill, 2000). As *E. coli* LPS failed to reproducibly synergise with leptin, this suggests that the canonical TLR/IL-1 intracellular signalling pathway may not regulate the synergy observed between leptin and IL-1/pam2CSK4 in hGFs. Alternatively, somewhere along the complex intracellular signalling pathways activated in IL-1 and pam2CSK4-stimulated hGFs molecules unique to these stimuli are activated or available which are not similarly utilised after *E. coli* LPS stimulation (except in hGFs from the responsive donor). For example, TLR2 and TLR4 have a differential ability to activate IRF transcription factors (Jung et al., 2005), and a differential requirement for TIR domain-containing adaptor proteins which interact with the intracellular tail of TLRs (Toshchakov et al., 2005).

Clearly, further studies that investigate the likely multiple and complicated intracellular mechanisms behind these synergies are warranted. One approach to investigate this would be to use chemical inhibitors or siRNA to specifically target molecules within intracellular signalling pathways in the aim to identify which pathways/molecules are required to enhance MMP gene expression in hGFs stimulated with leptin and inflammatory mediators (Chapter 5).

In conclusion, the results in this chapter provide the first evidence that gingival fibroblast-mediated ECM remodelling may be synergistically enhanced during hyperleptinaemia and inflammation. These results support the hypothesis that leptin is a molecular link between conditions associated with hyperleptinaemia (e.g. obesity, T2DM) and periodontitis.
Further lines of work could be pursued in relation to the results presented in this chapter. Numerous other endogenous and exogenous molecules (including anti-inflammatory molecules) regulate MMP and TIMP production by gingival fibroblasts (Domeij et al., 2002; Matsushita et al., 2006; Sukkar et al., 2007; Zhang et al., 2011; Kuo et al., 2012; Ujii et al., 2012). Therefore, it is unknown whether the synergistic regulation of MMP-1 and MMP-3 by leptin and IL-1/TLR2 agonists in gingival fibroblasts is relevant to gingival ECM remodelling in vivo where other stimuli will also be present. This could be addressed by using in vivo or 3D tissue models where a more genuine inflammatory response could be induced (Chapter 7).
Table 4.1: MMP and TIMP expression in hGFs after stimulation with leptin (0.1 μg/ml), IL-1 and OSM.

Supernatants were collected and cDNA was prepared from hGFs after stimulation with leptin (0.1 μg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 0.1 μg/ml, IL-1α 0.05 ng/ml), OSM (5 ng/ml), leptin+OSM (leptin 0.1μg/ml, OSM 5 ng/ml), IL-1α+OSM (IL-1α 0.05 ng/ml, OSM 5 ng/ml) and leptin+IL-1α+OSM (leptin 0.1 μg/ml, IL-1α 0.05 ng/ml, OSM 5 ng/ml) for 24 h. Real-time RT-PCR was used to assess MMP/TIMP and RNAP gene expression. Relative MMP/TIMP gene expression was determined using the 2−ΔΔCt method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean±SD or median (IQR) (n=12) from three donors stimulated in independent experiments. MMP-13, MMP-9 and MMP-7 gene expression was not detected in hGFs after any treatment conditions (n=4, one donor). ND – not detected. Statistics: T tests for independent samples or Mann-Whitney U test (Bonferroni-corrected). For clarity no indications of significance are displayed in this table; however, selected p values are indicated in the relevant body text or figure.

<table>
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<th>0.1 µg/ml leptin</th>
<th>IL-1</th>
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<th>IL-1+OSM</th>
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<td></td>
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<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Collagenase</td>
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</tr>
<tr>
<td>MMP-1</td>
<td>1.22 ±1.65</td>
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<td>(0.37-2.45)</td>
<td>(3.80-8.89)</td>
<td>(4.72-11.6)</td>
<td>(3.56-10.4)</td>
<td>(3.47-12.7)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>(1.81-4.87)</td>
<td>(1.81-5.82)</td>
<td>(1.45-6.73)</td>
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<td>ND</td>
<td>ND</td>
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<td>MMP-14</td>
<td>1.29 ±0.47</td>
<td>0.994</td>
<td>1.16</td>
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<td>(0.37-2.45)</td>
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<tr>
<td>TIMP-1</td>
<td>1.13 ±0.47</td>
<td>0.994</td>
<td>1.16</td>
<td>1.13</td>
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<td>(0.37-2.45)</td>
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<td>(1.77-2.62)</td>
<td>(1.81-4.87)</td>
<td>(1.45-6.73)</td>
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</tbody>
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Table 4.2: MMP and TIMP expression in hGFs after stimulation with leptin (10 µg/ml), IL-1 and OSM.

Supernatants were collected and cDNA was prepared from hGFs after stimulation with leptin (10 µg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml), OSM (5 ng/ml), leptin+OSM (leptin 10 µg/ml, OSM 5 ng/ml), IL-1α+OSM (IL-1α 0.05 ng/ml, OSM 5 ng/ml) and leptin+IL-1α+OSM (leptin 10 µg/ml, IL-1α 0.05 ng/ml, OSM 5 ng/ml) for 24 h. Real-time RT-PCR was used to assess MMP/TIMP and RNAP gene expression. Relative MMP/TIMP gene expression was determined using the 2^(-dcT) method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean±SD or median (IQR) (n=12) from three donors stimulated in independent experiments. MMP-9 and MMP-7 gene expression was not detected in hGFs after any treatment conditions (n=4, one donor); MMP-13 was only detected in hGFs stimulated with leptin+IL-1 (indicated by +). ND – not detected. Statistics: T tests for independent samples or Mann-Whitney U test (Bonferroni-corrected). For clarity no indications of significance are displayed in this table; however, selected p values are indicated in the relevant body text or figure.

<table>
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<th>10 µg/ml leptin</th>
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<th>OSM</th>
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<td></td>
<td>MMP-13</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
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<td>Gelatinase</td>
<td>MMP-2</td>
<td>3.65 ±2.82</td>
<td>1.18 ±0.86</td>
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<td>(2.95-7.23)</td>
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<td>Matrilysin</td>
<td>MMP-14</td>
<td>3.64 ±2.10</td>
<td>1.25 ±0.45</td>
<td>3.78 ±1.19</td>
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<td>MT-MMP</td>
<td>(2.62-7.98)</td>
<td>(0.65-1.92)</td>
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<td>TIMPs</td>
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<td>0.994 ±0.398</td>
<td>0.920 ±0.408</td>
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Figure 4.1: MMP-1 gene expression in hGFs after stimulation with leptin and IL-1α.

hGFs were stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the 2^ΔΔCt method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Figure 4.2: MMP-1 protein secretion by hGFs after stimulation with leptin and IL-1α.

hGFs were stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml) for 24 h. Supernatants were collected and MMP-1 concentrations were assessed by ELISA. Data are shown as median+IQR (n=12) from three donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Table 4.3: MMP-1 and MMP-3 secretion by hGFs after stimulation with leptin (10 μg/ml), IL-1 and OSM.

Supernatants were collected and cDNA was prepared from hGFs after stimulation with leptin (10 μg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml), OSM (5 ng/ml), leptin+OSM (leptin 10 μg/ml, OSM 5 ng/ml), IL-1α+OSM (IL-1α 0.05 ng/ml, OSM 5 ng/ml) and leptin+IL-1α+OSM (leptin 10 μg/ml, IL-1α 0.05 ng/ml, OSM 5 ng/ml) for 24 h. Supernatants were collected and MMP1 and MMP-3 concentrations were assessed by ELISA. Data are shown as median (IQR) or mean ±SD (n=12) from three donors stimulated in independent experiments. ND – not detected. Statistics: T tests for independent samples or Mann-Whitney U test (Bonferroni-corrected). For clarity no indications of significance are displayed in this table; however, selected p values are indicated in the relevant body text or figure.

<table>
<thead>
<tr>
<th></th>
<th>10 μg/ml leptin</th>
<th>IL-1</th>
<th>OSM</th>
<th>IL-1+OSM</th>
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<td></td>
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<td>0.501</td>
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<tr>
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<td>0.211 ±0.062</td>
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Figure 4.3: MMP-3 gene expression in hGFs after stimulation with leptin, IL-1α and OSM.

hGFs were stimulated with leptin (0.1 or 10 μg/ml), IL-1α (0.05 ng/ml), OSM (5 ng/ml), leptin+IL-1α (leptin 0.1 or 10 μg/ml, IL-1α 0.05 ng/ml), leptin+OSM (leptin 0.1 or 10 μg/ml, OSM 5 ng/ml) or leptin+IL-1α+OSM (leptin 0.1 or 10 μg/ml, IL-1α 0.05 ng/ml, OSM 5 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Semi-quantitative RT-PCR was used to assess MMP-3 mRNA and 18S rRNA expression. The expected product size for MMP-3 was 150 bp, and for 18S rRNA was 85 bp. This experiment was performed in three donors (donors 2–4) and the results for each are displayed.
Figure 4.4: MMP-3 protein secretion by hGFs after stimulation with leptin and IL-1α.

hGFs were stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml) for 24 h. Supernatants were collected and MMP-3 concentrations were assessed by ELISA. Data are shown as mean+SD (n=12) from three donors stimulated in independent experiments. ND – not detected. Statistics: T tests for independent samples (Bonferroni-corrected) ***=p<0.001 for comparisons as indicated.
Figure 4.5: MMP-2 gene expression in hGFs after stimulation with leptin and OSM.

hGFs were stimulated with leptin (10 μg/ml), OSM (5 ng/ml) or leptin+OSM (leptin 10 μg/ml, OSM 5 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-2 and RNAP gene expression. Relative MMP-2 gene expression was determined using the 2^ΔΔCt method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05 as indicated.
Figure 4.6: MMP-14 gene expression in hGFs after stimulation with leptin and IL-1α.

hGFs were stimulated with leptin (10 µg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-14 and RNAP gene expression. Relative MMP-14 gene expression was determined using the 2^{-ΔΔCt} method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) **=p<0.01, ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Figure 4.7: TIMP-2 and MMP-10 gene expression in hGFs after stimulation with leptin, IL-1α and OSM.

hGFs from one donor were stimulated with leptin (0.1 or 10 μg/ml), IL-1α (0.05 ng/ml), OSM (5 ng/ml), leptin+IL-1α (leptin 0.1 or 10 μg/ml, IL-1α 0.05 ng/ml), leptin+OSM (leptin 0.1 or 10 μg/ml, OSM 5 ng/ml) or leptin+IL-1α+OSM (leptin 0.1 or 10 μg/ml, IL-1α 0.05 ng/ml, OSM 5 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Semi-quantitative RT-PCR was used to assess TIMP-2 and MMP-10 mRNA and 18S rRNA expression. The expected product size for TIMP-2 was 77 bp, for MMP-10 was 113 bp, and for 18S rRNA was 85 bp.
Table 4.4: MMP-1 and MMP-3 secretion by hGFs after stimulation with two concentrations of pam2CSK4 or *E. coli* LPS.

hGFs were stimulated with leptin (10 μg/ml), pam2CSK4 (50 or 100 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 or 100 ng/ml), *E. coli* LPS (10 or 1000 ng/ml) and leptin+*E. coli* LPS (leptin 10 μg/ml, *E. coli* LPS 10 or 1000 ng/ml) for 24 h. Supernatants were collected and MMP1 and MMP-3 concentrations were assessed by ELISA. Data are shown as mean ±SD or median (IQR) (n=12) fold control (MMP-1) or concentration in ng/ml (MMP-3) from three donors stimulated in independent experiments. Statistics: T tests for independent samples or Mann-Whitney U test (Bonferroni-corrected).

<table>
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<th></th>
<th>Basal</th>
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<td></td>
<td>10 μg/ml leptin</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Collagenase</td>
<td></td>
<td>1.00 ±0.16</td>
<td>5.59 ±2.90</td>
<td>5.60 ±2.85</td>
<td>19.8 ±4.5</td>
</tr>
<tr>
<td>Stromelysin</td>
<td>MMP-1</td>
<td>0.00 (0.00-0.377)</td>
<td>1.87 (0.00-2.24)</td>
<td>1.65 (0.00-2.52)</td>
<td>3.63 (0.15-3.93)</td>
</tr>
</tbody>
</table>
Table 4.5: MMP and TIMP expression in hGFs after stimulation with leptin, pam2CSK4 and E. coli LPS.

Supernatants were collected and cDNA was prepared from hGFs after stimulation with leptin (10 µg/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml), E. coli LPS (10 ng/ml) and leptin+E. coli LPS (leptin 10 µg/ml, E. coli LPS 10 ng/ml) for 24 h. Real-time RT-PCR was used to assess MMP/TIMP and RNAP gene expression. Relative MMP/TIMP gene expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. MMP-1 expression in LPS-stimulated hGFs was donor dependent and these results are presented in Table 4.7. Statistics: T tests for independent samples (Bonferroni-corrected). For clarity no indications of significance are displayed in this table; however, selected p values are indicated in the relevant body text or figure.

<table>
<thead>
<tr>
<th></th>
<th>pam2CSK4</th>
<th>E. coli LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml leptin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Collagenase MMP-1</td>
<td>9.76 ±2.34</td>
<td>8.67 ±6.47</td>
</tr>
<tr>
<td>MT-MMP MMP-14</td>
<td>2.17 ±2.07</td>
<td>0.97 ±0.74</td>
</tr>
<tr>
<td>TIMPs TIMP-1</td>
<td>1.14 ±0.43</td>
<td>1.01 ±0.33</td>
</tr>
</tbody>
</table>
Figure 4.8: MMP-1 gene expression in hGFs after stimulation with leptin and pam2CSK4.

hGFs were stimulated with leptin (10 μg/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the 2\(^{-\Delta\Delta C_T}\) method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05, ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Figure 4.9: MMP-1 protein secretion by hGFs after stimulation with leptin and pam2CSK4.

hGFs were stimulated with leptin (10 μg/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) for 24 h. Supernatants were collected and MMP-1 concentrations were assessed by ELISA. Data (fold unstimulated control) are shown as median+IQR (n=12) from three donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Table 4.6: MMP-3 protein secretion by hGFs stimulated with leptin and pam2CSK4.

hGFs were stimulated with leptin (10 µg/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. Supernatants were collected and analysed for MMP-3 by ELISA. Data is presented as the mean±SD (n=4) MMP-3 concentration (ng/ml) for each donor tested by treatment condition. ND – not detected. Statistics: T tests for independent samples (Bonferroni-corrected) **=p<0.01, ***=p<0.001 compared to unstimulated control; # p<0.05, ###=p<0.001 compared to leptin-stimulated hGFs; $$=$p<0.001 compared to pam2CSK4-stimulated hGFs.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Unstimulated control</th>
<th>Leptin</th>
<th>Pam2CSK4</th>
<th>Leptin+pam2CSK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 2</td>
<td>ND</td>
<td>0.44 ± 0.06</td>
<td>1.89 ± 0.40</td>
<td>4.06 ± 0.16 $$ $$</td>
</tr>
<tr>
<td>Donor 3</td>
<td>0.43 ± 0.07</td>
<td>1.76 ± 0.51**</td>
<td>2.19 ± 0.31***</td>
<td>3.65 ± 0.20 $$ $$</td>
</tr>
<tr>
<td>Donor 4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 4.10: MMP-14 gene expression in hGFs after stimulation with leptin and pam2CSK4.

hGFs were stimulated with leptin (10 μg/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-14 and RNAP gene expression. Relative MMP-14 gene expression was determined using the 2^(-ΔΔCt) method by using RNAP as the reference gene. Data are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01 as indicated.
Figure 4.11: TLR2 surface expression on hGFs.

Unstimulated hGFs were prepared and analysed for cell surface TLR2 expression by flow cytometry. Cells were stained with mouse anti-human TLR2 APC-conjugated monoclonal antibody or isotype control antibody. 10000 gated events were acquired using a FACSCalibur flow cytometer. This histogram displays the events for TLR2 staining (no fill) compared to an isotype control (grey fill) and is representative of results from 4 different hGF donors.
Table 4.7: MMP-1 production in hGFs after stimulation with leptin and *E. coli* LPS.

hGFs were stimulated with leptin (10 μg/ml), *E. coli* LPS (10 ng/ml) or leptin+*E. coli* LPS (leptin 10 μg/ml, *E. coli* LPS 10 ng/ml) for 24 h. [A] Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the 2<sup>-ΔΔCt</sup> method by using RNAP as the reference gene. Data (fold unstimulated control) are shown as mean+SD (n=4) for each donor. [B] Supernatants were collected and analysed for MMP-1 by ELISA. Data (fold unstimulated control) are shown as mean+SD (n=4) for each donor. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01, ***=p<0.001 compared to unstimulated control; #*=p<0.05, ##*=p<0.01 compared to leptin-stimulated hGFs; $*=p<0.05$ $**=p<0.01$ compared to LPS-stimulated hGFs.

[A] Gene expression (fold control)

<table>
<thead>
<tr>
<th>Donor 2</th>
<th>Leptin</th>
<th>LPS</th>
<th>Leptin+ LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.4 ± 2.4*</td>
<td>1.24 ± 0.24</td>
<td>13.7 ± 10.6</td>
<td></td>
</tr>
</tbody>
</table>
| Donor 3 | 9.78 ± 2.8* | 7.12 ± 4.72 | 41.1 ± 13.4$^*$^
| Donor 4 | 8.76 ± 2.1** | 1.30 ± 0.49 | 8.41 ± 2.7 |

[B] Protein (fold control)

<table>
<thead>
<tr>
<th>Donor 2</th>
<th>Leptin</th>
<th>LPS</th>
<th>Leptin+ LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.72 ± 0.56***</td>
<td>0.94 ± 0.12</td>
<td>4.53 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>Donor 3</td>
<td>5.22 ± 1.57**</td>
<td>4.79 ± 2.06**</td>
<td>11.9 ± 2.1##$$</td>
</tr>
<tr>
<td>Donor 4</td>
<td>8.45 ± 1.08***</td>
<td>1.07 ± 0.24</td>
<td>8.78 ± 0.90</td>
</tr>
</tbody>
</table>
Table 4.8: MMP-3 protein secretion by hGFs after stimulation with leptin and *E. coli* LPS.

hGFs were stimulated with leptin (10 μg/ml), *E. coli* LPS (10 ng/ml) or leptin+*E. coli* LPS (leptin 10 μg/ml, *E. coli* LPS 10 ng/ml) for 24 h. Supernatants were collected and analysed for MMP-3 by ELISA. Data (ng/ml) are shown as mean+SD (n=4) for each donor. ND – not detected. Statistics: T tests for independent samples (Bonferroni-corrected) **=p<0.01 compared to unstimulated control; ##=p<0.01 compared to leptin-stimulated hGFs; $$=p<0.05 compared to LPS-stimulated hGFs.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Unstimulated control</th>
<th>Leptin</th>
<th>LPS</th>
<th>Leptin+ LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>ND</td>
<td>0.44 ± 0.06</td>
<td>ND</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.43 ± 0.07</td>
<td>1.76 ± 0.51**</td>
<td>1.88 ± 0.42**</td>
<td>2.94 ± 0.31#$$</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 4.12: TLR4 surface expression on hGFs.

Unstimulated hGFs were prepared and analysed for cell surface TLR4 expression by flow cytometry. Cells were stained with mouse anti-human TLR4 APC-conjugated monoclonal antibody or isotype control antibody. 10000 gated events were acquired using a FACSCalibur flow cytometer. This histogram displays the events for TLR4 staining (no fill) compared to an isotype control (grey fill) and is representative of results from 4 different hGF donors.
Chapter 5. Investigation of intracellular signalling pathways which regulate MMP-1 expression in leptin-stimulated hGFs

5.1 Introduction

MMP production is regulated at multiple levels in an effort to ensure that ECM remodelling only occurs when necessary (Section 1.10). MMP-1 is highly regulated at the level of gene transcription and the MMP-1 promoter has binding sites for numerous transcription factors (e.g. NF-κB, STATs, AP-1, Ets, C/EBP) (Fanjul-Fernandez et al., 2010). These transcription factors are upregulated and/or activated downstream of intracellular signalling cascades. Several signalling pathways are implicated in promoting growth factor and cytokine-stimulated MMP-1 expression, including the NF-κB, MAPK and JAK/STAT pathways (Vincenti, 2001).

Many studies have shown that cytokines (e.g. IL-1, TNF-α, OSM) enhance MMP-1 production via the MAPK signalling pathway in fibroblasts and chondrocytes (Barchowsky et al., 2000; Mengshol et al., 2000; Han et al., 2001; Cortez et al., 2007; O’Kane et al., 2010). However, the particular MAPK signalling intermediates thus identified vary between studies. ERK and p38 MAPK activity are required for MMP-1 production in cytokine-stimulated synovial fibroblasts, chondrocytes and cardiac fibroblasts (Barchowsky et al., 2000; Mengshol et al., 2000; Cortez et al., 2007). JNK (and ERK) MAPK activity is required for enhancing cytokine-stimulated MMP-1 production in synovial fibroblasts isolated from individuals with RA (Han et al., 2001; Tagoe et al., 2008). This could suggest that different signalling pathway intermediates regulate MMP-1 production in synovial fibroblasts during RA; however, a pharmacological inhibitor of JNK has only relatively recently been available (Bennett et al., 2001). Therefore, earlier studies investigating MAPK signalling often only investigated the ERK and p38 arms of the MAPK signalling pathway; JNK activity was not explored (Barchowsky et al., 2000; Mengshol et al., 2000). Fibroblasts isolated from different sites require different MAPK intermediates to regulate MMP-1 production (Han et al., 2001; O’Kane et al., 2010). For example, synovial fibroblasts require JNK and ERK, but not p38, to regulate cytokine-stimulated MMP-1 production (Han et al., 2001); in contrast, p38, but not ERK or JNK, is required to regulate MMP-1 production by lung fibroblasts under inflammatory conditions (O’Kane et al., 2010). This suggests that the regulation of MMP-1 expression is not necessarily translatable
between different fibroblast populations, and supports investigations into the signalling pathway intermediates that regulate MMP-1 in distinct fibroblast populations.

The MAPK signalling pathway is not the only pathway that regulates MMP-1 production. Cytokine-stimulated MMP-1 expression in human RA synovial fibroblasts and cardiac fibroblasts requires NF-κB activity (Cortez et al., 2007; Tagoe et al., 2008). However, cytokine-stimulated MMP-1 production in rabbit synovial fibroblasts is not dependent on NF-κB, even though NF-κB is activated (Barchowsky et al., 2000). This suggests that the signalling pathways that regulate MMP-1 expression vary between species, and supports investigations into the signalling pathway intermediates that regulate MMP-1 in human cell populations. STAT3 activity regulates MMP-1 production in EGF-stimulated bladder epithelial cells (Itoh et al., 2006), and OSM-stimulated chondrocytes (Catterall et al., 2001). Furthermore, Itoh et al. (2006) found that only STAT3 and not STAT1 or STAT5 regulated EGF-stimulated MMP-1 production in bladder epithelial cells, suggesting that specific members of the STAT transcription factor family regulate MMP-1 production. OSM activates the PI3K/Akt signalling pathway and MMP-1 expression in chondrocytes (Litherland et al., 2008); however PI3K/Akt activity only regulates the synergistic increases in MMP-1 expression when chondrocytes are stimulated with OSM in combination with IL-1. This suggests that during the presence of multiple stimuli additional signalling pathways may play a role in regulating MMP-1 expression. This supports investigations into the signalling pathway intermediates that regulate MMP-1 during exposure to single stimuli and combinations of stimuli.

Leptin activates multiple intracellular signalling pathways, including the JAK/STAT and MAPK pathways (Section 1.2), which suggests that leptin signalling could regulate MMP production. Indeed, several studies have found that leptin regulates MMP production (Section 1.4.3). MAPK, NF-κB and PKC signalling are required for leptin-mediated MMP-1 production by human osteoarthritis cartilage (Koskinen et al., 2011). Similarly, MAPK (p38 and ERK) and PI3K/Akt signalling are required for leptin-mediated MMP-1 gene expression in primary human chondrocytes (Hui et al., 2012). The same mediators are required to regulate MMP-1 expression in chondrocytes synergistically stimulated by leptin+IL-1, suggesting that leptin and IL-1 synergistically regulate MMP-1 expression by amplifying the activity of the same intracellular signalling pathways. In contrast, the MAPK ERK and JAK2 (which is upstream of STAT transcription factor activation) are required to regulate leptin-mediated decreases
in MMP-1 production by HSCs (Cao et al., 2007). Together, these studies demonstrate that several signalling pathway intermediates regulate leptin stimulated-MMP-1 production, and that the intermediates required vary between cell types. This supports investigations into the signalling pathway intermediates that determine leptin-regulated MMP-1 expression in different cell types.

Gingival fibroblasts can be stimulated to increase the production of MMPs (Section 1.11.1), however relatively few studies have investigated which intracellular signalling pathway intermediates regulate this response. Pro-inflammatory cytokines and platelet-derived growth factor increase MMP-1 expression in gingival fibroblasts in a MAPK-dependent manner (Domeij et al., 2002; Kida et al., 2005; Ujii et al., 2012). In all of these studies p38 MAPK was required to regulate MMP-1 expression. Only Kida et al. (2005) investigated the role of other MAPKs in regulating MMP-1 production in gingival fibroblasts, and found that ERK and JNK are also required. Additionally, Kida et al. (2005) found that NF-κB signalling regulates MMP-1 expression in IL-1-stimulated gingival fibroblasts.

No studies to date have investigated intracellular signalling pathway activation in leptin-stimulated gingival fibroblasts, and whether these signalling events are involved in the regulation of MMP expression. Gingival fibroblasts express all four of the human leptin receptor isoforms at the mRNA level (Park et al., 2013). Additionally, leptin receptor protein is present at low levels in buccal connective tissue (Umeki et al., 2014). These studies suggest that gingival fibroblasts have the potential to respond to leptin directly.

The results presented thus far in this study suggest that leptin, alone or synergistically with IL-1 or pam2CSK4, increases MMP-1 mRNA levels and secretion (Chapters 3 and 4). Therefore, the aim of the experiments presented in this chapter was to investigate which signalling pathways are involved in leptin-mediated MMP expression in hGFs.

### 5.2 Leptin receptor expression by gingival fibroblasts

hGFs were lysed either under basal conditions or after stimulation with leptin (0.1 or 10 µg/ml) for 24 h. cDNA was prepared from the cell lysates and semi-quantitative RT-PCR was used to determine whether hGFs expressed the long isoform of the leptin receptor (Figure 5.1). The long isoform of the leptin receptor was expressed in all donors tested (n=7).
hGFs were prepared and analysed for cell surface leptin receptor expression by flow cytometry (Section 2.2.7) (Figure 5.2). hGFs isolated from all donors tested expressed leptin receptor on the cell surface (n=4). Across the donors tested, the MFI increased 2.2±0.2-fold in samples stained for leptin receptor compared to the isotype control.

5.3 Activation of intracellular signalling pathways in gingival fibroblasts stimulated with leptin, IL-1, OSM and pam2CSK4

The pathways investigated in this study were chosen because they are activated by leptin and can regulate MMP expression (Sections 1.2 and 1.10). hGFs were stimulated with leptin (10 μg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) or OSM (5 ng/ml) for 20 min. Cell lysates were prepared for SDS-PAGE. Selected intracellular signalling pathway intermediates were assessed by Western blot (Section 2.11.3). A 20 min time point was chosen as phosphorylation of MAPK, JAK/STAT and PI3K signalling pathway intermediates is detected at this time point after leptin and IL-1 stimulation of chondrocytes (Hui et al., 2012). Additionally, in a preliminary time course experiment leptin enhanced STAT3 and ERK (p42/44) phosphorylation at 10 and 30 min time points (albeit at very low levels) (Figure G.1). Unstimulated hGFs served as a negative control. OSM-stimulated hGFs were used to identify the signalling pathways activated by an IL-6-family cytokine, and were compared to the responses generated by leptin.

5.3.1 Leptin activation of the MAPK signalling pathway in hGFs

Leptin increased the phosphorylation of the MAPKs p38, ERK (p42/44) and JNK in hGFs isolated from all three donors compared to the unstimulated control (Figure 5.3). Increased levels of p38, ERK (p42/44) and JNK phosphorylation were also observed in hGFs stimulated with leptin+IL-1 or leptin+pam2CSK4 compared to the unstimulated control (Figure 5.3). The levels of p38 and ERK (p42/44) phosphorylation were higher in hGFs stimulated with leptin+IL-1 compared to those stimulated with leptin or IL-1 alone (in donors 4 and 7); a similar effect was observed for JNK phosphorylation (in donors 4 and 6) (Figure 5.3). This suggests that the phosphorylation of p38, ERK (p42/44) and JNK in hGFs is increased synergistically by leptin and IL-1. In hGFs isolated from two of the three donors (donors 6 and 7) the levels of p38 and JNK phosphorylation were higher after leptin+pam2CSK4 stimulation compared to those cells stimulated with leptin or pam2CSK4 alone, which suggests that the phosphorylation of p38 and JNK is synergistically increased by leptin and pam2CSK4.
No synergistic increases in ERK (p42/44) phosphorylation were detected after leptin+pam2CSK4 stimulation. OSM-stimulated hGFs had slightly increased levels of ERK (p42/44) phosphorylation compared to the unstimulated control but had little phosphorylated p38 or JNK at the 20 m time point.

Higher levels of p42 ERK were evident in hGFs in donors 6 and 7 across multiple treatment conditions (including unstimulated cells) compared to p44 ERK (Figure 5.3). In leptin or leptin+IL-1-stimulated hGFs from donor 7 higher levels of the 46 kDa JNK isoform were phosphorylated in comparison to the 54 kDa JNK isoform (Figure 5.3). In contrast, in leptin+pam2CSK4-stimulated hGFs from the same donor equal levels of phosphorylation between the JNK isoforms (46 and 54 kDa) were observed. Different JNK isoforms could not be distinguished in hGFs from the other donors; it is unclear whether the bands on these blots represent one JNK isoform or both JNK isoforms co-localised because of poor blot resolution.

5.3.2 Leptin activation of the JAK/STAT signalling pathway in hGFs

Leptin or OSM stimulation increased the tyrosine phosphorylation of STAT1 and STAT3 in hGFs isolated from all three donors compared to the unstimulated control (Figure 5.4). Increased levels of STAT1 and STAT3 tyrosine phosphorylation were also observed in hGFs stimulated with leptin+IL-1 or leptin+pam2CSK4 compared to the unstimulated control. The levels of STAT3 tyrosine phosphorylation were higher in hGFs stimulated with leptin+IL-1 compared to those stimulated with leptin or IL-1 alone in two of the three donors tested (donors 6 and 7); no similar effect was observed for STAT1 tyrosine phosphorylation (across all donors) (Figure 5.4). The levels of STAT3 tyrosine phosphorylation were higher in hGFs stimulated with leptin+pam2CSK4 compared to those stimulated with leptin or pam2CSK4 alone (also in donors 6 and 7). Together, this suggests that STAT3 tyrosine phosphorylation is synergistically increased by leptin and IL-1 or pam2CSK4 in hGFs. In hGFs isolated from donor 7 higher levels of STAT1 tyrosine phosphorylation were detected after leptin+pam2CSK4 stimulation compared to hGFs stimulated with leptin or pam2CSK4 alone; however, GAPDH levels were also higher in leptin+pam2CSK4-stimulated hGFs compared to hGFs stimulated with leptin or pam2CSK4 alone in this donor (Figure 5.4), suggesting that this effect could have been due to unequal protein levels between the samples.
Serine-phosphorylated STAT3 was not detected across any treatment conditions in hGFs isolated from two of the three donors tested (donors 4 and 7). In hGFs isolated from donor 6 the levels of STAT3 serine phosphorylation detected were low, but were increased in hGFs stimulated with leptin+IL-1, pam2CSK4 or leptin+pam2CSK4 compared to the unstimulated control (Figure 5.4). Native STAT3 protein levels were consistent across treatment conditions in hGFs (only assessed in donor 4). Leptin had no effect on STAT1 serine phosphorylation in hGFs (across all donors) compared to the unstimulated control; the levels of serine-phosphorylated STAT1 between the other treatment conditions and the unstimulated control were variable between donors (Figure 5.4).

In leptin (±IL-1 or pam2CSK4)-stimulated hGFs from donors 6 and 7 higher levels of the 91 kDa STAT1α isoform were tyrosine phosphorylated in comparison to the 84 kDa STAT1β isoform (Figure 5.4).

5.3.3 Leptin activation of the PI3K signalling pathway

Leptin, leptin+IL-1, leptin+pam2CSK4 or OSM stimulation increased the serine phosphorylation of Akt in hGFs isolated from all three donors compared to the unstimulated control (Figure 5.5). Increased levels of threonine-phosphorylated Akt were detected after leptin, leptin+IL-1, leptin+pam2CSK4 or OSM stimulation in one of the two donors tested (donor 4). Additionally, the levels of threonine-phosphorylated Akt in hGFs isolated from donor 4 were higher after leptin+pam2CSK4 stimulation compared to cells stimulated with leptin or pam2CSK4 alone (Figure 5.5). In contrast, no threonine-phosphorylated Akt was detected in the other donor assessed (donor 6) across all treatment conditions.

In one donor (donor 6), the levels of Akt serine phosphorylation were higher in leptin+IL-1-stimulated hGFs compared to hGFs stimulated with leptin or IL-1 alone; however, this was not observed in hGFs isolated from the other donors (Figure 5.5). In two of the three donors (donors 6 and 7) leptin+pam2CSK4-stimulated hGFs had higher levels of Akt serine phosphorylation compared to hGFs stimulated with leptin or pam2CSK4 alone, which suggests that serine phosphorylation of Akt is increased synergistically by leptin and pam2CSK4.

5.3.4 Leptin activation of the NF-κB signalling pathway

Leptin and OSM did not increase the levels of serine-phosphorylated NF-κB p65 in hGFs compared to the unstimulated control in the one donor tested (Figure 5.6). In
contrast, the levels of serine-phosphorylated NF-κB p65 were higher in hGFs stimulated with IL-1 or pam2CSK4 (with or without leptin) compared to the unstimulated control.

5.4 Signalling pathway intermediates which regulate MMP-1 expression in leptin-stimulated gingival fibroblasts

The signalling pathways chosen to be inhibited were selected based on whether they were activated by leptin, IL-1 or pam2CSK4 in Section 5.3. hGFs were pre-treated for 30 min with chemical pathway inhibitors; a full description of the inhibitors and the concentrations used is presented in Table 5.1. An additional pre-treatment condition, 0.1 % v/v DMSO, was used to control for non-specific effects as the inhibitors were dissolved in DMSO. After pre-treatment, hGFs were stimulated with leptin (10 µg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 µg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. hGFs were lysed and cDNA prepared by reverse transcription. Real-time RT-PCR was used to assess how the relative levels of MMP-1 gene expression in stimulated hGFs were affected by inhibiting specific signalling pathway intermediates.

In a preliminary experiment, hGFs were pre-treated for 30 m with either 0.1 % v/v DMSO or serum-free DMEM (SFM) prior to stimulation as above. Real-time RT-PCR was used to assess whether the relative levels of MMP-1 gene expression in stimulated hGFs were affected by 0.1 % v/v DMSO. DMSO pre-treatment (0.1 % v/v) had no significant effect on MMP-1 gene expression for any of the stimulatory conditions tested (Appendix H).

Bay 11-7085 at a concentration of 10 µM did not cause any toxicity in hGFs as described in the methods (Section 2.2.6.1). However, hGFs from all the subsequent donors treated with 10 µM Bay 11-7085 were subject to extensive toxicity as evidenced by loss of attachment and shrinkage upon visual inspection at the 24 h end point of the stimulation. Therefore, MMP-1 gene expression was not assessed in these samples.

5.4.1 MEK/ERK regulate leptin-stimulated MMP-1 gene expression in hGFs

Inhibition of MEK/ERK signalling in leptin-stimulated hGFs caused a 3.0-fold decrease (p<0.01) in MMP-1 gene expression (Figure 5.7). Similarly, 5.4-fold and 3.2-fold decreases in MMP-1 gene expression in IL-1 and leptin+IL-1-stimulated hGFs respectively were observed after MEK/ERK inhibition (both p<0.001). Additionally, inhibition of MEK/ERK signalling in pam2CSK4 and leptin+pam2CSK4-stimulated
hGFs caused 2.5-fold and 3.8-fold decreases respectively in MMP-1 gene expression (pam2CSK4 p<0.05, leptin+pam2CSK4 p<0.001).

5.4.2 JNK regulation of MMP-1 gene expression in hGFs
JNK inhibition did not significantly alter MMP-1 gene expression in hGFs stimulated with leptin or pam2CSK4 (Figure 5.8). However, MMP-1 gene expression was reduced 2.3-fold after JNK inhibition in hGFs stimulated with leptin+pam2CSK4 (p<0.05). Interestingly, inhibition of JNK reduced MMP-1 gene expression 2.3-fold and 36 % in hGFs stimulated with IL-1 and leptin+IL-1 respectively (both p<0.01).

5.4.3 p38 regulation of MMP-1 gene expression in hGFs
Inhibition of p38 signalling did not significantly alter MMP-1 gene expression in hGFs stimulated with leptin, IL-1, pam2CSK4 or leptin+pam2CSK4 (Figure 5.9). In contrast, MMP-1 gene expression was reduced 2.3-fold in hGFs stimulated with leptin+IL-1 after inhibition of p38 signalling.

5.4.4 STAT3 regulation of MMP-1 gene expression in hGFs
STAT3 inhibition did not significantly alter MMP-1 gene expression in hGFs stimulated with leptin or pam2CSK4 (Figure 5.10). However, MMP-1 gene expression was reduced 2.3-fold after STAT3 inhibition in hGFs stimulated with leptin+pam2CSK4 (p<0.05). Inhibition of STAT3 signalling reduced MMP-1 gene expression 2.3-fold and 39 % in hGFs stimulated with IL-1 and leptin+IL-1 respectively (both p<0.05).

5.4.5 AKT regulation of MMP-1 gene expression in hGFs
AKT inhibition did not significantly alter MMP-1 gene expression in hGFs under any of the treatment conditions tested (Figure 5.11).

5.5 Discussion
The increased production of the collagenase MMP-1 under inflammatory conditions is implicated in deleterious collagen and ECM remodelling, as occurs in osteoarthritis, rheumatoid arthritis (RA) and periodontitis (Burrage et al., 2006; Sorsa et al., 2006). Leptin (alone and in synergy with pro-inflammatory mediators) increases MMP-1 production in chondrocytes and cartilage (Koskinen et al., 2011; Hui et al., 2012), and in gingival fibroblasts in this study (Chapters 3 and 4). Multiple signalling pathway intermediates are implicated in regulating MMP-1 gene expression (Section 1.10), but the individual signalling mediators that are involved vary depending on cell type and input stimuli. No studies of leptin-stimulated intracellular signalling in gingival
fibroblasts have been previously reported; therefore it was unknown how leptin signalling might affect MMP-1 expression in gingival fibroblasts. The results presented in this chapter show that leptin activates the MAPK, JAK/STAT, and PI3K signalling pathways in gingival fibroblasts. Additionally, the MAPK and JAK/STAT signalling pathways were found to be required for increasing MMP-1 gene expression in gingival fibroblasts after stimulation with leptin, IL-1 and/or pam2CSK4.

The long leptin receptor isoform is expressed by gingival fibroblasts as demonstrated by Park et al. (2013), and in this study. Cell surface expression of the leptin receptor has been detected in human cells previously (Zarkesh-Esfahani et al., 2004), and in gingival fibroblasts for the first time in this study. One of the limitations of measuring cell surface leptin receptor by flow cytometry is that the extracellular region of the leptin receptor is the same for the different isoforms, and therefore it is unclear which of the isoforms are present on the cell surface. All the short isoforms of the leptin receptor are expressed at the mRNA level by gingival fibroblasts (Park et al., 2013), so it is likely that the detected leptin receptor on the surface on gingival fibroblasts consists of multiple leptin receptor isoforms. This could affect the degree of intracellular signalling stimulated by leptin, because only the long isoform leptin receptor can facilitate the activation of all the intracellular signalling pathways that leptin stimulates (Bjorbaek et al., 1997).

In this study enhanced STAT3 Y705, STAT1 Y701, MAPK (ERK, JNK, p38) and Akt phosphorylation was detected in gingival fibroblasts after leptin stimulation for the first time; these are some of the well characterised signalling responses activated downstream of the long leptin receptor isoform (Coppari and Bjorbaek, 2012).

Together, these data suggest that functional long leptin receptor isoform is present on the surface of fibroblasts within the gingiva, and that one of the functions of gingival fibroblasts is to directly sense local leptin concentrations. Further experiments that aim to specifically block leptin/leptin receptor interaction are required to confirm this. In the current study several experiments using a leptin antagonist (ProSpec, Ness-Ziona, Israel) were performed. The leptin antagonist is recombinant leptin but with three mutations (L39A, D40A, F41A) that competitively binds to the leptin receptor, but does not activate STAT3 signalling (Shpilman et al., 2011). The leptin antagonist had no ability to reduce MMP-1 production (protein) or STAT3 tyrosine phosphorylation by
hGFs stimulated with leptin, possibly due to the use of an ineffective leptin:antagonist ratio (Appendix I).

Leptin failed to increase NF-κB p65 phosphorylation in gingival fibroblasts, although this conclusion should be viewed with caution as hGFs from only one donor were tested. This result, however, contradicts a previous study investigating leptin signalling in synovial fibroblasts from individuals with RA (Tong et al., 2008). Tong et al. (2008) investigated leptin signalling at a similar time point and using a similar concentration of leptin to this study, suggesting that this discrepancy is due to the different cell types tested or disease status of the cells. Further studies of NF-κB signalling in hGFs isolated from more donors are required to confirm this.

STAT1 and STAT3 tyrosine phosphorylation is a feature of leptin receptor and OSM receptor signalling (e.g. type I cytokine receptor signalling) (Fantuzzi and Faggioni, 2000). STAT1 and STAT3 tyrosine phosphorylation was observed in this study in gingival fibroblasts after stimulation with OSM or leptin, but not IL-1 or pam2CSK4. This provides some support to the theory that leptin and OSM do not synergise to regulate MMP production because they activate similar signalling pathways (Section 4.4).

Differences in the relative abundance and phosphorylation of ERK MAPK isoforms have been described previously (Pages and Pouyssegur, 2004), and were observed in this study. Interestingly, ERK2 (p42) knockout mice are embryonic lethal while ERK1 (p44) knockout mice are viable (Yao et al., 2003), which suggests that the ERK isoforms have different functions. Furthermore, ERK2 promotes the proliferative responses of mouse embryonic fibroblasts (Vantaggiato et al., 2006), while ERK1 acts as an inhibitor of ERK2 presumably by competing for MEK (the kinase directly upstream of and responsible for phosphorylating ERK) (Mazzucchelli et al., 2002; Vantaggiato et al., 2006). Together with the higher levels of total and phosphorylated ERK2 compared to ERK1 in gingival fibroblasts observed in this study, this suggests that ERK2-mediated responses may predominate over ERK1 responses in gingival fibroblasts. The inhibitor used in this study, U0126 inhibits MEK, and therefore inhibits both ERK isoforms; future investigations using siRNA to target ERK2 and ERK1 individually may help to delineate the functional importance of the different ERK isoforms in gingival fibroblasts.
An increase in the relative abundance of p46 compared to the p54 molecular weight form of phosphorylated JNK MAPK was observed in this study. Differences in the downstream targets of p46 JNK and p54 JNK have been described previously (Liu et al., 2004; Singh et al., 2009); for example, p46 JNK (but not p54 JNK) is essential for phosphorylating the transcription factors c-Jun and ATF-2 in mouse fibroblasts (Liu et al., 2004). Together, this suggests that there is more p46 JNK activity than p54 JNK activity in leptin-stimulated hGFs, and that this might be affecting the phosphorylation of downstream transcription factors. However, there are three JNK genes in humans and 10 different JNK isoforms due to differential mRNA splicing; all of these isoforms have a molecular weight of approximately 46 or 54 kDa (Bennett et al., 2001). It would be interesting to determine by RT-PCR which of the JNK isoforms are expressed in (leptin-stimulated) gingival fibroblasts as a first step in attempting to understand whether the different JNK isoforms are responsible for different gingival fibroblast responses.

STAT1 has two isoforms which are generated by alternative splicing: STAT1α (91 kDa) and STAT1β (84 kDa) (Darnell, 1997). Both STAT1 isoforms were tyrosine phosphorylated in leptin-stimulated gingival fibroblasts in this study, and there was a greater relative abundance of pSTAT1α Y701 compared to pSTAT1β Y701. STAT1α and STAT1β regulate different transcriptional responses, thought to be partly due to the lack of a transactivation domain (which is a binding site for transcriptional activators) in STAT1β that is present in STAT1α (Darnell, 1997; Najjar et al., 2008). This suggests that STAT1α activity may predominate over STAT1β activity in leptin-stimulated gingival fibroblasts. Plasmid-mediated overexpression of STAT1 isoforms has been used previously to determine the differential roles of STAT1α and STAT1β in B cells (Najjar et al., 2008), and may be a technique amenable to further investigation of the relative functions of STAT1 isoforms in hGFs.

The MAPK signalling pathway is activated by leptin, and is required to regulate MMP-1 production in chondrocytes and cartilage (Koskinen et al., 2011; Hui et al., 2012), and in gingival fibroblasts in this study. The MAPK signalling pathway regulates MMP production in several ways (Burrage et al., 2006). ERK and JNK MAPK phosphorylate components of the dimeric transcription factor AP-1 (e.g. c-Fos and c-Jun), thereby activating AP-1 and promoting AP-1 binding to DNA (Mengshol et al., 2002). The promoter region for MMP-1 contains several binding sites for AP-1; AP-1 is considered a key transcription factor for MMP-1 gene expression (Burrage et al., 2006).
Additionally, ERK and JNK-activated c-Jun can bind to the c-Jun and c-Fos promoters, thereby increasing the levels of AP-1 protein and promoting MMP gene expression in an indirect manner (Burra et al., 2006). ERK and p38 MAPK are also implicated in activating the transcription factor Ets which can directly enhance MMP-1 gene expression by binding to PEA-1 sites on the MMP-1 promoter (Burra et al., 2006). Therefore, MAPK signalling can regulate MMP-1 gene expression by directly activating transcription factors that bind to the MMP-1 promoter, or by indirectly upregulating the transcription of genes like c-Fos and c-Jun that make up transcription factors that regulate MMP-1 expression.

In this study, leptin and pam2CSK4 required ERK MAPK signalling to increase MMP-1 gene expression in hGFs. A similar requirement for ERK in regulating MMP-1 expression was observed in a study of leptin-stimulated chondrocytes (Hui et al., 2012). However, Hui et al. (2012) also found that p38 and JNK regulated leptin-stimulated MMP-1 gene expression in chondrocytes; no significant requirement for these MAPK mediators was observed regarding leptin (or pam2CSK4)-stimulated MMP-1 expression in gingival fibroblasts in this study. This discrepancy may be due to the different cell types studied.

IL-1-stimulated gingival fibroblasts require MAPK signalling to regulate MMP-1 production as observed in this and other studies (Domeij et al., 2002; Kida et al., 2005). In the present study ERK and JNK, but not p38, were required to regulate IL-1-stimulated MMP-1 gene expression; however, Kida et al. (2005) found that all three of these MAPK mediators were required to regulate MMP-1 secretion in IL-1-stimulated gingival fibroblasts. The activation of MAPK signalling is regulated by the strength of the stimuli (Raman et al., 2007). The discrepancy between this study and that of Kida et al. (2005) could be due to a dose-dependent effect of IL-1 (in this study 0.05 ng/ml IL-1 was used, while Kida et al. (2005) used a higher concentration of 2.5 ng/ml). Additionally, Domeij et al. (2002) found that p38 was required for IL-1-stimulated MMP-1 secretion by gingival fibroblasts. Both Domeij et al. (2002) and Kida et al. (2005) investigated MMP-1 secretion, not gene expression, which suggests that p38 may regulate MMP-1 production at a level downstream of gene transcription and RNA stability in gingival fibroblasts.

Taken together, these results suggest that ERK regulates non-synergistic increases in MMP-1 gene expression in leptin and pam2CSK4-stimulated gingival fibroblasts, while
ERK and JNK regulate IL-1-stimulated MMP-1 expression. Mechanistically, MMP-1 expression in leptin, IL-1 and pam2CSK4-stimulated hGFs could be enhanced by ERK/JNK dependent increases in the activity and protein levels of the transcription factor AP-1, and by ERK-dependent increases in the activity of the transcription factor Ets.

MAPK intermediates partially regulated MMP-1 gene expression in hGFs after stimulation with leptin+IL-1 (ERK, JNK and p38), or leptin+pam2CSK (ERK and JNK). This suggests that multiple arms of the MAPK signalling pathway are required to generate the levels of MMP-1 gene expression observed during the synergy between these stimuli. Therefore, it is likely that a broad amplification and activation of the MAPK signalling pathway is one mechanism by which leptin and IL-1 or pam2CSK4 synergistically regulate MMP-1 expression. For example, similar to ERK and JNK, p38 can increase the expression of c-Fos and c-Jun, thereby increasing AP-1 protein levels and potentially enhancing MMP-1 expression (Vincenti and Brinckerhoff, 2002) (as discussed above). Thus, the synergistic upregulation of MMP-1 expression in hGFs by leptin+IL-1 could be mediated by an additional increase in c-Jun and c-Fos expression due to p38 activity.

In this study MMP-1 expression in hGFs after stimulation with IL-1±leptin or leptin+pam2CSK4 required STAT3 signalling. This suggests that the MAPK signalling pathway is not the only intracellular signalling cascade required for MMP-1 expression in hGFs. However, STAT3 may only play a role in promoting the higher levels of MMP-1 expression observed in hGFs. The MMP-1 promoter contains a STAT-binding site (Barchowsky et al., 2000; Itoh et al., 2006), and therefore STAT3 may directly regulate MMP-1 expression in hGFs. In IL-1+OSM-stimulated chondrocytes, STAT3 acts in an indirect manner to regulate MMP-1 expression by increasing c-Fos expression (Catterall et al., 2001); the c-Fos promoter also contains a STAT-binding site. As such, STAT3 may indirectly regulate MMP-1 expression in hGFs. Alternatively, given the concomitant activation of the MAPK and JAK/STAT pathway in leptin+IL-1/pam2CSK4-stimulated hGFs, it is possible that cross-talk between the JAK/STAT and MAPK signalling pathways, as characterised previously (Wisler et al., 2011), could also regulate the synergy between leptin+IL-1/pam2CSK4 that increases MMP-1 expression in hGFs. Another possible mechanism by which JAK/STAT signalling may affect MMP expression includes regulating chromatin remodelling to increase promoter availability (Bezbradica and Medzhitov, 2009). For example, in murine T cells IFN-γ production
requires JAK3/STAT5-mediated acetylation of the IFN-γ promoter (Shi et al., 2008). Finally, the importance of STAT activity in regulating MMP expression in gingival fibroblasts may be underestimated in this study due to functional redundancy between STATs (Murray, 2007). For example, when STAT3 was inhibited the functions of STAT3 may have been performed by STAT1, which was similarly tyrosine phosphorylated by leptin in this study.

In gingival fibroblasts in this study and in chondrocytes in a previous study, IL-1 did not increase STAT3 tyrosine phosphorylation (Hui et al., 2012). Therefore, it was somewhat unexpected that IL-1-stimulated hGFs required STAT3 to regulate MMP-1 expression. However, IL-1 can stimulate STAT3 tyrosine phosphorylation in murine osteoblasts (Mori et al., 2011). Perhaps IL-1 similarly stimulates STAT3 tyrosine phosphorylation in hGFs, but at a time point other than the 20 min assessed in this study. Further studies with a more comprehensive range of time points may help to identify how STAT3 (and other STATs) regulate MMP-1 expression in gingival fibroblasts, particularly in the presence of IL-1 and TLR agonists which have been reported to inhibit JAK/STAT signalling previously (Ahmed and Ivashkiv, 2000). STAT3 serine phosphorylation is increased in leptin-stimulated chondrocytes at 60 min (Hui et al., 2012); therefore, a comprehensive time course study may also help elucidate the profile of STAT3 serine phosphorylation in leptin-stimulated gingival fibroblasts which was inconclusive in the present study.

The results showing that Akt had no ability to regulate MMP-1 expression in leptin-stimulated gingival fibroblasts are in agreement with another study in human chondrocytes using the same inhibitor (Hui et al., 2012). A lower concentration of the Akt inhibitor had to be used in this study compared to Hui et al. (2012) to prevent toxicity. Therefore, the Akt inhibitor may only have been inhibiting Akt1 and Akt2 (not Akt3) isoforms according to in vitro kinase assays as performed by the manufacturer. To investigate whether Akt3 regulates MMP-1 expression in gingival fibroblasts siRNA-mediated knockdown of Akt3 would be required. Nonetheless, these results suggest that Akt1 and Akt2 are not involved in regulating MMP-1 expression in gingival fibroblasts. Intriguingly, these results contradict those in a previous study of chondrocytes, where Akt1 was required to regulate IL-1+OSM-stimulated MMP-1 expression (Litherland et al., 2008). This discrepancy could either be due to the different stimuli tested or could suggest that Akt signalling after pro-inflammatory stimulation is cell-type specific. In support of the latter idea, the 10 µM concentration of
Akt VIII inhibitor used by Litherland et al. (2008) on chondrocytes would have killed the gingival fibroblasts in the present study (Figure A.1B). The ability of hGFs to regulate collagen phagocytosis is Akt-dependent (Takahashi et al., 2008). Therefore, while Akt may not regulate MMP-1 expression, Akt may still be able to regulate collagen degradation by gingival fibroblasts in an alternative way.

It would also be interesting to determine what functional significance, if any, leptin-stimulated Akt phosphorylation has on gingival fibroblast function, beyond regulating hGF proliferation as observed in this study (Figure A.1B). Signalling via the PI3K/Akt pathway is implicated in a number of inflammatory diseases including inflammatory bowel disease (IBD) and RA (Wisler et al., 2011). For example, Akt is one of a number of signalling pathway intermediates that regulates the expression of the chemokine CXCL8 in leptin-stimulated synovial fibroblasts (Tong et al., 2008). No previous studies have investigated the outcomes of leptin-stimulated Akt activity in gingival fibroblasts. Akt signalling regulates the expression of IL-23 (Zhu et al., 2011), TLR2 (Gutiérrez-Venegas and Arreguín-Cano, 2011) and CXCL10 (Hosokawa et al., 2010a) in gingival/periodontal fibroblasts, and therefore, future studies investigating leptin-stimulated Akt activity in hGFs are warranted.

One limitation with the approach used in this study is that chemical pathway inhibitors may inhibit multiple intracellular targets. Indeed, in this study the JNK inhibitor SP600125 (which inhibits JNK1-3) also reduced ERK phosphorylation (Figure B.1B). Therefore, future studies using siRNA approaches, which can target the RNAs of individual protein or isoforms for degradation, would help to confirm the requirement of specific signalling molecules in regulating MMP-1 expression in gingival fibroblasts. The MMP-1 and MMP-3 promoters are similar as both promoters have AP-1, Ets and NF-κB binding sites; however, the MMP-3 promoter does not have a STAT binding site (Fanjul-Fernandez et al., 2010). Therefore, it would be interesting in future studies to determine whether the synergistic regulation of MMP-3 production observed in leptin+IL-1 or leptin+pam2CSK4-stimulated hGFs (Chapter 4) is dependent on the same or different intracellular signalling pathways as observed for MMP-1.

In summary, the current study presents the first evidence that leptin activates the MAPK, JAK/STAT and PI3K signalling pathways in gingival fibroblasts. Additionally, the results presented in this chapter add mechanistic information regarding the synergy between leptin and IL-1/pam2CSK4 that enhances MMP-1 production in gingival
fibroblasts. Overall, these results demonstrate that leptin and pro-inflammatory mediators stimulate specific and coordinated gingival fibroblast signalling events that determine the production of the collagenase MMP-1.
Figure 5.1: Long isoform leptin receptor gene expression in hGFs.

hGFs were either unstimulated (donors 1, 3, and 4) or were stimulated with leptin (0.1 or 10 μg/ml) (donor 5) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. The amount of RNA used for reverse transcription differed between donors. Semi-quantitative RT-PCR was used to assess leptin receptor (long isoform) and β2m gene expression. The expected product size for the leptin receptor (long isoform) was 428 bp, and for β2m was 435 bp. Long isoform leptin receptor expression was also confirmed in three other hGF donors. A dose-dependent increase in leptin receptor expression was also confirmed in another donor. LEPR – leptin receptor, β2m – β2-microglobulin.
Figure 5.2: Leptin receptor surface expression on hGFs.

Unstimulated hGFs were prepared and analysed for cell surface leptin receptor expression by flow cytometry. Cells were stained with mouse anti-human leptin receptor PE-conjugated monoclonal antibody or isotype control antibody. 10000 gated events were acquired using a FACSCalibur flow cytometer. This histogram displays the events for leptin receptor staining (no fill) compared to an isotype control (grey fill) and are representative of results from 4 different hGF donors.
Figure 5.3: Phosphorylation status of MAPK signalling pathway intermediates in hGFs after stimulation with leptin, IL-1, pam2CSK4 and OSM.

hGFs were stimulated with leptin (10 μg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) or OSM (5 ng/ml) for 20 min. Cells were lysed and prepared for SDS-PAGE. Extracts were immunoblotted with antibodies against phospho(p)-p38 (T180/Y182), pJNK (T183/Y185), pERK (p42/44) (T202/Y204), ERK (p42/44) and GAPDH as a loading control (the amino acids highlighted in these brackets represent the site/s of phosphorylation in the phospho-epitopes detected by these antibodies). The results from the three donors tested are displayed.
Figure 5.4: Phosphorylation status of STAT1 and STAT3 in hGFs after stimulation with leptin, IL-1, pam2CSK4 and OSM.

hGFs were stimulated with leptin (10 μg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) or OSM (5 ng/ml) for 20 min. Cells were lysed and prepared for SDS-PAGE. Extracts were immunoblotted with antibodies against phospho(p)-STAT3 (Y705), pSTAT3 (S727), pSTAT1 (Y701), pSTAT1 (S727), STAT3 and GAPDH as a loading control (the amino acids highlighted in these brackets represent the site/s of phosphorylation in the phospho-epitopes detected by these antibodies). The results from the three donors tested are displayed.
Figure 5.5: Phosphorylation status of Akt in hGFs after stimulation with leptin, IL-1, pam2CSK4 and OSM.

hGFs were stimulated with leptin (10 μg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) or OSM (5 ng/ml) for 20 min. Cells were lysed and prepared for SDS-PAGE. Extracts were immunoblotted with antibodies against phospho(p)-Akt (S473), pAkt (T308) and GAPDH as a loading control (the amino acids highlighted in these brackets represent the site/s of phosphorylation in the phospho-epitopes detected by these antibodies). The results from the three donors tested are displayed. Phospho-Akt T308 was also assessed in donor 6 but no bands could be resolved for any treatment condition.
Figure 5.6: Phosphorylation status of NF-κB p65 in hGFs after stimulation with leptin, IL-1, pam2CSK4 and OSM.

hGFs from one donor were stimulated with leptin (10 μg/ml), IL-1 (0.05 ng/ml), leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), pam2CSK4 (50 ng/ml), leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) or OSM (5 ng/ml) for 20 min. Cells were lysed and prepared for SDS-PAGE. Extracts were immunoblotted with antibodies against phospho(p)-p65 (S536) and GAPDH as a loading control (the amino acid highlighted in this bracket represents the site of phosphorylation in the phospho-epitopes detected by these antibodies).
Table 5.1: The inhibitors of intracellular signalling pathway intermediates used in this study.

The inhibitors used were chosen as the intracellular targets that they inhibit are activated by leptin, IL-1 or pam2CSK4. The concentrations of inhibitors used did not cause toxicity in hGFs, and where tested inhibited their respective intracellular target (Section 2.2.6.1).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>Intracellular target</th>
</tr>
</thead>
<tbody>
<tr>
<td>U0126</td>
<td>7.5</td>
<td>MEK/ERK</td>
</tr>
<tr>
<td>SP600125</td>
<td>10</td>
<td>JNK</td>
</tr>
<tr>
<td>SB203580</td>
<td>10</td>
<td>p38</td>
</tr>
<tr>
<td>STAT3 inhibitor VI</td>
<td>100</td>
<td>STAT3</td>
</tr>
<tr>
<td>Akt inhibitor VIII</td>
<td>0.3</td>
<td>Akt</td>
</tr>
<tr>
<td>Bay 11-7085</td>
<td>10</td>
<td>NF-κB</td>
</tr>
</tbody>
</table>
Figure 5.7: The role of MEK/ERK in regulating MMP-1 gene expression by stimulated hGFs.

hGFs were either pre-treated with 0.1 % v/v DMSO or the MEK inhibitor U0126 (7.5 µM) for 30 min and then stimulated with leptin (10 µg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. Data (fold unstimulated DMSO control) are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01, ***=p<0.001 as indicated.
Figure 5.8: The role of JNK in regulating MMP-1 gene expression by stimulated hGFs.

hGFs were either pre-treated with 0.1 % v/v DMSO or the JNK inhibitor SP600126 (10 μM) for 30 min and then stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the \(2^{\Delta\Delta Ct}\) method by using RNAP as the reference gene. Data (fold unstimulated DMSO control) are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01 as indicated.
hGFs were either pre-treated with 0.1 % v/v DMSO or the p38 inhibitor SB203580 (10 µM) for 30 min and then stimulated with leptin (10 µg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. Data (fold unstimulated DMSO control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) **=p<0.01 as indicated.

**Figure 5.9: The role of p38 in regulating MMP-1 gene expression by stimulated hGFs.**
Figure 5.10: The role of STAT3 in regulating MMP-1 gene expression by stimulated hGFs.

hGFs were either pre-treated with 0.1 % v/v DMSO or STAT3 inhibitor VI (100 µM) for 30 min and then stimulated with leptin (10 µg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the $2^{-\Delta\Delta C_t}$ method by using RNAP as the reference gene. Data (fold unstimulated DMSO control) are shown as mean±SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected) *=p<0.05 as indicated.
hGFs were either pre-treated with 0.1 % v/v DMSO or AKT inhibitor VIII (0.3 µM) for 30 min and then stimulated with leptin (10 µg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 µg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 µg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the 2^{-ΔΔCt} method by using RNAP as the reference gene. Data (fold unstimulated DMSO control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected).
6 Genome-wide expression profiling of gingival fibroblasts after stimulation with leptin and IL-1

6.1 Introduction

Gingival fibroblasts are implicated in maintaining the structural integrity of the gingiva by regulating the production of ECM-remodelling enzymes, as evidenced by the results of this study and by others previously (Chapters 3 and 4 and Section 1.11.1). However, gingival fibroblasts are also immunocompetent cells that are implicated in regulating inflammation; for example, gingival fibroblasts are a source of chemokines, cytokines and growth factors (Section 1.11).

Leptin regulates a broad range of inflammatory responses in a number of cell types (Section 1.4). In this study it was demonstrated that gingival fibroblasts stimulated with leptin (and IL-1) activated multiple intracellular signalling pathways (Chapter 5). These signalling pathways (MAPK, JAK/STAT, PI3K/Akt, NF-κB) are implicated in regulating the production of a broad range of molecules in gingival fibroblasts, including molecules implicated in inflammation (Bodet et al., 2007; Bage et al., 2010; Hosokawa et al., 2010b; Hosokawa et al., 2010a; Gutiérrez-Venegas and Arreguín-Cano, 2011). For example, NF-κB signalling is required to enhance LPS-stimulated IL-6 gene expression in gingival fibroblasts (Jin et al., 2012). Therefore, it can be hypothesised that the effects of leptin on gingival fibroblast phenotype are widespread, and may affect inflammatory responses in these cells.

Aside from the current study, only one other study has investigated how leptin regulates gingival fibroblast responses (Park et al., 2013). Together, these studies have only investigated the regulation of a limited profile of MMPs, TIMPs, and inflammatory mediators (IL-6 and CXCL8) in leptin-stimulated gingival fibroblasts. Therefore, a holistic understanding of the leptin(±IL-1)-stimulated gingival fibroblast phenotype is lacking.

Proteomic and genomic array-based technologies have been used to study gingival fibroblast responses previously (Han and Amar, 2002; Bodet et al., 2007; Vardar-Sengul et al., 2009; Bage et al., 2010; Ebisawa et al., 2011). Gene expression arrays have been used to compare gingival fibroblast phenotypes to other cells such as PDL fibroblasts, gingival epithelial cells, and dermal fibroblasts (Han and Amar, 2002; Abiko et al., 2004; Ebisawa et al., 2011). Using a DNA microarray with over 9000
unique probes, Han and Amar (2002) identified over 100 genes that are differentially expressed between PDL and gingival fibroblasts, thereby highlighting the intrinsic differences between these cells. Similarly, Ebisawa et al. (2011) found that only 5% of the approximately 5000 genes represented on a DNA microarray were differentially expressed between dermal and gingival fibroblasts; the authors suggest that understanding the differences in gene expression profiles between distinct fibroblast populations may assist in the development of cell-based therapies. Additionally, gene expression arrays have been used to compare the phenotype of cytokine (TNF-α, IL-1β) stimulated gingival fibroblasts to unstimulated controls (Vardar-Sengul et al., 2009; Bage et al., 2010; Davanian et al., 2012). For example, Bage et al. (2010) used a DNA microarray containing >37000 probes to determine that genes whose products are implicated in the JNK and NF-κB signalling pathways are significantly overrepresented in TNF-α stimulated gingival fibroblasts compared to unstimulated controls. Subsequently, Bage et al. (2010) used chemical inhibitors of JNK and NF-κB to show that these pathways regulate the increased production of PGE$_2$ by TNF-α stimulated gingival fibroblasts, thereby identifying intracellular signalling pathways that could be targeted to modulate the inflammatory responses of gingival fibroblasts.

In this study, Illumina beadchip microarray technology was chosen to profile the gingival fibroblast transcriptome after stimulation with leptin and IL-1 (Section 2.8.2 and Appendix E). Illumina microarrays contain oligonucleotides that are bound to microbeads, and these microbeads are randomly dispensed onto the microarray. This is in contrast to the other main array platforms (e.g. Affymetrix microarrays) which directly spot oligonucleotides onto the microarray. Another difference between the two microarray platforms is in the number and sequences of oligonucleotides used. For example, Affymetrix microarrays contain several different oligonucleotide probes for each gene (and oligonucleotides with one base mis-matched to control for non-specific hybridisation); in contrast, Illumina beadchip microarrays dispense multiple beads labelled with the same oligonucleotide. The advantage of this regarding the Illumina platform is that it allows for an assessment of technical variation for each oligonucleotide. Nonetheless, both platforms are widely used. (Barnes et al., 2005)

The genome-wide expression microarray approach chosen in this study has several advantages to expression arrays with a more limited number of probes (e.g. 96-gene TLDA). Not only does the genome-wide expression array contain probes for genes which are hypothesised to be of interest (e.g. inflammatory mediators for this study),
but it also allows for the identification of novel genes (with known or unknown function) which may be regulated by leptin in gingival fibroblasts. Additionally, the genome-wide expression microarray contains probes for all of the MMPs and TIMPs that have been assessed in this study previously. Therefore, this approach may help to confirm the changes in MMP expression in leptin-stimulated gingival fibroblasts that have already been observed. Finally, the data acquired from the genome-wide expression analysis can be analysed using bioinformatics tools to identify biological processes or pathways that are differentially regulated in leptin-stimulated gingival fibroblasts (Section 2.8.2). These bioinformatics tools are used to highlight potential functions that are occurring under the conditions in which the samples were obtained (Huang da et al., 2009). These processes and pathways may help to identify the biological functions that leptin(+IL-1)-stimulated gingival fibroblasts are performing.

The aims of the experiments presented in this chapter were to discover the genome-wide expression profile of gingival fibroblasts after stimulation with leptin (±IL-1), and thereby to identify whether leptin (±IL-1) affects the inflammatory phenotype of gingival fibroblasts. A sub-aim of the experiments presented in this chapter was to identify molecules relevant to ECM remodelling expressed by gingival fibroblasts stimulated by leptin (±IL-1) that may add to those previously studied (Chapters 3 and 4).

6.2 Expression of immune function genes in leptin-stimulated hGFs – pilot study

6.2.1 Gene expression analysis by TLDA
hGFs were stimulated with leptin (0.1, 1 or 10 μg/ml) for 24 h, or left unstimulated. Cells were lysed, RNA extracted and cDNA was prepared. cDNA was loaded onto a human immune panel TLDA (Section 2.8.1), and gene expression was determined by real-time RT-PCR, to identify what effect leptin had on the expression of genes implicated in immune function in hGFs. The relatively small number of genes on this array allowed for simple and quick data analysis, and was therefore beneficial for this pilot investigation.

6.2.1.1 Chemokines and CSFs
The chemokines CXCL10, CXCL8, CCL5, CXCL11 and CCL2, the chemokine receptor CCR4, and CSF1 were expressed in leptin-stimulated hGFs (Table 6.1), which suggests that leptin-stimulated hGFs have the potential to regulate leukocyte chemotaxis and
proliferation. The chemokine CXCL10 was most highly differentially expressed (+8.2-fold) after 1 μg/ml leptin stimulation compared to unstimulated cells.

6.2.1.2 **Cell surface markers**
The cell surface markers CD8A, CD40, CD68, CD38 and CD4 were expressed by leptin-stimulated hGFs (Table 6.1), which indicates that leptin-stimulated hGFs may express a number of proteins on the cell surface which function in immune responses. Expression of the cell surface marker CD80 was induced in hGFs stimulated with 1 μg/ml leptin compared to unstimulated cells. HLA-DRA1 (major histocompatibility complex, class II, DRα) and ICAM1 were expressed in leptin-stimulated hGFs (Figure D.1A). HLA-DRA1 and ICAM1 expression levels were higher in hGFs stimulated with 10 μg/ml leptin compared to those stimulated with 0.1 μg/ml leptin.

6.2.1.3 **Intracellular signalling molecules**
STAT3, NFKB2 (NF-κB2) and IKBKB (inhibitor of κB kinase-β) were expressed in leptin-stimulated hGFs (Table 6.1). Interestingly, STAT3 expression was increased 2.4-fold in hGFs stimulated with 1 μg/ml leptin compared to unstimulated cells, which suggests that leptin enhances the ability of hGFs to signal via STAT3.

6.2.1.4 **Immune and inflammatory mediators**
The interleukins IL6, IL15, IL12A, IL7 and IL18 were expressed by leptin-stimulated hGFs (Table 6.1), which suggests that leptin-stimulated hGFs may be a source of cytokines in the gingiva. IL6 was most highly differentially expressed interleukin (+8.0-fold) after stimulation with 1 μg/ml leptin compared to unstimulated cells. C3 (complement component 3) and TGFβ1 (TGF-β1) were expressed in leptin-stimulated hGFs (Figure D.1B). The expression levels of both C3 and TGFβ1 were higher in hGFs stimulated with 10 μg/ml leptin compared to those stimulated with 0.1 μg/ml leptin.

6.2.1.5 **Other genes**
VEGF, ACE (angiotensin 1 converting enzyme), ECE1 (endothelin converting enzyme 1) and EDN1 (endothelin 1), whose gene products are implicated in vascular functions, were expressed in leptin-stimulated hGFs (Figure D.1C). COL4A5 (collagen, type IV, α5) and FNI (fibronectin 1), whose gene products comprise ECM components, were expressed by hGFs after stimulation with leptin (Figure D.1D).
6.2.2 Leptin-stimulated hGFs increase IL-6 secretion

Protein levels of IL-6 were investigated in hGF supernatants to identify whether the leptin-stimulated increase in IL-6 mRNA levels translated to IL-6 secretion. In addition, hGFs were stimulated with IL-1 to determine whether leptin and IL-1 synergistically regulate IL-6 production similar to that observed regarding MMP-1 and MMP-3 production (Chapter 4). hGFs were stimulated with leptin (0.1, 1 or 10 μg/ml) with or without IL-1α (0.05 ng/ml) for 24 h. IL-6 concentrations in the supernatants were measured by ELISA. Unstimulated hGFs served as a negative control. No statistical analysis was performed because only 2 experimental replicates were analysed.

Leptin appeared to dose-dependently increase IL-6 secretion by hGFs (Figure 6.1). IL-1-stimulated hGFs also appeared to produce higher levels of secreted IL-6 compared to the unstimulated control (IL-1: 34±0.4 pg/ml, unstimulated: 12±0.8 pg/ml). Interestingly, at least 8-fold higher levels of IL-6 protein were produced by hGFs stimulated with leptin+IL-1 compared to either IL-1 or leptin alone, indicating that MMPs are not the only molecules synergistically upregulated by leptin and IL-1 in hGFs.

6.3 Genome-wide expression analysis in hGFs stimulated with leptin and IL-1

Several preliminary studies were required to be performed prior to genome-wide expression analysis in hGFs. These studies were performed to:

1. Assess whether IL-1α and IL-1β similarly stimulated hGFs, as IL-1β was available to be used to stimulate hGFs in genome-wide expression experiments.
2. Check that the RNA samples prepared for the microarray were from successful stimulations.
3. Ensure sample RNA yield and quality were suitable for microarray analysis.

6.3.1 IL-1α and IL-1β similarly regulate MMP-1 expression in hGFs

hGFs were stimulated with IL-1α or IL-1β (both 0.05 ng/ml) for 24 h. Unstimulated hGFs served as a control. Cells were lysed and cDNA was prepared by reverse transcription. MMP-1 gene expression was assessed by real-time RT-PCR, and MMP-1 concentrations in the hGF supernatants were assessed by ELISA, to determine whether IL-1α and IL-1β stimulate hGFs in a comparable manner. Additionally, proliferation was assessed to determine whether IL-1α and IL-1β differentially regulate hGF proliferation as per Section 2.5.1 (Figure J.1).
Both IL-1α and IL-1β significantly increased MMP-1 gene expression and protein secretion compared to unstimulated hGFs (all p<0.001) (Figure 6.2 and Figure 6.3). No significant difference in MMP-1 gene expression was detected between IL-1α and IL-1β stimulated hGFs. However, IL-1β-stimulated hGFs secreted 79% more MMP-1 compared to IL-1α-stimulated hGFs (p<0.001). IL-1α and IL-1β had no significantly different ability to regulate hGF proliferation (Figure J.1). These results confirm that IL-1α and IL-1β regulate MMP-1 gene expression and proliferation in hGFs to a similar extent. The finding that IL-1β promotes higher MMP-1 secretion than IL-1α suggests that IL-1α and IL-1β have a differential ability to regulate post-transcriptional processing of MMP-1 in hGFs.

6.3.2 Generation of successful stimulations and high quality RNA

hGFs were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng.ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h (6-well and 96-well formats). Unstimulated hGFs served as a control. 96-well format stimulations were performed in parallel to allow for statistical analysis; the number of cells required for 6-well format stimulations prohibited the seeding of >2 wells per treatment condition. Cells were lysed and cDNA was prepared by reverse transcription. MMP-1 gene expression was assessed by real-time RT-PCR. As well as RNA being extracted from the samples, the supernatants were collected to allow for the study of secreted products by hGFs.

Stimulations were designated successful if leptin and IL-1β treated hGFs significantly (p<0.05) increased MMP-1 gene expression compared to the unstimulated control, and if leptin+IL-1β treated hGFs had significantly (p<0.05) increased MMP-1 gene expression compared to hGFs stimulated with leptin and IL-1β alone (e.g. synergy evident) as observed previously (Section 4.2.1). Successful stimulations were generated from hGFs isolated from donors 2, 3 and 4 (Figure 6.4). The same patterns of MMP-1 expression were detected for samples generated from the parallel 6-well stimulations (Figure J.2).

RNA isolated from hGFs (6-well format) was assessed for yield and quality by spectrophotometer (Table J.1). The minimum concentration for microarray analysis was 50 ng/μl. The RNA samples generated for microarray analysis had concentrations ≥ 125 ng/μl. Similarly, the RNA absorbance ratios A260/280 and A260/230 should be above 1.8. This was fulfilled for all but two of the RNA samples generated (donor 3, leptin and
IL-1β stimulations) (Table J.1). These samples were sent for genome-wide expression analysis at CGS where all RNA samples passed additional QC (Section 2.8.2).

6.3.3 Overview of genome-wide expression results
The dataset acquired was normalised, subject to QC and corrected for unwanted variation (Section 2.8.2). Differentially expressed genes were then identified according to the methods (Section 2.8.2).

The profile (displayed as a heat map) of the relative expression levels of differentially expressed genes in hGFs stimulated with leptin, IL-1 or leptin+IL-1 were compared to the unstimulated control by hierarchical clustering (Figure E.3). Hierarchical clustering is a data analysis approach that groups samples depending on how similar each sample dataset is to the datasets of the other samples. These heat maps show that for each comparison the samples group by treatment condition. Also, in hGFs stimulated with leptin, IL-1 and leptin+IL-1 both increased and decreased levels of expression of the differentially expressed genes were observed compared to unstimulated hGFs. Additionally, these heat maps showed that the relative expression levels between different genes for each RNA sample were highly variable.

Leptin, IL-1 and leptin+IL-1-stimulated hGFs had 1599 genes in common that were expressed at a significantly different level (p<0.01) to unstimulated hGFs (Figure 6.5). Leptin+IL-1-stimulated hGFs had more unique genes expressed at a significantly different level (p<0.01) than leptin or IL-1 stimulated hGFs (leptin+IL-1 2105 genes, leptin 392 genes, IL-1 548 genes). IL-1 and Leptin+IL-1 stimulated hGFs shared more genes expressed at a significantly different level (p<0.01) than leptin+IL-1 and leptin stimulated hGFs (583). Leptin and IL-1 stimulated hGFs shared 123 unique genes expressed at a significantly different level (p<0.01) compared to leptin+IL-1 stimulated hGFs or the unstimulated control.

6.3.4 Differentially expressed genes in hGFs stimulated with leptin
Leptin-stimulated hGFs had 111 differentially expressed genes compared to unstimulated hGFs (Table 6.2). CCL2, IL18BP (IL-18 binding protein) and IL6 were among the genes most highly upregulated in leptin-stimulated hGFs; while ADAM19 (ADAM metallopeptidase domain 19) was one of only a few genes that were downregulated by leptin treatment. Interestingly, ADAM19 is a membrane-anchored protein that has extracellular protease activity (Qi et al., 2009), and therefore, this
finding adds evidence to the earlier results (Chapter 3) that suggest leptin plays a role in regulating hGF-mediated ECM remodelling.

**MMP1** was not differentially expressed in leptin-stimulated hGFs compared to unstimulated hGFs. Further profiling of the dataset revealed that MMP-1 expression was increased 2.7-fold in leptin-stimulated hGFs compared to unstimulated hGFs. However, the adjusted p value was 0.017 and therefore, **MMP1** did not make the p value cut-off of 0.01.

GO analysis identified 44 significant processes (p<0.001) differentially regulated in leptin-stimulated hGFs compared to unstimulated hGFs (Table 6.3). The processes highlighted were relevant to immune function such as cytokine responses, and cell activation. KEGG pathway analysis identified 13 pathways significantly (p<0.01) overrepresented in leptin-stimulated hGFs compared to unstimulated hGFs (Table 6.4). The pathways identified were relevant to immune function such as cytokine and TLR signalling, and metabolism. These enrichment analyses suggest that leptin enhances the immune function of hGFs.

### 6.3.5 Differentially expressed genes in hGFs stimulated with IL-1

IL-1-stimulated hGFs had 263 differentially expressed genes compared to unstimulated hGFs (Table 6.5). **IL8**, **IL6** and **CXCL6** were among the genes most highly upregulated in IL-1-stimulated hGFs. Similar to leptin-stimulated hGFs, **ADAM19** was one of relatively few genes that were downregulated in IL-1-stimulated hGFs compared to unstimulated hGFs. In contrast to leptin-stimulated hGFs, **MMP1** was differentially expressed in IL-1-stimulated hGFs (+4.3-fold, p=0.00086 compared to unstimulated hGFs); this is in agreement with the earlier results in this study (Section 4.2), and supports the suggestion that IL-1 stimulates hGF-mediated collagen remodelling. Other genes relevant to ECM remodelling, namely **MMP3** and **COL8A1** (collagen, type VIII, α1) were differentially expressed in IL-1-stimulated hGFs (Table 6.5).

GO analysis identified 153 significant processes (p<0.001) differentially regulated in IL-1-stimulated hGFs compared to unstimulated hGFs (Table 6.6). The processes highlighted were relevant to immune responses, inflammation, and responses to wounding. KEGG pathway analysis identified 10 pathways significantly (p<0.01) overrepresented in IL-1-stimulated hGFs compared to unstimulated hGFs (Table 6.7). The pathways identified were relevant to metabolism and immune function (e.g. innate
pathogen recognition and cytokine/chemokine signalling). These enrichment analyses suggest that IL-1 enhances the immune and inflammatory functions of hGFs.

6.3.6 **Differentially expressed genes in hGFs stimulated with leptin+IL-1**

Leptin+IL-1-stimulated hGFs had 637 differentially expressed genes compared to unstimulated hGFs (Table 6.8). Similar to IL-1-treated hGFs, *IL8, IL6* and *CXCL6* were among the genes most highly upregulated in leptin+IL-1-stimulated hGFs. *COL6A3* (collagen, type VI, α3) and *TIMP3* whose protein products function in ECM homeostasis and the gene encoding the cytokine TGF-β3 (*TGFβ3*) were among the genes downregulated in leptin+IL-1-stimulated hGFs compared to unstimulated hGFs. The genes encoding several proteases (*MMP3, MMP1, CTSL* – cathepsin L, *PLAT* – tissue plasminogen activator, *MMP8, MMP2, MMP14, MMP12*) were upregulated in leptin+IL-1 stimulated hGFs compared to unstimulated hGFs. In contrast, compared to unstimulated hGFs the genes encoding several collagen proteins (*COL8A2, COL5A1, COL15A1, COL14A1*) were downregulated in leptin+IL-1 stimulated hGFs, which suggests that the production of some collagen proteins is reduced in hGFs exposed to leptin and IL-1.

GO analysis identified 255 significant processes (p<0.001) differentially regulated in leptin+IL-1 stimulated hGFs compared to unstimulated hGFs (Table 6.9). The processes highlighted were relevant to wound responses and inflammation, immune system function, and anatomical development. KEGG pathway analysis identified 17 pathways significantly (p<0.01) overrepresented in leptin+IL-1-stimulated hGFs compared to unstimulated hGFs (Table 6.10). The pathways identified were related to immune responses and immune pathology, metabolism, interactions with the ECM, and cancer. The genes overrepresented in the RA pathway are displayed as an example (Figure 6.6). Interestingly, the products of the genes overrepresented in the RA pathway are implicated in immune cell infiltration (CCL2, CCL20, CXCL1, CXCL5, and CXCL8), inflammation (IL-6) and joint destruction (MMP-1, MMP-3). These enrichment analyses suggest that leptin+IL-1 enhance immune and inflammatory functions in hGFs, but also affect ECM homeostasis.

6.3.7 **Genes synergistically regulated by leptin+IL-1 in hGFs**

Several similarities emerged from the analysis of the differentially expressed genes in leptin, IL-1 and leptin+IL1 stimulated hGFs. For example, in each list *IL6* was one of the most highly upregulated genes (Table 6.2, Table 6.5 and Table 6.8). Similarly,
immune system processes and pathways were significantly overrepresented in GO analysis and KEGG pathway analysis for leptin, IL-1 and leptin+IL-1 stimulated hGFs compared to unstimulated hGFs.

Two additional differentially expressed gene lists were created: leptin v leptin+IL-1, and IL-1 v leptin+IL-1 (Section 2.8.2). Using these lists a new list of genes potentially synergistically regulated by leptin and IL-1 in hGFs was created (Table 6.11). Genes were selected for this list if they fulfilled the following properties:

1. The gene was differentially expressed in leptin+IL-1 stimulated hGFs compared to the unstimulated control.
2. The fold change in gene expression was greater after leptin+IL-1 stimulation than either leptin or IL-1 stimulation alone, or the gene was not present in the leptin or IL-1 gene lists.
3. The gene encodes a protein which has a function implicated in immunity or ECM remodelling as determined by literature search.

The genes in Table 6.11 that were differentially expressed in both of the additional genes lists were highlighted as significantly synergistically regulated by leptin+IL-1 in hGFs in this genome-wide expression analysis. For example, using this analysis MMP3, IL24 and CXCL5 were synergistically regulated by leptin+IL-1 in hGFs. MMP1 was also synergistically regulated by leptin+IL-1 in hGFs in this microarray analysis, which is in agreement with previous real-time RT-PCR analyses of MMP-1 gene expression in this study (Chapter 4). Interestingly, several other MMP genes (MMP8, MMP2, MMP14, and MMP12) were identified in Table 6.11; however only MMP8 was confirmed as being synergistically regulated by leptin+IL-1.

6.3.8 Further analysis of selected genes by real-time RT-PCR

Several genes identified in the genome-wide expression analysis were selected for further study by real-time RT-PCR, namely MMP3, IL24, MMP8, MMP2, MMP14, MMP12, TIMP3, COL6A3. These genes were selected firstly to validate the microarray, and secondly because either they were significantly synergistically regulated by leptin and IL-1 in hGFs (MMP3, IL24, MMP8); or because these genes were potentially synergistically regulated by leptin and IL-1 in hGFs (e.g. present in Table 6.11), and their protein product is implicated in ECM homeostasis (MMP2, MMP14, MMP12, TIMP3, COL6A3). MMP-1 gene expression was not further studied as this was
performed prior to the microarray (Section 6.3.2). The same RNA generated in Section 6.3.2 was freshly converted to cDNA by reverse transcription.

Leptin and IL-1 synergised to increase MMP-3, IL-24, MMP8 and MMP-12 gene expression in hGFs (Figure 6.7A-D). Leptin+IL-1 stimulated hGFs increased MMP-3 gene expression 316-fold and 5.2-fold compared to leptin and IL-1 stimulated hGFs respectively (both p<0.001). Similarly, leptin+IL-1 stimulated hGFs increased IL-24 gene expression 292-fold and 375-fold compared to leptin and IL-1 stimulated hGFs respectively (both p<0.01). Leptin+IL-1 stimulated hGFs increased MMP-8 gene expression 32-fold and 15-fold compared to leptin and IL-1 stimulated hGFs respectively (both p<0.001). Also, Leptin+IL-1 stimulated hGFs increased MMP-12 gene expression 58-fold and 5.9-fold compared to leptin and IL-1 stimulated hGFs respectively (leptin p<0.001, IL-1 p<0.01).

No synergy between leptin and IL-1 was observed regarding MMP-14 and MMP-2 expression (Figure 6.7E-F). Only leptin (±IL-1) stimulated MMP-14 gene expression in hGFs compared to the unstimulated control (p<0.05), which is in agreement with previous results in this study (Section 4.2.3). In contrast, only IL-1 (±leptin) stimulated hGFs significantly increased MMP-2 gene expression compared to the unstimulated control (p<0.01).

Interestingly, leptin and IL-1 synergised to decrease COL6A3 expression in hGFs (Figure 6.7G). Leptin+IL-1 stimulated hGFs decreased COL6A3 expression 55 % and 35 % compared to leptin and IL-1 stimulated hGFs respectively (leptin p<0.001, IL-1 p<0.01). In contrast, no significant differences in TIMP-3 gene expression were observed after any combination of leptin and IL-1 stimulation in hGFs compared to the unstimulated control (Figure 6.7H).

6.3.9 IL-24 secretion by hGFs stimulated with leptin and IL-1

A literature search of IL-24 revealed that no previous studies had identified differential IL-24 gene expression in gingival fibroblasts, or after leptin stimulation in any cells. To further investigate this novel finding, IL-24 secretion by hGFs was assessed by measuring the concentration of IL-24 protein by ELISA in the supernatants from the samples used for genome-wide expression analysis (Sections 6.3).

Leptin and IL-1 synergistically regulated IL-24 secretion by hGFs (Figure 6.8). No IL-24 was detected in the supernatants of unstimulated hGFs or cells stimulated with IL-1
but, IL-24 was detected in the supernatants of leptin-stimulated hGFs. Interestingly, the concentration of IL-24 in leptin+IL-1-stimulated hGF supernatants was 68-fold higher than that detected in the supernatants of leptin-stimulated hGFs (p<0.01).

6.4 Discussion
Leptin regulates immune and inflammatory responses by altering cellular immunocompetence (Matarese et al., 2005). Gingival fibroblast phenotypes are altered by inflammatory mediators, but the particular phenotype generated depends on the stimuli gingival fibroblasts are exposed to (Mahanonda et al., 2007; Uehara and Takada, 2007; Belibasakis and Guggenheim, 2011). I have shown that gingival fibroblasts express cell surface leptin receptor, and respond to leptin stimulation by activating intracellular signalling pathways and increasing the production of several MMPs (Chapters 3-5). Only one study has investigated the ability of leptin to regulate the production of inflammatory mediators by gingival fibroblasts, but only two mediators were studied (Park et al., 2013). No genome-wide expression analysis has been reported in leptin-stimulated hGFs or indeed in leptin-stimulated fibroblasts from any anatomical site, therefore the results presented in this chapter are entirely novel. The results presented in this chapter suggest that leptin enhances the immunocompetence of hGFs, by promoting the expression of several mediators that are implicated in inflammation. In addition, leptin and IL-1 synergistically stimulated the expression of a number of mediators that are implicated in inflammation and tissue remodelling, such as chemokines, cytokines and MMPs. These results suggest that the way in which leptin regulates hGF phenotype differs in the absence and presence of IL-1, and add to the body of evidence that gingival fibroblasts are an important regulator of immune responses and inflammation in the gingiva (Section 1.11).

In this study leptin increased the expression of three genes in hGFs whose products are implicated in antigen processing and presentation (HLA-DRA, HLA-DMA, and CTSL). Increased expression levels of the MHC class II molecule HLA-DRA and cathepsin L have been observed in cytokine-stimulated gingival fibroblasts previously (Cox et al., 2006; Mahanonda et al., 2008). HLA-DRA encodes the α-subunit of one of the cell surface MHC class II molecules, and functions to present exogenous peptide antigens to T cells (Blum et al., 2013). HLA-DMA encodes the α-subunit of one of the intracellular MHC class II molecules which assists in antigen presentation by promoting the binding of peptides to the cell surface MHC class II molecules (e.g. HLA-DR) (Brocke et al., 2002). The lysosomal protease cathepsin L assists in antigen processing by producing
peptides that can be bound to MHC class II molecules by degrading proteins in the endocytic pathway (Blum et al., 2013). Together, these results suggest that leptin may increase the ability of gingival fibroblasts to act as professional antigen presenting cells, thereby assisting in the activation of lymphocytes in the gingiva. However, only the α-subunit of these MHC class II molecules was upregulated in this study. This suggests that the increased expression levels of HLA-DRA and HLA-DMA may not translate to HLA-DR and HLA-DM protein levels because the protein is a heterodimer consisting of both α- and β-subunits.

Cathepsin L is also implicated in the turnover of ECM molecules, and the activation of MMPs (Uitto et al., 2003). Two other enzymes with proteolytic activity were differentially regulated in leptin-stimulated hGFs in this study (ADAMTS1 was upregulated, ADAM19 was downregulated). ADAM19 is a transmembrane protein that is implicated in cleaving a variety of substrates including ECM components, growth factors and cytokines (Qi et al., 2009). ADAMTS1 is a secreted protein, often associated with ECM components, which cleaves a wide range of protein substrates and is upregulated by leptin in murine ovarian follicles (Barkan et al., 2005). These results add to the earlier findings in this study that suggest that leptin affects the ECM degrading potential of hGFs (Chapter 3 and 4). However, in the microarray analysis performed in this study, leptin alone had no significant ability to alter the expression of any of the MMPs previously investigated. This discrepancy is likely due to the stringent fold change and p value cut offs selected during analysis of the microarray expression dataset (as determined for MMP-1 in Section 6.3.4). Further studies are required to determine whether leptin-stimulated alterations in protease expression by gingival fibroblasts play a functional role in ECM remodelling in the gingiva.

In this study leptin increased the expression of genes whose products are cell surface receptors for molecules implicated in inflammation and immunity (CD14, IL13RA1, IL1R1, and FAIM3). The transmembrane protein CD14 binds LPS and promotes TLR4 signalling (Lu et al., 2008). I have shown that TLR4 is expressed on hGFs (Section 4.3.2.4). Together, this suggests that leptin may promote LPS-stimulated TLR4 signalling in hGFs, although perhaps only in individuals who are responsive to LPS given the donor-dependent differences in LPS-stimulated MMP production observed previously (Section 4.3.2). IL13RA1 encodes one of the subunits of the heterodimeric IL-13 receptor which can bind the Th2-type cytokines IL-13 and IL-4 (Preshaw and Taylor, 2011). This suggests that leptin-stimulated hGFs have an increased ability to
detect these Th2-type cytokines. However, the other subunit of the IL-13 receptor (IL4RA) was not similarly upregulated by leptin in hGFs, suggesting that the increased expression of IL13RA1 detected in this study may not translate to increased IL-13 receptor protein levels. The type I IL-1 receptor is encoded by IL1R1, and can bind IL-1α, IL-1β and the IL-1 receptor antagonist (Preshaw and Taylor, 2011). However, signal transduction downstream of the type I IL-1 receptor requires an accessory protein (IL1RAP) (Bowie and O'Neill, 2000), which was not similarly increased after leptin stimulation. Therefore, it is unclear whether leptin-stimulated gingival fibroblasts would have an increased ability to activate IL-1 signalling. FAIM3 has recently been found to encode an Fc receptor for IgM (Kubagawa et al., 2009). IgM is the first antibody isotype produced by plasma cells and is thought to be present at only low levels outside of the circulation. However, there is evidence that GCF IgM concentrations increase during the course of experimental gingivitis and during gingival trauma (Griffiths et al., 1997). Together with the increases in expression of genes relevant to antigen presentation discussed earlier, this suggests that leptin increases the ability of gingival fibroblasts to bind IgM, potentially preparing gingival fibroblasts for antigen sampling and presentation should an injury or inflammatory state arise in the gingiva.

Several genes (e.g. BCL3, BCL6, STAT1, ATF3, MAP3K8, SOCS3) encoding molecules involved in intracellular signalling and gene transcription were upregulated in leptin-stimulated hGFs in this study. STAT1, ATF3 and BCL6 encode transcription factors while BCL3 encodes a protein that regulates NF-κB signalling; all are implicated in regulating widespread biological processes as they regulate the expression of a range of genes (Hess et al., 2004; Murray, 2007; Oeckinghaus and Ghosh, 2009; Basso and Dalla-Favera, 2012). SOCS3 is an inhibitor of the JAK/STAT signalling pathway and is upregulated after leptin stimulation (Section 1.2.3); however, this is the first description of leptin-stimulated SOCS3 expression in gingival fibroblasts. Therefore, SOCS3 may be acting to dampen the gingival fibroblast response to leptin (and other cytokines that signal via JAK/STAT molecules). Interestingly, mice deficient in the upstream MAPK signalling mediator MAP3K8 have a reduced severity of ligature-induced periodontitis (as evidenced by alveolar bone loss) compared to wild-type animals, and MAP3K8 is required for TNF-α expression in the gingiva (Ohnishi et al., 2010). Further studies investigating the targets of these molecules in leptin-stimulated gingival fibroblasts may help to understand whether they have a functional importance in regulating gingival fibroblast responses during exposure to leptin.
The genes encoding two chemokines (CCL2 and CXCL1) were upregulated by leptin in the genome-wide expression analysis performed in this study. CCL2 and CXCL1 are secreted proteins which bind to the cell surface receptors CCR2 and CXCR2 respectively (Fernandez and Lolis, 2002). This suggests that leptin-stimulated gingival fibroblasts may promote chemokine signalling and responses in CCR2+ and CXCR2+ cells. Chemokines are characterised by their role in promoting leukocyte infiltration into peripheral tissues; for example, CCL2 promotes monocyte infiltration and macrophage migration, while CXCL1 promotes neutrophil chemotaxis (Fernandez and Lolis, 2002).

However, chemokines also regulate other processes in inflammation and immunity (e.g. angiogenesis, leukocyte survival and differentiation); for example, CCL2 is implicated in regulating Th2 polarisation and regulatory T cell function (Deshmane et al., 2009). Leukocyte infiltration is an important part of homeostatic tissue immunosurveillance, but increased chemokine levels and leukocyte infiltration are associated with numerous diseases including periodontitis (Silva et al., 2007). Overall, these results suggest that leptin-stimulated gingival fibroblasts promote CCL2 and CXCL1 responses; however, it is unclear whether these responses would be beneficial or pathogenic.

Of note, the chemokines upregulated in the TLDA expression analysis did not correspond to those upregulated in the genome-wide expression analysis in this study. This discrepancy could be due to the different expression analysis technologies used (PCR versus microarray), data generated (expression relative to reference gene versus no reference gene), or differences in experimental design (number of hGF donors tested and concentration of leptin). Therefore, validation of the microarray results for chemokine expression at the mRNA and protein level should be performed before any further studies of chemokines based on the results of this study are performed.

Leptin increased the expression of genes that encode three extracellular immune mediators in hGFs (IL18BP, IL6, and IDO). IL-18 binding protein inhibits the activity of the IL-1 family cytokine IL-18; IL-18 is implicated in promoting Th1-type immune responses (Preshaw and Taylor, 2011). This suggests that leptin-stimulated gingival fibroblasts may dampen Th1-polarised responses in the gingiva. The enzyme IDO converts the essential amino acid tryptophan to kynurenine, thereby reducing tryptophan availability, and is upregulated in gingival fibroblasts stimulated with TLR agonists (Mahanonda et al., 2007). Tryptophan is required for cell metabolism and growth, and therefore in regard to immune function, IDO is implicated in inhibiting both microbe and T cell proliferation (King and Thomas, 2007). This suggests that gingival
fibroblasts may regulate infection and immunity in the gingiva by producing IDO, and that leptin is one mediator that regulates this process. The results of this study confirm the work of Park et al. (2013) who found that leptin stimulates the production of the cytokine IL-6 by gingival fibroblasts. IL-6 has pleiotropic functions in regard to inflammation and immunity including stimulating cells to produce inflammatory mediators such as cytokines, chemokines, vasoactive mediators and proteases (Scheller et al., 2011). Also, IL-6 regulates the proliferation, activation and differentiation of numerous cell types, and enhances the production of leukocytes in the bone marrow (Scheller et al., 2011). IL-6 binds to the IL-6 receptor, which is either found on the cell surface or in a soluble form (Mihara et al., 2012). Intracellular signal transduction stimulated by IL-6 requires the formation of a complex consisting of the IL-6 receptor and another cell surface receptor (gp130/IL-6 signal transducer) (Mihara et al., 2012). Interestingly, cells that express only gp130 can still respond to IL-6 if IL-6 is bound to the soluble form of the IL-6 receptor (Scheller et al., 2011). IL-6 is implicated in the pathogenesis of RA as evidenced by reduced disease activity after treatment with an anti-IL-6 monoclonal antibody; however, individuals treated with this antibody had an increased risk of infection, which supports an important role for IL-6 in controlling and clearing infections (Emery et al., 2008). The results in this study demonstrate that fibroblasts are a likely source of IL-6 in the gingiva, and support the hypothesis that gingival fibroblasts regulate inflammation and immunity (Section 1.11). However, it is unclear what the functional outcomes of leptin-stimulated increases in IL-6 production by gingival fibroblasts are, and whether these outcomes would be beneficial or deleterious in vivo.

Several of the GO terms overrepresented in leptin-stimulated gingival fibroblasts were relevant to IFN signalling and responses. Two possibilities exist to explain this result: either leptin upregulated the production of IFN-γ at an early time point which had an autocrine effect on hGFs evident at 24 h; alternatively, IFN-γ signalling and responses were highlighted after leptin stimulation because both leptin and IFN-γ activate similar signalling pathways and upregulate a similar profile of genes (Section 1.2) (Schroder et al., 2004; Myers et al., 2008). Further work investigating gene expression at other time points and IFN-γ receptor expression on the surface of gingival fibroblasts would help to address the first possibility.

The results in this study support the existing evidence that IL-1 promotes an inflammatory gingival fibroblast phenotype (Section 1.11). In this study IL-1 stimulated
a higher number of differentially expressed genes in hGFs in comparison to leptin, and
the differential level of gene expression for many of the genes whose products have
inflammatory function was also higher in hGFs stimulated with IL-1 compared to hGFs
stimulated with leptin. For example, *IL6* was expressed at a level in IL-1 stimulated
hGFs over 10 times that observed in leptin-stimulated hGFs. This suggests that IL-1 has
a stronger stimulatory effect on the inflammatory gingival fibroblast phenotype in
comparison to leptin (at least regarding the present experimental design). The finding
that IL-1 and leptin+IL-1 stimulated hGFs had more differentially expressed genes in
common compared to leptin and leptin+IL-1 stimulated hGFs, suggests that IL-1 has a
more dominant effect on hGF phenotype than leptin. Nonetheless, leptin and IL-1
synergistically regulated the expression of several genes in hGFs whose products are
implicated in inflammation and ECM remodelling. These results add to the finding that
leptin+IL-1 synergistically regulate the production of MMPs by hGFs identified earlier
in this study (Chapter 4), and suggest that the actions of leptin and IL-1 on gingival
fibroblast phenotype are not limited to ECM remodelling.

hGFs can express a number of chemokines (Section 1.11.3), and at least one chemokine
(CXCL10) is synergistically upregulated by hGFs stimulated with IL-1 and OSM
(Hosokawa *et al.*, 2010a). In this study leptin and IL-1 synergistically increased the
expression of genes that encode chemokines in hGFs (*CXCL5, CXCL9, CCL7* and
*CCL20*). Several other chemokines genes were significantly upregulated in leptin+IL-1
stimulated hGFs but not synergistically regulated by these stimuli (*CXCL8, CXCL6,
*CCL8, CCL2* and *CXCL1*). As discussed earlier, chemokines are implicated in
leukocyte recruitment into tissues but also affect angiogenesis, leukocyte activation and
differentiation (Fernandez and Lolis, 2002). This suggests that leptin+IL-1 stimulated
hGFs (in comparison to leptin-stimulated hGFs) have a greater ability to stimulate
chemokines responses in the gingiva. The chemokines encoded by the genes
upregulated in leptin+IL-1 stimulated hGFs are implicated in the chemotaxis of
neutrophils (via *CXCL5, CXCL1, CXCL6, CXCL8*), monocytes, basophils and NK
cells (via *CCL7, CCL2, CCL8*), lymphocyte subsets (via *CXCL9, CCL7, CCL20,
CCL2, CCL8*) and DCs (via *CCL7, CCL20, CCL8*) (Murphy *et al.*, 2011). Broadly
speaking, this suggests that leptin+IL-1 stimulated hGFs may promote the infiltration of
all the main circulating leukocyte subsets into the gingiva. Additionally, leptin+IL-1
stimulated hGFs synergistically increased the expression of *CSF2*, which encodes the
cytokine granulocyte-macrophage CSF. This implies that hGFs exposed to leptin and
IL-1 not only increase leukocyte infiltration, but also stimulate haematopoiesis of granulocytes and monocytes in the bone marrow. CCL20 only binds one chemokine receptor (CCR6), and this receptor is highly expressed on the Th17 subset of lymphocytes and promotes their transendothelial migration (Grodecki-Pena et al., 2013). This suggests that in the presence of leptin and IL-1 gingival fibroblasts may enhance the recruitment of Th17 cells into the gingiva, which is interesting because Th17 cells are implicated in the pathogenesis of chronic inflammatory diseases including periodontitis (Miossec and Kolls, 2012; de Aquino et al., 2014). Chemokines do not solely act on leukocytes; for example, fibroblast-derived CCL7 enhances the proliferation, invasion and migration of oral squamous carcinoma cells (Jung et al., 2010). Perhaps gingival fibroblasts exposed to leptin and IL-1 enhance gingival epithelial cell proliferation and migration via CCL7, which could play a role in both wound healing and the epithelial proliferation observed in periodontitis. Further studies that identify whether non-cancerous gingival epithelial cells are responsive to CCL7 would help to address this hypothesis.

The expression levels of only two other genes which encode cytokines were synergistically upregulated in hGFs by leptin+IL-1 in this study (IL6, IL24); however, several other cytokine genes were differentially expressed in leptin+IL-1 stimulated hGFs (IL32, IL33, LIF, TGFB3, CSF3). This suggests that the cytokine networks active in the gingiva will be altered if gingival fibroblasts are exposed to both leptin and IL-1. The results in this study support the previous finding that the IL-10 family cytokine IL-24 is expressed in oral fibroblasts (Costea et al., 2013). To my knowledge, this is the first study to show that leptin, alone and in synergy with IL-1, increases IL-24 production in any cells. IL-24 is a relatively newly described cytokine, and its functional relevance is still to be fully determined (Wang and Liang, 2005). However, clinical studies have found that increased expression levels of IL-24 are positively associated with chronic inflammatory diseases including IBD and RA (Wang and Liang, 2005; Kragstrup et al., 2008; Andoh et al., 2009). Additionally, IL-24 promotes the production of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ by peripheral blood mononuclear cells in vitro (Caudell et al., 2002). These studies suggest that IL-24 plays a role in inflammatory processes.

Lower expression levels of IL-24 in rat gingiva are detected 7 days after ligature-induced periodontitis is initiated in Zucker (leptin receptor deficient) rats compared to wild-type controls (Soboku et al., 2014). This suggests that the reduced levels of IL-24
observed by Soboku et al. (2014) could be due to a lack of leptin signalling, which is in agreement with the results in the current study that leptin(+IL-1) enhances IL-24 production by gingival fibroblasts. However, differential IL-24 expression levels between Zucker and wild-type rats were only detected at the later of the two time points studied. Therefore, the functional relevance of differentially expressed IL-24 in ligature-induced periodontitis in the leptin receptor deficient rat is unclear.

In another study, increased IL-24 expression levels were detected in mucoperiosteal (oral mucosa and periosteum) connective tissue from individuals with refractory compared to stable periodontitis (Kim et al., 2006). In this study, refractory periodontitis was diagnosed if individuals required multiple rounds of treatment for periodontitis, or because they experienced multiple tooth loss after initial treatment for periodontitis. In comparison, individuals who required only maintenance therapy after initial periodontitis treatment were classified as having stable periodontitis. This suggests that IL-24 could play a role in the pathogenesis of refractory periodontitis. Interestingly, two of the other genes highly upregulated in refractory periodontitis connective tissue in this study were MMP1 and MMP3 which, along with IL24, were synergistically upregulated by leptin and IL-1 in hGFs in the current study. Taken together, these suggest that leptin and IL-1 could drive the gingival fibroblast phenotype towards that of one observed during refractory periodontitis. However, mucoperiosteal connective tissue would have contained multiple cell types, not just gingival fibroblasts. Additionally, no patient information (e.g. sex, BMI, age) was reported in this study beyond not having any significant medical history nor taking medications that might interfere with the study. Leptin levels vary between sex and BMI (Maffei et al., 1995), and therefore, in the study by Kim et al. (2006) it is impossible to determine whether differences in leptin levels may have been associated with refractory periodontitis and the observed gene expression profile. Overall, the studies by Soboku et al. (2014), Kim et al. (2006) and now the current study, suggest that IL-24 is a gingival fibroblast-derived cytokine that is associated with gingival inflammation and periodontitis. It would be interesting to perform further studies to identify which cells in the inflamed gingiva express the IL-24 receptors as a first step to investigate the targets and functions of IL-24 in the inflamed gingiva.

The gene encoding prostaglandin-endoperoxide synthase 2 (PTGS2) (alternatively known as COX-2) is upregulated in IL-1 stimulated hGFs (Båge et al., 2011), and in this study was synergistically upregulated by leptin and IL-1. It is well established that
the intracellular enzyme PTGS2 is upregulated during inflammation. PTGS2 converts
the fatty acid arachidonic acid to PGH₂. PGH₂ is subsequently converted into lipid mediators classified as the prostanoids (prostaglandins, thromboxanes and prostacyclins). Between them, the prostanoids have a wide range of pro- and anti-
inflammatory functions, and can regulate the production of MMPs (Domeij et al., 2002; Smyth et al., 2009). This suggests that gingival fibroblasts exposed to IL-1 and leptin could regulate inflammatory processes and ECM remodelling in the gingiva by producing prostanoids. Interestingly, only one of the genes (PTGES) that encodes an
enzyme that converts PGH₂ to a prostanoid was upregulated in leptin+IL-1 stimulated hGFs. PTGES encodes PGE synthase and converts PGH₂ to PGE (Smyth et al., 2009). This suggests that gingival fibroblasts exposed to leptin and IL-1 may increase the production of PGE in particular. Immunoassays that detect prostanoids in supernatants from in vitro cell cultures are commercially available and could be used to confirm this.

Several genes (LBP, CD14, CFB, C1R, and FAIM3) whose products are implicated in
immune system processes were upregulated by leptin+IL-1 in hGFs in this study. FAIM3 has been discussed earlier but it was interesting that the mRNA level of this Fc receptor was synergistically upregulated by leptin+IL-1 in this study, suggesting that gingival fibroblasts may be even more sensitive to IgM when exposed to both leptin+IL-1 compared to either stimuli alone. LBP which encodes LPS binding protein (LBP) was also synergistically upregulated by IL-1 and leptin in hGFs. At low concentrations LBP promotes cellular responses to LPS by facilitating the interaction of LPS with CD14 and TLR4; at high concentrations LBP appears to be able to neutralise LPS, thereby reducing the threat of endotoxaemia in the circulation or preventing microbial invasion in epithelial tissues (Gutsmann et al., 2001). Interestingly, the gene encoding CD14 was only upregulated in hGFs stimulated with leptin(±IL-1). Therefore, after confirming if CD14 and LBP are altered at the protein level, it would be interesting to determine whether there is any difference in TLR4 signalling in gingival fibroblasts pre-treated with leptin, IL-1 or leptin+IL-1 given the differences in the regulation of CD14 and LBP expression observed between these conditions.

The genome-wide expression analysis performed in this study identified several
differentially expressed genes (MMP1, MMP3, MMP8, MMP12, MMP14, MMP2,
PLAT, TIMP3, COL6A3, COL8A2, COL5A1, COL15A1, and COL14A1) in gingival fibroblasts after leptin+IL-1 treatment whose products are relevant to ECM remodelling. I confirmed that leptin and IL-1 synergistically upregulate the expression of MMP-1
and MMP-3 by gingival fibroblasts as observed earlier in this study (Chapter 4), but also identified that MMP-8 and MMP-12 were similarly synergistically regulated adding to the earlier findings in this study which suggest that leptin and IL-1 promote an ECM-degrading gingival fibroblast phenotype. The results presented in this chapter suggest that under inflammatory conditions leptin substantially enhances gingival fibroblast-mediated collagen remodelling by increasing the production of the collagenases MMP-1 and MMP-8. As discussed previously, the collagenase MMP-8 is thought to play an important role in tissue destruction during periodontitis (Section 3.5). The elastase MMP-12 degrades numerous ECM components, but can also activate other MMPs, including MMP-2 and MMP-3 (Chen, 2004). Interestingly, MMP-12 can activate itself through autolytic processing, which suggests that gingival fibroblast-derived MMP-12 could initiate the activation of other MMPs produced by gingival fibroblasts in the inactive pro-enzyme form.

Significant decreases in the expression levels of several collagen genes (COL6A3, COL8A2, COL5A1, COL15A1, and COL14A1) were detected in hGFs after leptin+IL-1 stimulation in this study; however, only COL6A3 was synergistically downregulated by leptin and IL1. COL6A3 encodes one of the three proteins which form collagen type VI microfibrils. These microfibrils bind both fibrillar collagens and proteoglycans, and are therefore implicated in the organisation of ECMs (Bonnemann, 2011). Collagen type VI is found in the gingiva (Shikata et al., 1993), which together with the current results suggests that gingival fibroblasts are a cellular source of this collagen. The finding that leptin+IL-1 decreased the expression of several collagen genes in hGFs suggests that under proinflammatory and hyperleptinaemic conditions gingival fibroblasts are synthesising less collagen.

Overall, the increased expression of ECM-targeting proteases in combination with the reduced expression of several collagen genes and the lack of change in TIMP expression in gingival fibroblasts after leptin+IL-1 stimulation, suggests that under hyperleptinaemic and pro-inflammatory conditions gingival fibroblasts alter the balance of ECM homeostasis towards ECM degradation.

Interestingly, the RA pathway was one of the most significantly over-represented in leptin, IL-1 and leptin+IL-1-stimulated hGFs. The pathogenesis of RA bears several similarities to periodontitis; fibroblasts are implicated in both diseases, both diseases are positively associated with obesity, and chronic inflammation and excessive ECM
degradation are involved in both diseases (Kornman, 2008; McInnes and Schett, 2011). Periodontitis currently has no unique KEGG pathway and therefore, the RA pathway is probably one of the best matches for periodontitis given the similarities between these conditions.

In conclusion, the genome-wide expression analysis performed in this study demonstrated that leptin changes the expression profile of hGFs, and suggests that these changes may function to regulate immune system processes and inflammatory pathways. In particular these results suggest that leptin enhances the immunocompetence of gingival fibroblasts and may improve immunosurveillance in the gingiva. Additionally, leptin enhanced the potent inflammatory and ECM-degrading phenotype stimulated by IL-1 in hGFs, which suggests that the presence of high concentrations of leptin in the inflamed gingiva may promote a stronger inflammatory response and ECM destruction. This supports the hypothesis that leptin is a mechanistic link underpinning the increased risk of developing periodontitis in conditions associated with hyperleptinaemia.

Several limitations must be addressed regarding the results presented in this chapter. Gene expression at only one time point was assessed in this microarray study. Therefore, genes regulated at other time points by leptin and IL-1 may not have been identified, and this may have affected the interpretation of the gingival fibroblast expression profile after leptin and IL-1 stimulation. Additionally, two decisions used in the analysis of the microarray dataset may have affected the results presented in this chapter. Firstly, to generate data from microarray experiments certain assumptions have to be made. In this study the datasets from the samples of one donor were somewhat different to those of the other two donors (Figure E.2E). It is important, and appropriate, to address this kind of variation in large datasets (Leek and Storey, 2007). The assumption was made that this variation was due to a combination of unknown or non-measurable variation (likely both technical and biological). The surrogate variable analysis is one approach which is used to eliminate this kind of variance between datasets. However, there are other approaches that could have addressed this variation. For example, a paired analysis approach could have been performed. In this approach an assumption could have been made that each of the datasets derived from an individual donor are related, to account for donor-dependent variation yet maintain the statistical power of the experiment. It would be interesting to compare the results of a paired analysis to the surrogate variable analysis performed in this study. Secondly, the
identification of differentially expressed genes used for gene lists, GO and KEGG pathway analysis was based on a raw fold change of 2, and a corrected p value of <0.01. Cut-offs like this are required in microarray analysis, but have the statistical potential to generate false negative or positive results. For example, in this study leptin-stimulated MMP-1 expression in hGFs was not significantly upregulated in the microarray analysis, in contrast to the real-time RT-PCR analysis. Nonetheless, the focus of this study was to identify strongly differentially expressed genes in hGFs, particularly those regulated by leptin and IL-1 in synergy. Therefore, a stringent p-value cut-off was selected to eliminate false positives. Finally, the microarray only identified gene expression levels in gingival fibroblasts and further work would be required (as was performed for IL-24 in this study) to determine whether these differential expression levels related to protein levels. Additionally, it is not possible to determine how the observed changes in hGF phenotype are relevant to function in the gingiva; in vivo, ex vivo, or 3D models of normal or inflamed gingival tissue would be required to address this. For this reason, a gingival tissue explant model was developed and the results of this work are presented in Chapter 7.
Table 6.1: Expression levels of genes on the immune TLDA in leptin-stimulated hGFs.

hGFs were stimulated with 1 μg/ml leptin for 24 h or left unstimulated as a control. Cells were lysed and cDNA was prepared by reverse transcription. cDNA was loaded onto a human immune panel TLDA and gene expression was determined by real-time RT-PCR. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method by using 18S rRNA as the reference gene. Data (n=1) are shown as fold unstimulated control. Genes with >2-fold change in gene expression are highlighted in bold. ND – not detected in leptin or unstimulated hGFs, + - only detected in leptin-stimulated hGFs.

<table>
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<th>Gene</th>
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Figure 6.1: IL-6 secretion by hGFs after stimulation with leptin and IL-1

hGFs from one donor were either unstimulated or stimulated with leptin (0.1, 1, 10 μg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 0.1, 1, 10 μg/ml, IL-1α 0.05 ng/ml) for 24 h. Supernatants were collected and IL-6 concentrations were assessed by ELISA. Data (ng/ml) are shown as mean+SD (n=2). Leptin (10 μg/ml) +IL-1α was above the top standard (300 ng/ml).
Figure 6.2: MMP-1 gene expression in hGFs after stimulation with IL-1α and IL-1β.

hGFs were stimulated with IL-1α (0.05 ng/ml) or IL-1β (0.05 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the 2^{-ΔΔCt} method by using RNAP as the reference gene. Data are shown as median+IQR (n=20) from two donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control.
Figure 6.3: MMP-1 protein secretion by hGFs after stimulation with IL-1α and IL-1β.

hGFs were stimulated with IL-1α (0.05 ng/ml) or IL-1β (0.05 ng/ml) for 24 h. Supernatants were collected and MMP-1 concentrations were assessed by ELISA. Data (fold unstimulated control) are shown as median+IQR (n=15-16) from two donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Figure 6.4: MMP-1 gene expression in hGFs after stimulation with leptin and IL-1β.

hGFs from three donors were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h in independent experiments (96-well format). Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the $2^{-\Delta\DeltaCT}$ method by using RNAP as the reference gene. Data (fold unstimulated control) on each graph are shown as mean+SD (n=4) from one donor as indicated. Statistics: T test or Mann-Whitney U test for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01, ***=p<0.001 compared to unstimulated control unless otherwise indicated.
Figure 6.5: Venn diagram displaying the relationship between differentially expressed genes in hGFs after stimulation with leptin, IL-1 or leptin+IL-1

hGFs from three donors were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. The 12 samples generated were used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were subject to bioinformatics analysis. Using the corrected dataset differentially expressed gene lists were generated for the comparisons control v leptin, control v IL1, control v leptin+IL-1 using an adjusted p-value cut off of 0.01, but no fold change cut-off. This Venn diagram displays the number of differentially expressed genes common or unique to the different treatment conditions.
Table 6.2: Selection of differentially expressed genes in hGFs after leptin stimulation

hGFs from three donors were stimulated with leptin (10 μg/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v leptin (Section 2.8.2). 111 genes were differentially expressed; 20 genes relevant to inflammation, immunity, cell proliferation, leptin signalling and ECM homeostasis are displayed in this table. For each gene the full gene name, fold change and adjusted p-value are displayed. Downregulated genes are shaded in grey. CLL – chronic lymphocytic leukaemia, ADAM – a disintegrin and metalloproteinase.

<table>
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<th>Fold change</th>
<th>p-value</th>
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<td>BCL6</td>
<td>B-cell CLL/lymphoma 6</td>
<td>2.72</td>
<td>1.1E-06</td>
</tr>
<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1, 91kDa</td>
<td>2.69</td>
<td>4.5E-03</td>
</tr>
<tr>
<td>IL13RA1</td>
<td>interleukin 13 receptor, alpha 1</td>
<td>2.69</td>
<td>7.7E-07</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)</td>
<td>2.58</td>
<td>7.5E-03</td>
</tr>
<tr>
<td>FAIM3</td>
<td>Fas apoptotic inhibitory molecule 3</td>
<td>2.54</td>
<td>5.9E-05</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>major histocompatibility complex, class II, DM alpha</td>
<td>2.50</td>
<td>1.1E-04</td>
</tr>
<tr>
<td>IDO1</td>
<td>indoleamine 2,3-dioxygenase 1</td>
<td>2.44</td>
<td>3.6E-04</td>
</tr>
<tr>
<td>IL1R1</td>
<td>interleukin 1 receptor, type 1</td>
<td>2.26</td>
<td>6.5E-07</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>ADAM metalloproteinase with thrombospondin type 1 motif, 1</td>
<td>2.23</td>
<td>7.8E-05</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signalling 3</td>
<td>2.17</td>
<td>1.8E-06</td>
</tr>
<tr>
<td>ATF3</td>
<td>activating transcription factor 3</td>
<td>2.15</td>
<td>4.4E-05</td>
</tr>
<tr>
<td>ADAM19</td>
<td>ADAM metalloproteinase domain 19</td>
<td>-2.10</td>
<td>8.9E-05</td>
</tr>
</tbody>
</table>
Table 6.3: Selected GO term processes significantly overrepresented in hGFs after leptin treatment.

hGFs from three donors were stimulated with leptin (10 μg/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control vs leptin (Section 2.8.2). GO analysis was performed and the most significant processes (10 of 44 with p<0.001) (as indicated by adjusted p-value) are displayed in this table.

<table>
<thead>
<tr>
<th>Name</th>
<th>Process</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0034097</td>
<td>response to cytokine stimulus</td>
<td>4.43E-07</td>
</tr>
<tr>
<td>GO:0002376</td>
<td>immune system process</td>
<td>4.43E-07</td>
</tr>
<tr>
<td>GO:0034341</td>
<td>response to interferon-gamma</td>
<td>2.70E-06</td>
</tr>
<tr>
<td>GO:0071345</td>
<td>cellular response to cytokine stimulus</td>
<td>2.70E-06</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>defence response</td>
<td>5.69E-06</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>immune response</td>
<td>7.31E-06</td>
</tr>
<tr>
<td>GO:0050865</td>
<td>regulation of cell activation</td>
<td>8.87E-06</td>
</tr>
<tr>
<td>GO:0019221</td>
<td>cytokine-mediated signalling pathway</td>
<td>1.42E-05</td>
</tr>
<tr>
<td>GO:0050863</td>
<td>regulation of T cell activation</td>
<td>1.42E-05</td>
</tr>
<tr>
<td>GO:0060337</td>
<td>type I interferon-mediated signalling pathway</td>
<td>1.42E-05</td>
</tr>
</tbody>
</table>
Table 6.4: KEGG pathways significantly overrepresented in hGFs after leptin treatment.

hGFs from three donors were stimulated with leptin (10 μg/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for whole-wide genome expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v leptin (Section 2.8.2). KEGG pathway analysis was performed and the significant pathways identified (p<0.01) are displayed in this table.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>4.1E-05</td>
</tr>
<tr>
<td>Cytokine:cytokine receptor interaction</td>
<td>7.0E-04</td>
</tr>
<tr>
<td>Graft-versus-host disease</td>
<td>8.8E-04</td>
</tr>
<tr>
<td>Haematopoietic cell lineage</td>
<td>1.5E-03</td>
</tr>
<tr>
<td>Intestinal immune network for IgA production</td>
<td>1.6E-03</td>
</tr>
<tr>
<td>JAK-STAT signalling pathway</td>
<td>3.4E-03</td>
</tr>
<tr>
<td>Tryptophan metabolism</td>
<td>3.6E-03</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>4.8E-03</td>
</tr>
<tr>
<td>Primary bile acid biosynthesis</td>
<td>6.0E-03</td>
</tr>
<tr>
<td>Asthma</td>
<td>7.1E-03</td>
</tr>
<tr>
<td>TLR signalling pathway</td>
<td>7.7E-03</td>
</tr>
<tr>
<td>Amoebiasis</td>
<td>8.1E-03</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>9.7E-03</td>
</tr>
</tbody>
</table>
Table 6.5: Selection of differentially expressed genes in hGFs after IL-1 stimulation

hGFs from three donors were stimulated with IL-1 (0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control vs IL-1 (Section 2.8.2). 263 genes were differentially expressed; 20 genes relevant to inflammation, immunity, and ECM homeostasis are displayed in this table. For each gene the full gene name, fold change and adjusted p-value are displayed. Downregulated genes are shaded in grey. CLL – chronic lymphocytic leukaemia, ADAM – a disintegrin and metalloproteinase.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>146</td>
<td>9.1E-07</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6 (interferon, beta 2)</td>
<td>69.6</td>
<td>1.0E-07</td>
</tr>
<tr>
<td>CXCL6</td>
<td>chemokine (C-X-C motif) ligand 6</td>
<td>65.4</td>
<td>6.1E-08</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)</td>
<td>52.8</td>
<td>1.0E-07</td>
</tr>
<tr>
<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
<td>42.4</td>
<td>5.2E-08</td>
</tr>
<tr>
<td>CFB</td>
<td>complement factor B</td>
<td>14.0</td>
<td>1.0E-05</td>
</tr>
<tr>
<td>MMP3</td>
<td>matrix metallopeptidase 3 (stromelysin 1, progelatinase)</td>
<td>12.1</td>
<td>1.0E-04</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>10.9</td>
<td>1.3E-07</td>
</tr>
<tr>
<td>CXCL5</td>
<td>chemokine (C-X-C motif) ligand 5</td>
<td>7.74</td>
<td>2.3E-07</td>
</tr>
<tr>
<td>CCL7</td>
<td>chemokine (C-C motif) ligand 7</td>
<td>6.86</td>
<td>2.1E-06</td>
</tr>
<tr>
<td>CCL8</td>
<td>chemokine (C-C motif) ligand 8</td>
<td>6.60</td>
<td>1.0E-05</td>
</tr>
<tr>
<td>PTGES</td>
<td>prostaglandin E synthase</td>
<td>6.43</td>
<td>3.0E-06</td>
</tr>
<tr>
<td>CCL20</td>
<td>chemokine (C-C motif) ligand 20</td>
<td>5.95</td>
<td>2.3E-05</td>
</tr>
<tr>
<td>IL1RN</td>
<td>interleukin 1 receptor antagonist</td>
<td>4.46</td>
<td>2.3E-07</td>
</tr>
<tr>
<td>MMP1</td>
<td>matrix metallopeptidase 1 (interstitial collagenase)</td>
<td>4.32</td>
<td>8.6E-04</td>
</tr>
<tr>
<td>IL32</td>
<td>interleukin 32</td>
<td>3.20</td>
<td>3.5E-05</td>
</tr>
<tr>
<td>COL8A1</td>
<td>collagen, type VIII, alpha 1</td>
<td>2.71</td>
<td>6.9E-05</td>
</tr>
<tr>
<td>IL33</td>
<td>interleukin 33</td>
<td>2.14</td>
<td>9.1E-05</td>
</tr>
<tr>
<td>BCL6</td>
<td>B-cell CLL/lymphoma 6</td>
<td>2.09</td>
<td>5.2E-06</td>
</tr>
<tr>
<td>ADAM19</td>
<td>ADAM metallopeptidase domain 19</td>
<td>-2.61</td>
<td>5.9E-06</td>
</tr>
</tbody>
</table>
Table 6.6: Selected GO term processes significantly overrepresented in hGFs after IL-1 treatment.

hGFs from three donors were stimulated with IL-1 (0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v IL-1 (Section 2.8.2). GO analysis was performed and the most significant processes (10 of 153 with p<0.001) (as indicated by adjusted p-value) are displayed in this table.

<table>
<thead>
<tr>
<th>Name</th>
<th>Process</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006952</td>
<td>defence response</td>
<td>8.65E-17</td>
</tr>
<tr>
<td>GO:0051707</td>
<td>response to other organism</td>
<td>5.76E-14</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>inflammatory response</td>
<td>7.28E-14</td>
</tr>
<tr>
<td>GO:0009607</td>
<td>response to biotic stimulus</td>
<td>1.24E-13</td>
</tr>
<tr>
<td>GO:0042221</td>
<td>response to chemical stimulus</td>
<td>8.71E-13</td>
</tr>
<tr>
<td>GO:0009611</td>
<td>response to wounding</td>
<td>1.61E-12</td>
</tr>
<tr>
<td>GO:0002376</td>
<td>immune system process</td>
<td>1.91E-12</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>immune response</td>
<td>9.80E-11</td>
</tr>
<tr>
<td>GO:0009605</td>
<td>response to external stimulus</td>
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</tr>
<tr>
<td>GO:0050896</td>
<td>response to stimulus</td>
<td>1.24E-10</td>
</tr>
</tbody>
</table>
Table 6.7: KEGG pathways significantly overrepresented in hGFs after IL-1 treatment.

hGFs from three donors were stimulated with IL-1 (0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v IL-1 (Section 2.8.2). KEGG pathway analysis was performed and the significant pathways identified (p<0.01) are displayed in this table.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-like receptor signalling pathway</td>
<td>4.20E-07</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>8.10E-07</td>
</tr>
<tr>
<td>Cytokine:cytokine receptor interaction</td>
<td>8.30E-06</td>
</tr>
<tr>
<td>Chemokine signalling pathway</td>
<td>1.10E-05</td>
</tr>
<tr>
<td>Steroid hormone biosynthesis</td>
<td>1.80E-03</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>2.50E-03</td>
</tr>
<tr>
<td>RIG-1-like receptor signalling pathway</td>
<td>3.70E-03</td>
</tr>
<tr>
<td>TLR signalling pathway</td>
<td>4.50E-03</td>
</tr>
<tr>
<td>Cytosolic DNA-sensing pathway</td>
<td>5.20E-03</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>6.40E-03</td>
</tr>
</tbody>
</table>
Table 6.8: Selection of differentially expressed genes in hGFs after leptin+IL-1 stimulation

hGFs from three donors were stimulated with leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v leptin+IL-1 (Section 2.8.2). 637 genes were differentially expressed; 39 genes relevant to inflammation, immunity, and ECM homeostasis are displayed in this table. For each gene the full gene name, fold change and adjusted p-value are displayed. Downregulated genes are shaded in grey. TIMP – tissue inhibitor of metalloproteinase.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>167</td>
<td>1.4E-07</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6 (interferon, beta 2)</td>
<td>115</td>
<td>7.7E-09</td>
</tr>
<tr>
<td>CXCL6</td>
<td>chemokine (C-X-C motif) ligand 6</td>
<td>96.9</td>
<td>5.9E-09</td>
</tr>
<tr>
<td>MMP3</td>
<td>matrix metalloprotease 3 (stromelysin 1, progelatinase)</td>
<td>55.9</td>
<td>5.8E-07</td>
</tr>
<tr>
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<td>54.2</td>
<td>6.1E-09</td>
</tr>
<tr>
<td>CXCL1</td>
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<td>51.2</td>
<td>1.8E-08</td>
</tr>
<tr>
<td>IL24</td>
<td>interleukin 24</td>
<td>46.3</td>
<td>7.5E-10</td>
</tr>
<tr>
<td>CXCL5</td>
<td>chemokine (C-X-C motif) ligand 5</td>
<td>30.6</td>
<td>1.1E-09</td>
</tr>
<tr>
<td>CFB</td>
<td>complement factor B</td>
<td>29.0</td>
<td>3.3E-07</td>
</tr>
<tr>
<td>CCL7</td>
<td>chemokine (C-C motif) ligand 7</td>
<td>20.9</td>
<td>1.1E-08</td>
</tr>
<tr>
<td>CCL20</td>
<td>chemokine (C-C motif) ligand 20</td>
<td>20.3</td>
<td>7.5E-08</td>
</tr>
<tr>
<td>MMP1</td>
<td>matrix metalloprotease 1 (interstitial collagenase)</td>
<td>13.0</td>
<td>3.3E-06</td>
</tr>
<tr>
<td>CCL8</td>
<td>chemokine (C-C motif) ligand 8</td>
<td>12.5</td>
<td>2.1E-07</td>
</tr>
<tr>
<td>PTGES</td>
<td>prostaglandin E synthase</td>
<td>10.1</td>
<td>9.1E-08</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>9.07</td>
<td>3.8E-08</td>
</tr>
<tr>
<td>IL32</td>
<td>interleukin 32</td>
<td>4.07</td>
<td>2.0E-06</td>
</tr>
<tr>
<td>IL18BP</td>
<td>interleukin 18 binding protein</td>
<td>3.87</td>
<td>2.1E-05</td>
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<tr>
<td>CSF2</td>
<td>colony stimulating factor 2 (granulocyte-macrophage)</td>
<td>3.66</td>
<td>1.3E-08</td>
</tr>
<tr>
<td>CTSL</td>
<td>cathepsin L</td>
<td>3.31</td>
<td>2.0E-07</td>
</tr>
<tr>
<td>OSMR</td>
<td>oncostatin M receptor</td>
<td>3.28</td>
<td>1.9E-08</td>
</tr>
<tr>
<td>IL33</td>
<td>interleukin 33</td>
<td>2.99</td>
<td>1.4E-06</td>
</tr>
<tr>
<td>IL1R1</td>
<td>interleukin 1 receptor, type I</td>
<td>2.95</td>
<td>4.9E-09</td>
</tr>
<tr>
<td>IL1RN</td>
<td>interleukin 1 receptor antagonist</td>
<td>2.88</td>
<td>8.0E-07</td>
</tr>
<tr>
<td>CSF3</td>
<td>colony stimulating factor 3 (granulocyte)</td>
<td>2.80</td>
<td>5.1E-06</td>
</tr>
<tr>
<td>C1R</td>
<td>complement component 1, r subcomponent</td>
<td>2.68</td>
<td>5.8E-06</td>
</tr>
<tr>
<td>CXCL9</td>
<td>chemokine (C-X-C motif) ligand 9</td>
<td>2.65</td>
<td>2.5E-08</td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
<td>2.61</td>
<td>2.5E-05</td>
</tr>
<tr>
<td>PLAT</td>
<td>plasminogen activator, tissue</td>
<td>2.59</td>
<td>4.6E-05</td>
</tr>
<tr>
<td>MMP8</td>
<td>matrix metalloprotease 8 (neutrophil collagenase)</td>
<td>2.45</td>
<td>1.4E-06</td>
</tr>
<tr>
<td>MMP2</td>
<td>matrix metalloprotease 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)</td>
<td>2.43</td>
<td>2.2E-06</td>
</tr>
<tr>
<td>C1S</td>
<td>complement component 1, s subcomponent</td>
<td>2.41</td>
<td>1.6E-06</td>
</tr>
<tr>
<td>IL6ST</td>
<td>interleukin 6 signal transducer (gp130, oncostatin M receptor)</td>
<td>2.30</td>
<td>1.2E-06</td>
</tr>
<tr>
<td>MMP14</td>
<td>matrix metalloprotease 14 (membrane-inserted)</td>
<td>2.03</td>
<td>7.5E-07</td>
</tr>
<tr>
<td>MMP12</td>
<td>matrix metalloprotease 12 (macrophage elastase)</td>
<td>2.00</td>
<td>7.4E-04</td>
</tr>
<tr>
<td>COL8A2</td>
<td>collagen, type VIII, alpha 2</td>
<td>-2.02</td>
<td>9.7E-06</td>
</tr>
<tr>
<td>COL5A1</td>
<td>collagen, type V, alpha 1</td>
<td>-2.02</td>
<td>8.5E-08</td>
</tr>
<tr>
<td>COL15A1</td>
<td>collagen, type XV, alpha 1</td>
<td>-2.05</td>
<td>6.8E-06</td>
</tr>
<tr>
<td>COL14A1</td>
<td>collagen, type XIV, alpha 1</td>
<td>-2.18</td>
<td>2.4E-06</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>transforming growth factor, beta 3</td>
<td>-2.18</td>
<td>4.2E-07</td>
</tr>
<tr>
<td>TIMP3</td>
<td>TIMP metalloprotease inhibitor 3</td>
<td>-2.28</td>
<td>1.1E-03</td>
</tr>
<tr>
<td>COL6A3</td>
<td>collagen, type VI, alpha 3</td>
<td>-2.80</td>
<td>4.3E-04</td>
</tr>
</tbody>
</table>
Table 6.9: Selected GO term processes significantly overrepresented in hGFs after leptin+IL-1 treatment.

hGFs from three donors were stimulated with leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v lep+IL-1 (Section 2.8.2). GO analysis was performed and the most significant processes (10 of 255 with p<0.001) (as indicated by adjusted p-value) are displayed in this table.

<table>
<thead>
<tr>
<th>Name</th>
<th>Process</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0009611</td>
<td>response to wounding</td>
<td>7.66E-18</td>
</tr>
<tr>
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<tr>
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<td>response to chemical stimulus</td>
<td>7.97E-16</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>inflammatory response</td>
<td>7.97E-16</td>
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<td>system development</td>
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<td>single-multicellular organism process</td>
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</tr>
<tr>
<td>GO:0002376</td>
<td>immune system process</td>
<td>5.48E-15</td>
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<tr>
<td>GO:0048856</td>
<td>anatomical structure development</td>
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hGFs from three donors were stimulated with leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v leptin+IL-1 (Section 2.8.2). KEGG pathway analysis was performed and the significant pathways identified (p<0.01) are displayed in this table.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value</th>
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<tr>
<td>Cytokine:cytokine receptor interaction</td>
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</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>7.10E-07</td>
</tr>
<tr>
<td>JAK-STAT signalling pathway</td>
<td>1.30E-06</td>
</tr>
<tr>
<td>Amoebiasis</td>
<td>4.70E-05</td>
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<tr>
<td>Haematopoietic cell lineage</td>
<td>2.50E-05</td>
</tr>
<tr>
<td>Malaria</td>
<td>1.50E-04</td>
</tr>
<tr>
<td>Metabolism of xenobiotics by cytochrome P450</td>
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</tr>
<tr>
<td>NOD-like receptor signalling pathway</td>
<td>4.90E-04</td>
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<tr>
<td>ECM-receptor interaction</td>
<td>2.10E-03</td>
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<tr>
<td>Chemokine signalling pathway</td>
<td>2.60E-03</td>
</tr>
<tr>
<td>Drug metabolism - cytochrome P450</td>
<td>4.80E-03</td>
</tr>
<tr>
<td>Histidine metabolism</td>
<td>5.60E-03</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>7.80E-03</td>
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<tr>
<td>Tryptophan metabolism</td>
<td>8.70E-03</td>
</tr>
<tr>
<td>Glycolysis/Gluconeogenesis</td>
<td>8.80E-03</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>9.10E-03</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>9.90E-03</td>
</tr>
</tbody>
</table>
Figure 6.6: Leptin+IL-1 stimulated differentially expressed genes in hGFs represented on the RA KEGG pathway.

hGFs from three donors were stimulated with leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset a differentially expressed gene list was generated for the comparison unstimulated control v leptin+IL-1 (Section 2.8.2). KEGG pathway analysis was performed on this gene list. This schematic diagram displays the RA KEGG pathway and highlights where gene products are functionally implicated. Differentially expressed genes in the leptin+IL-1 gene list were overrepresented on this pathway as indicated by colour (green/grey/red). Genes coloured green were downregulated, genes coloured grey were upregulated at a low level, and genes coloured red were upregulated at a high level by leptin+IL-1 treatment.
Table 6.11: Selected differentially expressed genes in hGFs potentially synergistically regulated by leptin+IL-1

hGFs from three donors were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA was used for genome-wide expression analysis (Illumina human Ht12 v4 beadchip). The microarray datasets were used for bioinformatics analysis. Using the corrected dataset differentially expressed gene lists were generated for the comparisons unstimulated control v leptin, unstimulated control v IL-1, unstimulated control v leptin+IL-1, leptin v leptin+IL-1 and IL-1 v leptin+IL-1 (Section 2.8.2). 42 genes relevant to inflammation, immunity, and ECM homeostasis are displayed in this table. For each gene the full gene name and the fold changes in gene expression after leptin, IL-1 and leptin+IL-1 stimulation are displayed. Genes highlighted in bold (and shaded grey) were significantly differentially expressed in the leptin v leptin+IL-1 and IL-1 v leptin+IL-1 gene lists indicating a significant synergistic relationship. ND – not detected (no differential gene expression was detected) TIMP – tissue inhibitor of metalloproteinase, TRAF – TNF receptor-associated factor.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Fold change (leptin+IL-1)</th>
<th>Fold change (leptin)</th>
<th>Fold change (IL-1)</th>
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<tbody>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>166.74</td>
<td>ND</td>
<td>146</td>
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<tr>
<td>IL6</td>
<td>interleukin 6 (interferon, beta 2)</td>
<td>115.04</td>
<td>3.74</td>
<td>69.6</td>
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<td>CXCL6</td>
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<td>96.90</td>
<td>ND</td>
<td>65.4</td>
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<td>MMP3</td>
<td>matrix metallopeptidase 3 (stromelysin 1, progelatinase)</td>
<td>55.86</td>
<td>ND</td>
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<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
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<td>7.98</td>
<td>42.4</td>
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<td>IL24</td>
<td>interleukin 24</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>CXCL5</td>
<td>chemokine (C-X-C motif) ligand 5</td>
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<td>ND</td>
<td>7.74</td>
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<td>complement factor B</td>
<td>28.97</td>
<td>ND</td>
<td>14.0</td>
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<td>PTGS2</td>
<td>prostaglandin-endoperoxide synthase 2</td>
<td>19.43</td>
<td>ND</td>
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<td>MMP1</td>
<td>matrix metallopeptidase 1 (interstitial collagenase)</td>
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<td>ND</td>
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<td>LBP</td>
<td>lipopolysaccharide binding protein</td>
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<td>ND</td>
<td>ND</td>
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<td>10.06</td>
<td>ND</td>
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<td>2.54</td>
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<td>CD14 molecule</td>
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<td>CSF2</td>
<td>colony stimulating factor 2 (granulocyte-macrophage)</td>
<td>3.66</td>
<td>ND</td>
<td>ND</td>
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<td>OSMR</td>
<td>oncostatin M receptor</td>
<td>3.28</td>
<td>ND</td>
<td>ND</td>
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<td>JAK2</td>
<td>Janus kinase 2</td>
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<td>interleukin 33</td>
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<td>ND</td>
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<td>suppressor of cytokine signalling 3</td>
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<td>IL1R1</td>
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<td>ND</td>
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<td>colony stimulating factor 3 (granulocyte)</td>
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<tr>
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<td>vascular endothelial growth factor A</td>
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<td>LIF</td>
<td>leukaemia inhibitory factor</td>
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<td>ND</td>
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<td>PLAT</td>
<td>plasminogen activator, tissue</td>
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<td>ND</td>
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<td>MMP8</td>
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<td>matrix metallopeptidase 2</td>
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<td>ND</td>
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<td>transforming growth factor, beta 3</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TIMP3</td>
<td>TIMP metallopeptidase inhibitor 3</td>
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<td>ND</td>
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<tr>
<td>COL6A3</td>
<td>collagen, type VI, alpha 3</td>
<td>-2.80</td>
<td>ND</td>
<td>ND</td>
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[G] COL6A3/RNAP (% control)

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<tr>
<th></th>
<th>Leptin</th>
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</tr>
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<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

[***] [**]

[H] TIMP3/RNAP (% control)

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<th>Leptin</th>
<th>IL-1β</th>
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</tbody>
</table>

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Figure 6.7: Real-time PCR analysis of genes chosen for further study from microarray analysis.

hGFs were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h [A-F] 6-well format, [G-H] 96-well format. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess [A] MMP-3, [B] IL-24, [C] MMP-8, [D] MMP-12, [E] MMP-14, [F] MMP-2, [G] collagen 6A1, [H] TIMP-3 and RNAP gene expression. [A, C-H] Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. [B] Relative gene expression was determined by normalising to the reference gene RNAP. Data are shown as mean±SD ([A-F] n=6, [G-H] n=12) from three donors stimulated in independent experiments. Statistics: Mann-Whitney U test for independent samples (Bonferroni-corrected) *=p<0.05, **=p<0.01 ***=p<0.001 compared to unstimulated control unless indicated otherwise.
Figure 6.8: IL-24 secretion by hGFs after stimulation with leptin and IL-1

hGFs were either unstimulated or stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h. Supernatants were collected and IL-24 concentrations were assessed by ELISA. Data (pg/ml) are shown as mean±SD (n=6) from three donors stimulated in independent experiments. Statistics: T test for independent samples (Bonferroni-corrected) **=p<0.01 as indicated. ND – not detected.
Chapter 7. Development of an explant model to assess leptin+IL-1-mediated gingival tissue collagen degradation

7.1 Introduction
Fibroblasts are the predominant cell type in gingival connective tissue, and are surrounded by a dense collagen-rich ECM (Bartold and Narayanan, 2006). Numerous studies, including the current study (Chapters 3, 4 and 6), have found that gingival fibroblasts produce both ECM components and enzymes that degrade ECM such as MMPs (Zhang et al., 2011; Arancibia et al., 2012; Mah et al., 2014). As such, gingival fibroblasts are implicated in gingival tissue remodelling during health and disease.

Several studies have cultured gingival fibroblasts on or in collagen gel matrices to assess the production and activity of MMPs produced by gingival fibroblasts (Cox et al., 2006; Smith et al., 2009; Zhang et al., 2010; Zhang et al., 2011; Mah et al., 2014). For example, Smith et al. (2009) found that human gingival fibroblasts stimulated with TNF-α on a type I collagen matrix produce higher levels of MMP-14 protein compared to plastic surfaces, and that the culture supernatants contain increased levels of active MMP-2; no TNF-α-stimulated MMP-2 activity was observed in the supernatants of gingival fibroblasts cultured on a plastic surface.

In studies using hGFs seeded onto a central area of a type I collagen-coated surface, Zhang et al. found that hGFs could degrade collagen in the pericellular area constitutively (Zhang et al., 2010; Zhang et al., 2011). Interestingly, the entire collagen-coated area could be degraded when gingival fibroblasts were treated with P. gingivalis supernatant, suggesting that under this condition inter-territorial ECM degradation is promoted by gingival fibroblasts. This collagen degradation was totally inhibited by the MMP inhibitor GM6001, which suggests that MMPs were responsible for the collagen degradation observed, instead of P. gingivalis-derived proteinases. After stimulation with P. gingivalis supernatant, gingival fibroblasts secrete increased active MMP-2, MMP-1 and MMP-3; under the same stimulatory conditions secreted levels of TIMP-1 were not altered, while levels of TIMP-2 were decreased (Zhang et al., 2010; Zhang et al., 2011). Taken together, these findings suggest that gingival fibroblast-derived MMPs can degrade type I collagen, and that this ability may be regulated by disrupting the balance of active MMPs to TIMPs.
Over a culture period of several days IL-1β increases the concentration of hydroxyproline in supernatants from hGFs isolated from individuals with chronic periodontitis that were seeded onto a type I collagen matrix; the levels of active MMP-1, MMP-8 and MMP-13 tended to be higher in the supernatants of hGFs stimulated with IL-1β compared to unstimulated cells (Cox et al., 2006). This suggests that the pro-inflammatory cytokine IL-1β promotes gingival fibroblast-mediated collagen degradation by increasing the secretion and activation of collagenases.

Other studies have assessed the production of ECM-degrading enzymes by whole oral mucosal tissues, in an effort to determine whether this is an appropriate model for ECM remodelling in health and disease. In the 1980s, and therefore prior to the identification of specific MMP family members, pro and active forms of collagenases and gelatinases (as assessed by an ability to degrade collagen or gelatin substrates respectively) were detected in both human and porcine gingival tissue supernatants (Pettigrew et al., 1981; Heath et al., 1982). More recently, gingival tissues have been engineered from individual cell populations and man-made scaffolds for use in clinical tissue engineering applications and as a research tool (Andrian et al., 2004; Murata et al., 2008). Murata et al. (2008) developed a tissue model by culturing gingival fibroblasts in a collagen-containing matrix, which was then grafted over teeth that were subject to gingival recession; after the grafting surgery, significantly increased gingival tissue attachment was observed. Andrian et al. (2004) developed a tissue model by culturing primary human palatal fibroblasts in a type III collagen matrix, then seeding primary human palatal epithelial cells on top of the fibroblast layer. Both MMP-2 and MMP-9 were secreted from this engineered human oral mucosa (EHOM) constitutively; however, no consistent changes in MMP levels were detected when EHOMs were infected with P. gingivalis (Andrian et al., 2007). However, it is not yet clear whether cells within the oral mucosa (e.g. fibroblasts) can be stimulated to degrade collagen or other ECM components within the oral mucosal tissue in which they reside.

Studies of experimental periodontitis have been used to assess periodontal tissue destruction in vivo (Hasturk et al., 2006; Branch-Mays et al., 2008; Cantley et al., 2009; Cantley et al., 2011). Most of these studies have induced periodontitis in rodents (mice or rats), but rabbits and rhesus monkeys have also been used (Bjornsson et al., 2004; Hasturk et al., 2006; Branch-Mays et al., 2008; Ohnishi et al., 2010). In animal models periodontitis can be induced by placing a ligature around selected teeth; alternatively, periodontitis is induced by oral gavage with bacteria (P. gingivalis or A.
Actinomycetemcomitans (Bjornsson et al., 2004; Cantley et al., 2009; Trombone et al., 2009). The most common measurements of disease in experimental periodontitis are alveolar bone loss, markers relevant to osteoclastogenesis (RANKL, tartrate-resistant acid phosphatase) and histological analyses of inflammatory infiltrates (Serhan et al., 2003; Hasturk et al., 2006; Trombone et al., 2009). Additionally, in a study of experimental periodontitis in rhesus monkeys, periodontitis was characterised by clinical indices (gingival index, BOP, CAL, PD) similar to the human disease (Branch-Mays et al., 2008). Increased gingival MMP levels are detected after the induction of experimental periodontitis (Achong et al., 2003; Vardar-Sengul et al., 2008); whether the degradation of gingival tissue ECM is similarly increased was not investigated.

Leptin and IL-1 synergistically increase the expression of the collagenases MMP-1 and MMP-13 in chondrocytes (Hui et al., 2012). In this study, Hui et al. (2012) also utilised a fairly well established bovine nasal cartilage explant model to show that leptin+IL-1 synergistically increase cartilage collagen degradation after a 14 day stimulation period. Collagen degradation was assessed by measuring the proportion of the amino acid hydroxyproline in the cartilage supernatants. MMP activity was also assessed in these cartilage supernatants by gelatin zymography (to assess gelatinase activity) and by using a tritiated collagen substrate (to assess collagenase activity). As chondrocytes are the only cell type found within cartilage, Hui et al. (2012) concluded that leptin+IL-1 function to promote cartilage degradation via increasing the production (and activity) of MMPs by chondrocytes. No studies have investigated the functional ability of leptin (+ IL-1) to regulate periodontal tissue remodelling.

Previously, it was shown that leptin and IL-1 synergistically upregulate the production of MMPs by hGFs (Chapters 4 and 6). Additionally, it was shown that leptin and IL-1 had no similar ability to alter TIMP gene expression by hGFs, yet significantly reduced the levels of several collagen mRNAs. This suggested that leptin and IL-1 synergistically promote an ECM-degrading gingival fibroblast phenotype. However the functional significance of this phenotype in gingival tissue is yet to be explored. Several experimental approaches have been used to assess MMP production and ECM degradation in the periodontal tissues; however no studies have attempted to investigate whether leptin can regulate gingival tissue integrity. The aim of the results presented in this chapter was to investigate the role of leptin on tissue integrity by developing a gingival tissue explant model system.
7.2 Development of a gingival tissue explant model

A tissue explant-based approach was developed to investigate ECM degradation under inflammatory conditions (Section 2.2.9). The development process for this model consisted of:

1. Microscopic and histological analysis of gingival tissue
2. An assessment of gingival connective tissue (CT) viability and morphology after a range of *ex vivo* culture periods
3. Determining whether ECM degradation in the gingival CT explant could be measured after stimulation with the potent pro-inflammatory stimuli IL-1+OSM.

7.2.1 Characterisation of gingival tissue

Sections of human gingival tissue were stained with H+E for histological analysis (Figure 7.1). A thick epithelial layer was observed which was keratinised at the surface, and the epithelial basal cell layer could be seen at the basement membrane. Rete pegs were evident, as were numerous fibroblast cell nuclei in the connective tissue layer. The connective tissue subjacent to the epithelium appeared to have a denser collagen ECM compared to the connective tissue papillae (between the rete pegs).

Interestingly, light microscopic analysis of gingival CT identified areas rich in transparent, spherical cells characteristic of adipocytes (Figure 7.2A). Subsequently, similar monovacuolar cells, or potential adipocytes, were identified during histological analysis of gingival CT (Figure 7.2B). To provide further evidence that these transparent, spherical cells were adipocytes, gingival CT pieces were stained with a dye for triglycerides (Figure 7.3) (Section 2.2.8). The strongest triglyceride staining was observed within the monovacuolar cells of the gingival CT, which is in support of these cells being gingival tissue resident adipocytes.

These microscopic analyses confirmed that gingival tissue is complex; differences observed in ECM degradation may be affected by the composition of each gingival tissue piece. Therefore, dissection of gingival tissue into pieces for use in the explant model was performed such that tissue pieces from neighbouring areas were distributed across treatment conditions.

7.2.2 Morphology and viability of gingival CT explants

The gross morphology and pH of gingival CT explant cultures *ex vivo* were studied to determine whether they varied over time. Phenol red in the cell culture medium gives a
visual indication of the pH of the medium the tissue is suspended in. Over time, due to waste metabolites accumulating, tissue culture medium becomes more acidic and this can affect the viability of the tissue. In the one donor assessed, no changes in the gross morphology of the multiple gingival CT pieces cultured in SFM were observed by light microscopy over an 11 day culture period (daily observation). The medium was changed every 3-4 days and over this duration of incubation no medium acidification was observed. This practise was taken forward for use in subsequent stimulation experiments; all supernatants (e.g. all treatment conditions) were changed at the same time.

LDH activity was assessed in supernatants collected from gingival CT pieces incubated in SFM for 3, 7, 10, 14, 17 and 21 days as a measure of tissue viability. No LDH activity was quantified at any of these time points; absorbance values were below the lower limit of quantification. This suggests that the viability of these gingival CT pieces was maintained over the culture period.

Over the first few days of *ex vivo* culture some cells moved out of the gingival CT explant into the culture supernatant. Light microscopic analysis identified that these cells consisted mainly of red blood cells, and other small, non-adherent spherical cells (probably white blood cells). However, once the culture medium was changed after 3 days few other cells were identified in the culture supernatant. Unlike the protocol for fibroblast isolation (Section 2.2.1), adherence to cell culture plastic ware was not promoted for gingival CT pieces during *ex vivo* culture to reduce the development of fibroblast outgrowths. This was done by dispensing a larger volume of culture medium such that the tissue pieces were suspended.

### 7.2.3 Degradation of gingival CT ECM after stimulation with IL-1+OSM

Gingival CT pieces from one donor were prepared for *ex vivo* stimulation (Section 2.2.9). These tissue pieces were stimulated with 0.05 ng/ml IL-1 and 5 ng/ml OSM for 21 days. Unstimulated gingival CT pieces were used as a negative control. Supernatants were removed, stored for analysis and replaced with fresh SFM or IL-1+OSM as required. At the end of the stimulation period the gingival tissue pieces were enzymatically digested. GAG and hydroxyproline release from the gingival CT pieces were assessed to determine whether IL-1+OSM (which enhance MMP production by hGFs (Section 4.2)) could enhance gingival ECM degradation. MMP and LDH activities were also assessed in the culture supernatants.
IL-1+OSM had no significant effect on GAG release from gingival CT compared to the unstimulated control (Figure 7.4). By day 7 unstimulated and IL-1+OSM-stimulated gingival CT had released 42 % and 46 % GAG respectively; by day 21 almost all GAG had been released (unstimulated 94 %, IL-1+OSM 96 %).

Cumulative hydroxyproline release was detected in all of the IL-1+OSM-stimulated gingival CT pieces (mean ±SD day 7: 22 ±5 %, day 14: 45 ±6 %, day 21: 60 ±12 %) (Figure 7.5). In contrast, lower cumulative hydroxyproline release was detected in two of the three unstimulated pieces of gingival CT (mean±SD of 2 gingival tissue pieces, day 7: 2.0 ±0.1 %, day 14: 4.5 ±0.4 %, day 21: 6.4 ±0.2 %). However, in one of the unstimulated pieces of gingival CT, levels of hydroxyproline release similar to gingival CT pieces stimulated with IL-1+OSM were detected (day 7: 21 %, day 14: 40 %, day 21: 50 %).

Interestingly, the pieces of gingival CT that had a cumulative hydroxyproline release of > 10 % by day 21 contained a lower amount of total hydroxyproline than the other pieces of gingival CT (Table 7.1). This suggested that the total hydroxyproline content of a piece of gingival CT may affect the degree of hydroxyproline release; in subsequent stimulations gingival CT pieces with visibly different sizes (and therefore presumably different amounts of collagen) were distributed across treatment conditions. In an effort to generate pieces of gingival CT of an identical size a biopsy punch was trialled; however, these punches are designed for acquiring a piece of tissue embedded in a larger structure, and therefore were not as effective in dissecting gingival tissue as compared to a scalpel and forceps.

MMP activity in gingival CT supernatants was analysed in two ways (Figure 7.6). No significant differences in MMP activity (in RFU) were detected between IL-1+OSM-stimulated gingival CT pieces compared to the unstimulated control (Figure 7.6A). However, MMP activity tended to be higher in gingival CT treated with IL-1+OSM compared to the unstimulated controls at all time points studied. Due to the different amounts of hydroxyproline between the gingival CT pieces, MMP activity was also expressed relative to hydroxyproline for each sample (Figure 7.6B). MMP activity expressed in this manner resulted in a similar pattern of results to those generated for hydroxyproline release (Figure 7.5 and Table 7.1); e.g. samples with less total hydroxyproline had increased MMP activity. As collagen is the predominant ECM component of gingival CT (Bartold and Narayanan, 2006), MMP activity was presented
relative to total hydroxyproline levels in an effort to control for differences in tissue piece sizes in subsequent stimulations.

No LDH activity was detected in unstimulated or IL-1+OSM stimulated gingival CT supernatants at any time points as absorbance values were below the lower limit of quantification.

7.3 Regulation of collagen degradation in gingival CT by leptin and IL-1α
Gingival CT pieces were prepared for ex vivo stimulation (Section 2.2.9). Gingival CT pieces were either unstimulated or stimulated with 10 μg/ml leptin and 0.05 ng/ml IL-1α for 21 days. Supernatants were removed, stored for analysis and replaced with fresh SFM or leptin+IL-1α as required. At the end of the stimulation period the gingival CT pieces were enzymatically digested. Hydroxyproline release from the gingival CT pieces was assessed to determine whether leptin+IL-1α enhances gingival ECM degradation. MMP activity and LDH activity were also assessed in the culture supernatants.

The patterns of hydroxyproline release varied between the donors tested (Figure 7.7). In donor 8, significantly more hydroxyproline was released from leptin+IL-1α-stimulated gingival CT compared to the unstimulated control after a 21 day culture period. No similar significant difference was observed in this donor at earlier time points (day 7 and day 14), although leptin+IL-1α-stimulated gingival CT tended to have higher hydroxyproline release compared to the unstimulated control. In the other two donors tested no significant difference between leptin+IL-1α-stimulated tissue and the unstimulated control was observed at any time point assessed. Of note, over 40% hydroxyproline release was detected for both leptin+IL-1 and unstimulated gingival CT pieces at day 7 in donor 9. Coincident with this was a visible and extensive degradation of gingival CT and release of cells into the supernatant in this donor.

Gingival CT pieces from donor 10 were also stimulated with leptin (10 μg/ml) or IL-1 (0.05 ng/ml) alone to identify whether these stimuli had any effect on gingival CT collagen degradation in isolation (Figure 7.8). Leptin and IL-1 had no significant effect on hydroxyproline release at any time points tested (days 7, 14 and 21) as compared to the unstimulated control and leptin+IL-1-stimulated gingival CT pieces.

MMP activity in gingival CT supernatants varied between the two donors tested (Figure 7.9). No significant differences in MMP activity (corrected for tissue hydroxyproline
content) between leptin+IL-1-stimulated gingival CT and the unstimulated control were observed at any time point (days 7, 14 and 21) in both donors. MMP activity tended to be higher in leptin+IL-1-stimulated gingival CT from donor 9 at the day 7 and day 14 time points in comparison to the unstimulated control; however no similar trend was evident at the 21 day time point.

MMP activity was subsequently assessed in gingival CT after stimulation with leptin+IL-1 (leptin 10 μg/ml, IL-1α 0.05 ng/ml) in the absence or presence of the MMP inhibitor GM6001 (10 μM) to confirm that active MMP was present in gingival CT supernatants (Figure 7.10). Significantly lower levels of MMP activity (RFU) were detected in gingival CT pieces that were incubated with GM6001 in both the absence and presence of leptin+IL-1 at the day 3 time point assessed (unstimulated p<0.001, leptin+IL-1 stimulation p<0.01). No visible differences in GM6001-treated gingival CT was observed in comparison to un-treated samples.

LDH activity was assessed in gingival CT supernatants derived from two donors (donors 8 and 9). No LDH activity was quantified in unstimulated or leptin+IL-1 stimulated gingival CT supernatants at any time points as absorbance values were below the lower limit of quantification.

7.4 Discussion

Several experimental approaches have been developed to investigate collagen degradation in periodontal tissues, and by periodontal cells (Cox et al., 2006; Hasturk et al., 2006; Cantley et al., 2009; Zhang et al., 2010). Fibroblasts are the predominant cell in gingival connective tissue and are implicated in regulating gingival ECM integrity (Bartold and Narayan, 2006). It was previously shown that leptin and IL-1 synergistically promote an ECM-degrading gingival fibroblast phenotype (Chapter 4); however the functional relevance of this finding was unclear. However, leptin and IL-1 synergistically promote bovine cartilage collagen degradation (Hui et al., 2012), and it was hypothesised that leptin and IL-1 may have a similar function on collagen in gingival tissue. The results presented in this chapter showed that leptin and IL-1 had no significant ability to upregulate collagen degradation and MMP activity in gingival connective tissue explants. However, both collagen and GAG degradation was observed in gingival connective tissue explants, and MMP activity was detected. This suggests that further optimisation of these gingival connective tissue explants may be justified in an effort to model gingival ECM integrity during the presence of leptin and IL-1.
The size of the gingival connective tissue pieces used in this study was similar to those used previously to assess collagen degradation in human cartilage (Morgan et al., 2006). However, because cartilage is avascular, chondrocytes are required to survive in hypoxic and somewhat nutrient-deficient conditions, and rely on the diffusion of nutrients and stimuli through the tissue (Poole, 1997). In contrast, gingival tissue is vascular (Bartold and Narayanan, 2006), and hypoxic or nutrient-deficient conditions (e.g. due to disruptions in the gingival vasculature) may affect gingival cell responses and eventually result in cell and tissue death (Deschaumes et al., 2007; Kim et al., 2010a). This suggests that cartilage may be more amenable to explant culture where nutrients diffuse into the tissue than gingival connective tissue, at least in the absence of some kind of perfusion system. Interestingly, no indications of tissue damage (as assessed by LDH release) were identified for gingival tissue pieces over the period of incubation assessed in this study, although whether the pieces of gingival tissue were large enough to generate a quantitative LDH signal was undetermined. However, it is well established in tumour biology that a lack of nutrients and oxygen to cells in the centre of a tumour can promote cell death, while the peripheral cells remain viable and functional (Lowe and Lin, 2000). Perhaps cells in the centre of the gingival connective tissue explant may have released LDH during the process of cell death but no LDH was released into the culture supernatants. Similarly, due to a lack of perfusion it is possible that only the more external cells in the gingival connective tissue pieces were responding to leptin and IL-1; whether this was occurring and whether it would have had any effect on leptin+IL-1 stimulated collagen degradation is unclear. In a previous study aiming to assess the ability of IL-1 to promote collagen degradation by gingival fibroblasts seeded on a collagen matrix, higher concentrations of IL-1 (1-20 ng/ml) were used in comparison to the concentration used in this study (0.05 ng/ml) (Cox et al., 2006). Therefore, one possible alteration to the experimental design used in this study would be to use higher concentrations of leptin and IL-1.

Not only may the size of the gingival connective tissue pieces used in this study have affected tissue viability, but it may also have affected the reproducibility of the amount of collagen degradation observed between identically-treated gingival tissue pieces. Ideally, to correct for the influence of gingival tissue size on MMP activity in culture supernatants these measurements would have been corrected to total protein or tissue mass as has been used previously (Rosengren et al., 2003; Fowler et al., 2014). However, in this study, due to the papain digestion required for hydroxyproline
analysis, and the small size of tissue pieces used, neither the assessment of total protein or tissue mass were feasible. Collagen is the predominant ECM protein found in gingival connective tissue (Bartold and Narayanan, 2006), and hydroxyproline is one of the major amino acids that makes up fibrillar collagens (Gorres and Raines, 2010). Both hydroxyproline and total protein levels have been assessed in human gingiva previously, and there is some correlation between the two measurements (Akalin et al., 1993). Therefore, MMP activity was expressed in proportion to tissue hydroxyproline content in an effort to control for differences in tissue size. One potential problem with using hydroxyproline content to correct for tissue size is that collagen content of the gingiva varies, as observed by histologic analysis in this study and previously (Nanci, 2013). Therefore, the different amounts of hydroxyproline detected between gingival tissue pieces in this study may have represented the densities of collagen matrix and not tissue size. Nonetheless, in this study using GM6001 to inhibit MMP activity significant changes in MMP activity were observed without any correction for gingival tissue hydroxyproline content. This suggests that if leptin and IL-1 had a large differential effect on MMP activity as compared to unstimulated cells, that this would have been detected independent of tissue size or hydroxyproline content.

The H+E section of a piece of gingival tissue acquired in this study demonstrated the characteristic histological features of this complex tissue (Nanci, 2013). However, the nature of the processing used to generate multiple pieces of gingival tissue from the same donor may have affected the utility of this explant culture model by generating tissue pieces with an unrepresentative cell population. Fibroblasts are the predominant cell type in gingival connective tissue but other cell types are present (endothelial cells, resident leukocytes, neurones) (Bartold and Narayanan, 2006). Additionally, the original pieces of tissue acquired contained the gingival epithelium, which was subsequently removed using a scalpel. The basement membrane between the gingival epithelium and connective tissue is not flat, as evidence by the rete pegs and connective tissue processes identified in the gingiva by histology. Therefore, it was likely that some epithelial cells may have been present in the gingival tissue pieces used for explant culture. The enzyme dispase can detach the epithelium from connective tissue as dispase degrades type IV collagen which is found in basement membranes (Normand and Karasek, 1995); this approach was not optimised in this study. Fibroblasts bi-directionally interact with endothelial cells, leukocytes and epithelial cells (Yucel-Lindberg et al., 2001; Velazquez et al., 2002; Sorrell et al., 2008; Tandara and Mustoe,
suggesting that interactions between different cell types in gingival tissue may have altered fibroblast responsiveness. Endothelial cells, leukocytes and epithelial cells all also respond to leptin and IL-1 individually (no studies have investigated IL-1 and leptin in combination on these cell types) (Cozzolino et al., 1990; Eskan et al., 2008; Sukhotnik et al., 2009; Fernandez-Riejos et al., 2010; Saxena et al., 2013), and these cells are all implicated in regulating ECM integrity (Park et al., 2001; Delima and Van Dyke, 2003; Andrian et al., 2007). Functionally different fibroblast populations have also been identified within the periodontium (Jonsson et al., 2011), and within the gingiva (Irwin et al., 1994). Perhaps distinct fibroblast populations predominated in different gingival connective tissue pieces, and had differing abilities to regulate ECM remodelling. Together, this suggests that the measurement of leptin+IL-1 stimulated ECM degradation using gingival connective tissue explants as assessed in this study, would have been a complex measure of the collective responses of all of these cell types and subtypes.

Interestingly, ECM degradation has been observed in unstimulated human articular cartilage previously (Morgan et al., 2006), and was also observed in unstimulated gingival tissue in this study. Morgan et al. (2006) suggested that ECM degradation in unstimulated articular cartilage may have been because the cartilage used was donated by individuals who were about to undergo joint replacement surgery as a treatment for arthritis, and therefore these tissue pieces already had some integral active proteinase activity. This could also explain the ability of IL-1 to stimulate collagen degradation by hGFs cultured on a collagen matrix because the fibroblasts used were isolated from individuals with chronic periodontitis (Cox et al., 2006). As fibroblasts isolated from inflammatory sites have been shown to maintain an inflammatory phenotype in vitro (McGettrick et al., 2009), it is possible that the fibroblasts used by Cox et al. (2006) had an intrinsic matrix-degrading phenotype in contrast to the fibroblasts within the essentially healthy gingival tissue used in this study. It is unlikely that this is why ECM degradation occurred in unstimulated gingival tissue as the donors were essentially periodontally healthy. However, gingival tissue from one of the donors both disintegrated visually and had a correspondingly large release of collagen at the earliest time point assessed, suggesting that the gingival tissue acquired from this donor was more susceptible to ECM degradation than other donors. In agreement with donor dependent susceptibilities to gingival tissue degradation, are the clinical studies showing that certain individuals are more likely to develop periodontitis (Taylor et al., 2004;
Kornman, 2008; Preshaw et al., 2012), and the in vitro studies showing that hGFs from different donors respond variably (Tipton et al., 1991; Sukkar et al., 2007; Uehara and Takada, 2007). Although the scalpel has been shown to induce minimal tissue damage (Liboon et al., 1997), scalpel incisions still promote inflammation and wounding responses that take days to resolve (Homayounfar et al., 2012; Nanduri et al., 2013). Therefore, the tissue processing performed prior to gingival tissue culture may have promoted inflammatory responses in all gingival tissue pieces which masked any effect of leptin and IL-1 on gingival ECM degradation.

No previous studies have used gingival connective tissue explants to study ECM integrity during inflammatory conditions; the most similar model is probably the EHOM utilised by Andrian et al. (2007). However, the EHOM lacks several mucosal cell types and a physiological ECM, therefore the responses generated may not translate to in vivo oral mucosal tissue function. Similarly, studies using gingival fibroblasts cultured on a collagen matrix are likely to be even less representative of the gingival connective tissue in vivo for the same reasons (Cox et al., 2006; Zhang et al., 2010; Zhang et al., 2011). Indeed, fibroblasts in two-dimensional cell culture studies have an altered morphology to fibroblasts in three-dimensional culture and in vivo (Cukierman et al., 2001; Beningo et al., 2004). Gingival fibroblast responses also differ depending on whether the cells are cultured on plastic or on a collagen matrix (Smith et al., 2009). Therefore, the translatability of simple in vitro studies to more complex environments (such as in vivo) is questionable and any functional outcomes have to be interpreted with caution.

On the other hand, in vivo experimental periodontitis models have been used to understand the functional outcomes of inflammation in the periodontium. There are several potential difficulties in studying the ability of leptin to directly regulate gingival fibroblast-mediated ECM degradation in vivo. Firstly, while leptin or leptin receptor-deficient animals have been extensively studied (Myers et al., 2008), it is clear that these animals have wide-spread systemic alterations (Coleman, 2010; Soboku et al., 2014). Therefore, it would be difficult to assess whether any ability of gingival fibroblasts to regulate ECM degradation in these animals was due to a direct effect of leptin on gingival fibroblasts or an indirect effect due to deficient systemic leptin signalling. Usually, to investigate the function of leptin on a specific cell type in vivo, genetic manipulation studies are performed to selectively knockout leptin receptor expression; a process that has been used to success in studies of neurons and adipocytes.
(Cohen et al., 2001; Huan et al., 2003). However, this cell-type specific genetic manipulation requires a cell-type specific marker. As no unique but consistent fibroblast marker has been identified (Naylor et al., 2012), this approach is not yet feasible to study fibroblast functions in vivo. In the past, it has also been difficult to study MMP activity in vivo. However, recent studies have used a fluorescent cleavable MMP substrate similar to the one used in this study to monitor MMP activity, and how this relates to tissue degradation, over the course of experimental animal studies (Sheth et al., 2012; Thorek et al., 2013). Given the hypothesised importance of MMPs in the pathogenesis of periodontitis, it would be interesting to test whether this approach could be used to measure MMP activity in models of experimental periodontitis.

Finally, recent studies have shown that studies of inflammatory responses in animal models do not translate well to human inflammatory responses (Schroder et al., 2012; Seok et al., 2013), and similarly that inflammatory responses vary between animal strains (Uhlig and Powrie, 2009; Schroder et al., 2012). Furthermore, the effect that leptin deficiency has on growth in humans and mice is also different between species (Kelesidis et al., 2010). Therefore, while animal models may help to model systemic and temporal properties of health and disease in humans, these responses do not necessarily directly translate between species. Consequently, ex vivo studies modelling periodontitis in human tissue may be more functionally relevant to human periodontitis than studies of experimental periodontitis in animals.

If gingival connective tissue explants are to be used in future studies it will be important to attempt to generate reproducible pieces of gingival tissue (in terms of size and content), to alter the size or shape of explant pieces to ensure that all cells are exposed to nutrients and stimuli, and to minimise the damage generated during tissue processing. One possible approach is the use of precision-cut slicing; this technology uses a vibratome to generate viable 200 μm slices of tissues (de Graaf et al., 2010). This approach appears to show some promise regarding studies of liver function ex vivo (de Graaf et al., 2010), and a pilot experiment in this study found that gingival tissue could be similarly processed, and maintained the characteristic gingival structure as determined by histological analysis (Figure 7.2B). Future development of a gingival tissue explant model using precision-cut slicing is warranted and may provide a useful bridge between descriptive in vitro studies of human gingival cells and functional experimental animal studies of gingival and/or periodontal tissues in vivo.
Adipocytes have been reported (albeit rarely) in gingival tissues previously (Nanci, 2013), in agreement with the findings in this study. The adipocytes identified in the current study have the same unique morphological structure and stain strongly for triglycerides as identified in previous studies (Prattes et al., 2000; He et al., 2010). This supports the hypothesis that these cells are indeed adipocytes. Adipocytes are one of the main cell types that produce leptin (Zhang et al., 1994), and therefore this finding suggests that in some individuals there is a local source of leptin in the gingiva. Future studies to confirm whether these putative adipocytes do produce leptin are warranted, and may help to support a role for leptin in regulating gingival fibroblast function.

In conclusion, using gingival connective tissue explants no evidence was found to support the hypothesis that leptin and IL-1 increase gingival ECM degradation. However, while ECM components and MMP activity were measured in gingival connective tissue explants in this study, it is likely that in the current format gingival connective tissue explants are not a suitable model to investigate gingival tissue integrity under inflammatory conditions. A gap between in vitro and in vivo studies does exist regarding fibroblast function in the gingiva and may be bridged in the future by ex vivo models. Therefore, the results presented in this chapter highlight some of the issues that may need to be taken into account in future studies.
Figure 7.1: Histology of a piece of gingival tissue acquired in this study.

Gingival tissue acquired after canine exposure surgery was prepared for histological analysis. Sections of gingival tissue were stained with H+E. A representative image is shown which displays the keratinised gingival epithelium and connective tissue. Numerous fibroblast nuclei are present in the gingival connective tissue. The pink staining in the gingival connective tissue is indicative of a dense collagen extracellular matrix. Magnification 100x.
Figure 7.2: Potential adipocytes in gingival connective tissue.

Gingival tissue was acquired after canine exposure surgery. [A] Gingival tissue was dissected into small pieces (2 mm³), these tissue pieces were suspended in PBS and imaged by light microscopy. A representative image is shown featuring a group of spherical cells that may be adipocytes. Magnification 20 x. [B] Gingival tissue was dissected by precision cut slicing and prepared for histological analysis. Sections of gingival tissue were stained with H+E. A representative image is shown featuring a group of monovacuolar cells that may be adipocytes. Magnification 100 x.
Gingival tissue was acquired after canine exposure surgery. Gingival tissue was dissected into small pieces (2 mm$^3$). These tissue pieces were stained with the triglyceride dye AdipoRed for 10 minutes before imaging by light microscopy. A representative image is shown in which the putative adipocytes stain strongly for triglycerides. Magnification 100 x.
Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces (approximately 2 mm$^3$). These tissue pieces were either cultured in SFM or stimulated with 0.05 ng/ml IL-1α and 5 ng/ml OSM for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. The amounts of GAG in the culture supernatants were determined and were compared to the total amount of GAG in the gingival tissue. Data (mean+SD) are presented as cumulative % GAG release from gingival tissue (n=3) at three time points (day 7, day 14, day 21). Statistics: T tests for independent samples.

**Figure 7.4: Cumulative GAG release from gingival tissue explants over time.**
Figure 7.5: Cumulative hydroxyproline release from IL-1+OSM stimulated gingival tissue explants over time.

Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces (approximately 2 mm³). These tissue pieces were either cultured in SFM (unstimulated ●) or stimulated with 0.05 ng/ml IL-1α and 5 ng/ml OSM (I/O ▲) for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. The amounts of hydroxyproline in the culture supernatants were determined and were compared to the total amount of hydroxyproline in the comparative gingival tissue. Data are presented as cumulative % hydroxyproline release from each gingival tissue piece at three time points (day 7, day 14, and day 21). OHP – hydroxyproline.
Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces (approximately 2 mm$^3$). These tissue pieces were either cultured in SFM or stimulated with 0.05 ng/ml IL-1$\alpha$ and 5 ng/ml OSM for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. Hydroxyproline concentrations were assessed in culture supernatants and tissue digests. The total amount of hydroxyproline (μg) in each gingival tissue sample was calculated by adding the amount of hydroxyproline in each culture supernatant and the amount remaining in the tissue at day 21. MMP activity in the culture supernatants was determined (Section 2.14). This table displays the total hydroxyproline for each gingival tissue piece, whether the tissue was stimulated with IL-1+OSM, % hydroxyproline release at day 21 and MMP activity (RFU/μg hydroxyproline) at day 21.

<table>
<thead>
<tr>
<th>IL-1 + OSM</th>
<th>Total hydroxyproline (μg)</th>
<th>hydroxyproline release at day 21 (%)</th>
<th>MMP activity (RFU/μg hydroxyproline)</th>
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<tr>
<td>-</td>
<td>91.64</td>
<td>6.2</td>
<td>28.8</td>
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<td>48</td>
<td>242</td>
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</table>
Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces. These tissue pieces were either cultured in SFM or stimulated with 0.05 ng/ml IL-1α and 5 ng/ml OSM for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. MMP activity in the culture supernatants was determined (Section 2.14)). [A] Data (RFU) are presented as median+IQR (n=3) at three time points (days 7, 14 and 21). Statistics: Mann-Whitney U test. [B] Data (MMP activity in RFU) are presented per μg hydroxyproline in an effort to take account of the different tissue piece sizes. Unstimulated - ●, IL-1α+OSM (I/O) - ▲. The results for each individual piece of tissue are displayed at three time points (days 7, 14 and 21). OHP – hydroxyproline.
Figure 7.7: Cumulative hydroxyproline release from leptin+IL-1 stimulated gingival tissue explants over time.

Gingival tissue (from donors 8-10 as indicated) was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces. These tissue pieces were either cultured in SFM or stimulated with 10 μg/ml leptin and 0.05 ng/ml IL-1α for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. The amounts of hydroxyproline in the culture supernatants were determined and were compared to the total amount of hydroxyproline in the comparative gingival tissue. Data (mean+SD, donor 8 and 10 n=5/6, donor 9 n=5) are presented as cumulative % hydroxyproline release from gingival tissue at three time points (day 7, day 14, day 21). Statistics: T tests for independent samples: *=p<0.05 as indicated. OHP – hydroxyproline.
Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces. These tissue pieces were either cultured in SFM or stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml) or leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml) for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. The amounts of hydroxyproline in the culture supernatants were determined and were compared to the total amount of hydroxyproline in the comparative gingival tissue. Data (mean+SD, n=5) are presented as cumulative % hydroxyproline release from gingival tissue at [A] day 7, [B] day 14 and [C] day 21. Statistics: T tests for independent samples. OHP – hydroxyproline.
Figure 7.9: MMP activity in gingival tissue culture supernatants after stimulation with leptin and IL-1.

Gingival tissue (from donor 8 and donor 9 as indicated) was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces. These tissue pieces were either cultured in SFM or stimulated with 10 μg/ml leptin and 0.05 ng/ml IL-1α for 21 days. Culture supernatants were removed and replaced with fresh treatments as required. MMP activity in the culture supernatants was determined measuring the cleavage of a fluorescent MMP substrate. Data (mean±SD, donor 8 n=5/6, donor 9 n=5) are presented as MMP activity (RFU) per μg hydroxyproline at three time points (days 7, 14 and 21). OHP – hydroxyproline.
Figure 7.10: MMP activity in gingival tissue culture supernatants after stimulation with leptin+IL-1 and treatment with GM6001.

Gingival tissue from one donor was acquired after canine exposure surgery. Gingival tissue was washed, the epithelium removed by scalpel, and the resultant connective tissue dissected into small pieces. These tissue pieces were either cultured in SFM or stimulated with 10 µg/ml leptin and 0.05 ng/ml IL-1α for 3 days in the absence or presence of the MMP inhibitor GM6001 (10 µM). MMP activity in the culture supernatant was determined measuring the cleavage of a fluorescent MMP substrate. Data (MMP activity in RFU) are presented as mean+SD (n=3). Statistics: T tests for independent samples (Bonferroni-corrected): **=p<0.01, ***=p<0.001 as indicated.
Chapter 8. Discussion

The prevalences of obesity and T2DM are increasing at an alarming rate globally, presumably due to changes in lifestyle and diet (Malik et al., 2013). Obese and T2DM populations are at risk of developing co-morbidities, including conditions associated with destructive chronic inflammation such as periodontitis (Hotamisligil, 2006; Pischon et al., 2007; Preshaw et al., 2012). Circulating levels of the hormone leptin are often elevated in individuals who are obese or have T2DM (Coppari and Bjorbaek, 2012). Aside from functioning as a hormone, there is strong evidence that leptin can promote immune and inflammatory responses (Matarese et al., 2005).

The periodontium has a unique anatomy, and its exterior is constantly exposed to microbes (Bartold and Narayanan, 2006). It is for these reasons that the chronic inflammatory condition gingivitis is so common. The prevalence of periodontitis is increasing, and current treatment options are rarely fully successful (Newman et al., 2011; White et al., 2011). Fibroblasts are implicated in regulating the switch between acute and chronic inflammation by promoting leukocyte survival and retention in inflamed tissues (Buckley et al., 2001; Buckley, 2011). Fibroblasts within the periodontium are immunocompetent cells implicated in the pathogenesis of periodontitis, due to their ability to regulate ECM remodelling and the production of inflammatory mediators (Bartold and Narayanan, 2006) (Section 1.11). Instead of regulating the switch between acute and chronic inflammation, could it be possible that gingival fibroblasts regulate the switch between the resolvable, self-limiting inflammatory responses of gingivitis to the irreversible, unstable and destructive inflammatory responses of periodontitis? In this study, it was hypothesised that leptin is a molecular link between obesity, T2DM and periodontitis by promoting inflammatory responses in gingival fibroblasts.

This study has shown that leptin (alone and synergistically with IL-1) enhanced the expression of a range of MMPs, chemokines and other immune mediators by gingival fibroblasts. Additionally, and for the first time, it was shown that leptin synergised with a TLR2 agonist to increase the production of MMPs by gingival fibroblasts. Leptin-stimulated gingival fibroblasts activated multiple intracellular signalling pathways, but it was the MAPK pathway, and ERK in particular, which were required for leptin (+IL-1 or pam2CSK4) mediated MMP-1 expression. A gingival tissue explant model was developed in an effort to assess leptin+IL-1 stimulated collagen degradation; however, at present this model proved to be unsuitable for addressing this aim.
Two interesting questions arise from these findings: why do gingival fibroblasts express the leptin receptor, and what is the purpose of their response to leptin? I hypothesise that gingival fibroblasts express the leptin receptor because they need to be informed of the energy status of the organism in which they reside, and that their response to leptin regulates the structure and function of the gingival connective tissue in line with energy availability (Figure 8.1). Essentially, if energy resources are abundant (and therefore leptin levels are high) gingival fibroblasts can function effectively to maintain the structural integrity and immunocompetence of the gingival connective tissue. In contrast, if energy resources are depleted (and therefore leptin levels are low) gingival fibroblasts do not receive a signal to optimally maintain the structural integrity and immunocompetence of the gingival connective tissue. These functions which gingival fibroblasts regulate require energy which the organism needs to utilise elsewhere.

Finally, I hypothesise that the presence of leptin in the inflamed gingiva will enhance inflammatory gingival fibroblast responses, and that this will dose-dependently increase the risk of developing periodontitis.

There is much evidence that gingival fibroblasts regulate the structural integrity of the gingiva (discussed in chapters 3, 4, and 6). Similarly, it is not a new concept that gingival fibroblasts are immunocompetent cells (discussed in chapter 6); for example, class II MHC molecules are induced on the surface of gingival fibroblasts by cytokines (Mahanonda et al., 2008). As such, gingival fibroblasts, and indeed fibroblasts at other anatomical locations, are no longer thought of as inert cells whose only function is to produce and remodel ECMs (Section 1.11). Fibroblasts actively monitor their environment, and respond to changes in an integrated manner, as evidenced by this and other studies (Mahanonda et al., 2007; McGettrick et al., 2009; Mia et al., 2014). This supports the idea that leptin affects a range of responses in gingival fibroblasts. It has been difficult to pinpoint the roles of fibroblasts in health and disease due to the lack of consistency regarding the characteristics of these cells (Naylor et al., 2012); a problem possibly mediated by their functional plasticity. Nonetheless, a recent study did find that resistance to a chemotherapy drug is mediated by fibroblasts (Sun et al., 2014). In this study, human cancer-associated fibroblasts were genetically modified (stable knockdown of IL-6) ex vivo before being grafted into mice with cells of a human breast cancer line. Sun et al. (2014) determined that fibroblast-derived IL-6 mediated resistance to the chemotherapy drug tamoxifen. A more comprehensive understanding of gingival fibroblast function could help to elucidate the pathogenesis of periodontitis.
explain why existing treatments are not entirely effective, and even identify novel therapeutic targets.

Many different cell types, including gingival fibroblasts (Section 1.11.6), express the leptin receptor, and respond to leptin (Tartaglia et al., 1995; Sanchez-Margalet et al., 2003). What is striking is the variety of responses that leptin can regulate. For example, leptin regulates leukocyte proliferation (Sanchez-Margalet et al., 2003), the production of molecules involved in ECM remodelling by fibroblasts (Schram et al., 2011), the production of neuropeptides by neurones (Cohen et al., 2001), and fatty acid oxidation in muscle (Minokoshi et al., 2002). What is interesting is that these leptin-stimulated responses are among the expected responses of these cells (e.g. leptin stimulates neuropeptide production in neurones (Cohen et al., 2001), but did not in gingival fibroblasts in this study). This suggests that leptin enhances the diverse specialised responses of different cell types, and may explain the wide-ranging defects in humans with extremely low or absent leptin (Clement et al., 1998; Farooqi et al., 2002). It is already clear that leptin mediates the physiological adjustments that reduce metabolism as part of the starvation response (Kelesidis et al., 2010); however, much of this research has focussed on metabolism at the level of the whole organism. At a fundamental level, all cellular responses require and utilise energy in the form of ATP. It seems a logical progression from this that during periods of starvation, energy consumption is also reduced on a cellular level, and that this is also controlled by leptin. This could explain the diverse responses that leptin regulates, and in particular support the hypothesis that leptin acts to inform gingival fibroblasts of systemic energy levels.

As previously mentioned, gingival fibroblast responses depend on the stimuli these cells are exposed to. For example, in this study IL-1 stimulated the differential expression of a large number of genes in gingival fibroblasts compared to unstimulated cells (Section 6.3.5); additionally, leptin synergistically enhanced the expression of a number of those genes differentially expressed in gingival fibroblasts stimulated with IL-1. However, this response enhancement does not appear to be a feature unique to fibroblasts. Leptin enhances LPS-mediated TNF-α production by monocytes, but does not elicit this response in the absence of LPS (Jaedicke et al., 2013). Similarly, leptin enhances the proliferation of activated T cells, but cannot initiate T cell activation (Martin-Romero et al., 2000). This suggests that leptin has a permissive effect on cells under co-existing stimulatory conditions, and in particular supports the hypothesis that leptin enhances inflammatory responses in gingival fibroblasts.
Starvation is associated with a degree of immunodeficiency (Lord et al., 1998). Leptin promotes many processes relevant to inflammation, including the clearance of infections (Hsu et al., 2007), wound healing (Umeki et al., 2014), leukocyte infiltration (Vernooy et al., 2010), and ECM remodelling (Hui et al., 2012). Therefore, low levels of leptin are thought to be one mechanism that determines immunodeficiency during starvation, and evidence for this on a cellular level is starting to be generated (Saucillo et al., 2014). Inflammation and immune responses are complex processes involving a number of cell types, and as such require high levels of energy expenditure (Muehlenbein et al., 2010). This indicates that inflammatory responses are regulated in leptin-responsive cells to ensure that they occur at a level that can be supported by the available systemic energy stores. A comprehensive understanding of the functions of leptin on fibroblasts in the gingiva and elsewhere is important, because modulation of leptin has been utilised clinically, and remains a viable option for treating several conditions (most notably Type 1 diabetes mellitus and lipodystrophy) (Kelesidis et al., 2010) (Section 1.3). The wide-ranging cellular targets of leptin action, and the context-dependent responses, could affect the efficacy and safety of leptin as a therapeutic target.

In developed countries starvation (and therefore leptin deficiency) is rare. In contrast, the rapidly increasing obese (and T2DM) populations in developing countries indicates that there is an increasing population of hyperleptinaemic individuals (Malik et al., 2013). There is much evidence (including the results regarding gingival fibroblasts in this study) that leptin regulates cellular responses in a dose-dependent manner (Cao et al., 2007; Tong et al., 2008; Kato et al., 2011). For example, high concentrations of leptin stimulate higher production of cytokines and MMPs in mesenchymal cells than lower concentrations of leptin; this dose-dependent effect is also evident in the presence of IL-1 (Hui et al., 2012) (Chapters 3 and 4). This suggests that inflammatory mesenchymal cell responses are enhanced during hyperleptinaemia, and supports the original hypothesis in this study that leptin-stimulated inflammatory responses in gingival fibroblasts are mechanistically relevant to the increased risk of periodontitis in individuals who are obese or have T2DM.

In opposition to this, leptin resistance occurs in conditions of hyperleptinaemia (Section 1.3). Our understanding of leptin resistance is mainly based on in vivo comparisons of diet-induced obesity to healthy counterparts (Kelesidis et al., 2010; Mantzoros et al., 2011), and suggests that there is a threshold concentration of leptin above which leptin
signalling is saturated. However, differences in intracellular signalling pathway activation and biological exposure to leptin are implicated in a differential susceptibility to leptin resistance between different types of neurones (Münzberg et al., 2004; Coppari and Bjorbaek, 2012). There is evidence that leptin resistance can be somewhat corrected by dietary and therapeutic interventions (Roth et al., 2008; Kelesidis et al., 2010), suggesting that leptin resistance is not permanent. Finally, the ability for leptin to regulate energy metabolism and insulin sensitivity are differentially affected by leptin resistance (Coppari and Bjorbaek, 2012), which suggests that different functions regulated by leptin are not identically affected by leptin resistance. Together, this suggests that the threshold concentration for saturation of leptin signalling varies between cell types, anatomical niches, and functional responses. Therefore, it cannot be assumed that gingival fibroblasts are resistant to leptin under similar conditions to those which induce resistance in other cells types. Further studies are warranted to investigate leptin sensitivity and resistance in the gingiva during health and disease, in an effort to confirm whether leptin is relevant to the increased risk of periodontitis in the growing population of obese and T2DM (hyperleptinaemic) individuals.

In conclusion, the results presented in this thesis have greatly added to the current data regarding the role of leptin on fibroblasts in the gingiva. Specifically, it was shown that leptin enhances wide-ranging inflammatory responses in gingival fibroblasts, alone and synergistically in combination with inflammatory mediators. These results support the hypothesis that leptin is a molecular link between obesity, T2DM and periodontitis by synergistically promoting deleterious inflammatory responses in gingival fibroblasts, but also indicate that leptin may have a physiological role in regulating homeostatic gingival fibroblast function.

8.1 Future work
One limitation regarding the results presented in this thesis is whether they are translatable to gingival fibroblast responses in vivo. An important area of future research would be to assess the functional relevance of leptin on inflammatory responses in gingival fibroblasts. For example, the use of transwell filters in a similar approach to McGGettrick et al. (2010) could be used to assess whether leptin-stimulated gingival fibroblasts regulate leukocyte recruitment. Co-culture studies of gingival fibroblasts and leukocytes or epithelial cells, or the use of gingival fibroblast-conditioned media, could be used to determine whether leptin stimulated gingival fibroblasts affect leukocyte or epithelial cell survival, proliferation and differentiation. Further improvements to the ex
*vivo* gingival connective tissue explant system used in this study (e.g. precision cut slicing) may help to determine whether leptin-stimulated gingival fibroblasts play a role in gingival ECM degradation. Alternatively, human gingival (connective) tissue from individuals with periodontitis and healthy controls could be grafted onto severe combined immunodeficiency (SCID) mice in an effort to assess the responses of human gingival tissue to leptin without the problems encountered in *ex vivo* tissue culture (as discussed in Chapter 7). Although this would be a novel approach regarding gingival tissue, the technique of xenografting human tissue onto SCID mice has been utilised in the fields of cancer and rheumatoid arthritis to study human tissues in an *in vivo* setting (Morton and Houghton, 2007; Patel, 2010; Wythe *et al.*, 2013).

The results in this study indicated that leptin and OSM may regulate gingival fibroblast MMP production in the same manner (Chapter 4). It would be interesting to further investigate the similarity in gingival fibroblast responses stimulated by leptin and other IL-6 family cytokines to assess whether there is IL-6 family cytokine redundancy in gingival fibroblast responses. This could be assessed by genome-wide expression analysis in IL-1±IL-6/OSM stimulated gingival fibroblasts.

It would also be interesting to determine under what conditions gingival fibroblasts are resistant to leptin. To test whether gingival fibroblasts can become resistant to leptin *in vitro* gingival fibroblasts could be cultured over a longer time period with multiple exposures to leptin (and delayed stimulation with inflammatory mediators) prior to assessing gene expression and intracellular signalling responses.

Gingival fibroblasts, compared to fibroblasts from other anatomical sites, have a differential ability to remodel ECM (Mah *et al.*, 2014). Also, fibroblasts from distinct anatomical sites differ in their capacity to regulate immune and inflammatory responses (Buckley, 2011). It would be interesting to acquire and culture fibroblasts from other anatomical locations (e.g. synovium, skin, intestine) and to compare their responses to leptin with those of gingival fibroblasts.
Abundant energy reserves

↑ Leptin

Pro-inflammatory signals
(e.g. cytokines, TLR agonists)

hGF

Altered regulation of inflammatory processes
Extensive leukocyte recruitment
Deleterious ECM remodelling

Gingival connective tissue at increased risk of destructive inflammatory processes

Optimal gingival connective tissue structure and immunocompetence

Regulation of immunocompetence and inflammatory processes

Leukocyte recruitment

ECM remodelling

Sub-optimal gingival connective tissue structure and immunocompetence — starvation response

Deficient ECM remodelling

Deficient immunosurveillance

Altered regulation of immunocompetence and inflammatory processes

Depleted energy reserves

↓ Leptin
Figure 8.1: Schematic diagram illustrating the hypothetical role of leptin in regulating fibroblast function in the gingiva.

When energy reserves are abundant the circulating concentration of leptin will be high. Circulating leptin (or local sources of leptin e.g. gingival tissue adipocytes) may diffuse into the gingiva where it could bind the leptin receptor on gingival fibroblasts, thereby signalling the level of systemic energy stores. The response to leptin signalling in hGFs may affect ECM remodelling, leukocyte recruitment, and immunocompetence and inflammatory processes of both fibroblasts and other cells in the gingiva. If energy reserves are depleted, the circulating concentration of leptin will be low. In this instance lower amounts of leptin will bind to the leptin receptor on hGFs, possibly resulting in deficiencies in the structure and immunocompetence of the gingival connective tissue. This could be a peripheral feature of starvation. When gingival fibroblasts are exposed to leptin in the presence of pro-inflammatory signals, the inflammatory responses stimulated are enhanced. This could enhance the risk of destructive inflammation as observed in periodontitis. hGF – human gingival fibroblast.
A. Appendix A. The effect of chemical signalling pathway inhibitors on hGF proliferation.
hGFs (n=2) were treated for 24 h with a 3-fold dilution series of [A] STAT3 inhibitor S3I-201 (top concentration: 300 µM), [B] Akt inhibitor VIII (top concentration: 27 µM), [C] MEK inhibitor U0126 (top concentration: 15 µM), [D] MEK inhibitor PD98059 (top concentration: 100 µM), [E] p38 inhibitor SB203580 (top concentration: 30 µM), [F] JNK inhibitor SP600125 (top concentration: 10 µM), [G] IKK inhibitor VI (top concentration: 351 nM), [H] IKK inhibitor VIII (top concentration: 750 nM) and [I] IκB inhibitor Bay11-7085 (top concentration: 90 µM). hGF proliferation was assessed using a colourimetric assay (Section 2.5.1). hGF proliferation is expressed as % untreated control. A 4PL curve was fitted to the dose-response data except for U0126, SP600125 and IKK VI where no fits could be made. The dashed horizontal line displayed represents the proliferation observed for hGFs treated with a concentration of DMSO ≥ the highest concentration of DMSO in inhibitor-treated hGFs.
B. Appendix B. The effect of chemical signalling pathway inhibitors on intracellular signalling in hGFs.

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MW (kDa)

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MW (kDa)
hGFs were stimulated with IL-1α+OSM for 20 min. Prior to stimulation, hGFs were pre-treated for 30 min with DMSO or chemical pathway inhibitors: [A] 10 μM U0126, 10 μM PD98059 or 10 μM SB203580, [B] 0.5 μM or 10 μM SP600125, [C] 300 nM IKK VI, 700 nM IKK VIII or 10 μM Bay11-7085, [D] 300 nM Akt VIII, [E] 100 μM S3I-201. Lysates were prepared from hGFs and proteins (as labelled) were resolved by SDS-PAGE and immunoblotting. GAPDH was used as a loading control.
C. Appendix C. Real-time RT-PCR.

**Figure C.1: A representative amplification plot for real-time RT-PCR analysis.**

The magnitude of detected fluorescence (ΔRn) was plotted against PCR cycle number. In this plot the dark blue lines represent detection of MMP-1 while the green lines represent RNA polymerase II. A threshold (horizontal line shown in red) is fitted to cross each curve during the exponential phase of amplification. The cycle number where this threshold crosses the amplification curve (C_t) is used for relative gene expression analysis.
hGFs were stimulated with leptin (0 - 25 μg/ml) for 24 hours. hGFs were lysed in preparation for reverse transcription to generate cDNA. Real-time RT-PCR was performed to assess 18S rRNA and RNAP gene expression. [A] Data (n=4) are presented as mean C\textsubscript{t} values. 18S C\textsubscript{t} values (◊) are plotted against the left y-axis, while RNAP C\textsubscript{t} values (□) are plotted against the right y-axis. [B] Data (n=4) are presented as mean C\textsubscript{t} values for 18S and RNAP at each leptin concentration, and in combination (mean C\textsubscript{t} for all leptin concentrations). The standard deviation and % CV are also displayed. RNAP – RNA polymerase II, C\textsubscript{t} – threshold cycle, SD – standard deviation, cv – coefficient of variance.

**Figure C.2: Comparison of 18S rRNA and RNAP gene expression in hGFs.**
### D. Appendix D: The human immune panel TLDA.

#### Table D.1: List of genes on the human immune panel TLDA.

The assay ID (Life Technologies) indicates the specific primer/probe used for each gene. Reference genes are highlighted in bold.

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Figure D.1: Expression of selected genes on the immune TLDA in leptin-stimulated hGFs.

hGFs were stimulated with 0.1 or 10 μg/ml leptin for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. cDNA was loaded onto a human immune panel TLDA and gene expression was determined by real-time RT-PCR. Relative gene expression was determined by normalising to the reference gene β-actin. [A] Cell surface markers, [B] regulators of inflammation and immunity, [C] regulators of vascular function, [D] ECM components.
E. Appendix E. Human HT-12 v4 genome-wide expression beadchip.

A

Day 1
1. First strand cDNA synthesis
2. Second strand cDNA synthesis
3. cDNA purification
4. *In vitro* transcription

Day 2
5. cRNA purification and quantification
6. Set up hybridisation

Day 3
7. Wash and scan

B

Figure E.1: The direct hybridisation assay

[A] Flow diagram showing the workflow used to perform the direct hybridisation assay. This work was performed by CGS. [B] Schematic representation of the direct hybridisation assay technology. A 50 base gene-specific probe is linked to an address sequence and the bead. The probe hybridizes to the biotin-labeled cRNA generated from test samples. Figures [A] and [B] were adapted from Illumina Inc. (2014).
**Figure E.2: QC data for the HT-12 genome-wide microarrays.**

Illumina beadchip microarray technology was used to assess genome-wide expression profiles of leptin and IL-1 stimulated hGFs according to the methods described in Section 2.8.2. [A] Density plots of 8 of the microarray datasets. A calculated distribution of signal intensities for each bead (M) is plotted against the average signal intensity for each bead (A). Each plot represents one dataset (e.g. 1 treatment condition). Only 8 of 12 representative plots are shown. The majority of the data points on these graphs have a value for M near 0, which indicates that the variation in signal intensities between different beads and datasets is low. [B] A density plot showing a calculated form of the standard deviation of signal intensities across all the arrays against the ranked mean signal intensities post-normalisation. The red line represents the median SD; the horizontal nature of this line indicates that normalization was successful. [C] Box and whisker plots displaying the distribution of signal intensities for each array post-normalisation. The boxes for each array are well aligned, which supports the good quality of this experiment and the use of an appropriate normalisation technique. [D] A histogram showing the distribution of signal intensities for all arrays pre (i) and post (ii) normalisation. Each array is very well aligned which supports the good quality of this experiment. Alignment is even better after normalisation supporting the use of this normalisation technique. [E] 2-dimension principal component analysis (PCA) plots before (i) and after (ii) dataset correction. These plots are based on 29136 probes which had SD/mean>0.1. In (i) arrays group by treatment, not donor, across principal component dimension 1 (x-axis). However, arrays group by donor across principal component dimension 2 (y-axis). After the dataset was corrected (ii) the arrays grouped by treatment for both dimensions of the PCA.
Figure E.3: Genome-wide expression profiles of hGFs after stimulation with leptin and IL-1β.

Illumina beadchip microarray technology was used to assess genome-wide expression profiles of leptin and IL-1 stimulated hGFs according to the methods described in Section 2.8.2. Differentially expressed gene lists were generated for the comparisons unstimulated vs leptin [A], unstimulated vs IL-1 [B], and unstimulated vs leptin+IL-1 [C]. Both the genes and samples are shown on this heat map in relation to all other genes and samples respectively by means of hierarchical clustering analysis. The relative expression levels (log2 scale) of each gene are displayed using a colour key (red indicates lower levels of gene expression; blue indicates higher levels of gene expression).
F. Appendix F. hGF proliferation after stimulation with leptin and inflammatory mediators

[A]  

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<td>100</td>
</tr>
<tr>
<td>80</td>
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<tr>
<td>60</td>
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</table>

Leptin  
-  
+  
+  
-  

IL-1α  
-  
+  
+  
-  

FBS (10 %)  
-  
-  
-  
+  

[B]  

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Leptin  
+  
-  
+  
-  

OSM  
-  
+  
+  
-  

FBS (10 %)  
-  
-  
-  
+  

[C]  

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<td>60</td>
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</table>

Leptin  
+  
-  
+  
-  

IL-1+OSM  
-  
+  
+  
-  

FBS (10 %)  
-  
-  
-  
+  

284
**Figure F.1: hGF proliferation after stimulation with leptin, IL-1α and OSM**

hGFs were stimulated with [A] leptin (10 μg/ml), IL-1 (0.05 ng/ml) or leptin+IL-1 (leptin 10 μg/ml, IL-1 0.05 ng/ml), [B] leptin (10 μg/ml), OSM (5 ng/ml) or leptin+OSM (leptin 10 μg/ml, OSM 5 ng/ml), [C] leptin 10 μg/ml, IL-1+OSM (IL-1 0.05 ng/ml, OSM 5 ng/ml) or leptin+IL-1+OSM (leptin 10 μg/ml, IL-1 0.05 ng/ml, OSM 5 ng/ml) for 24 h. hGFs cultured in 10 % FBS-containing medium were used as a positive control. Proliferation was assessed using a tetrazolium compound-based assay. Data (mean+SD, n=9) are shown relative to the unstimulated control (% control) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected): *=p<0.05, **=p<0.01, ***=p<0.001 compared to unstimulated control. Only selected comparison are shown for clarity.
### [A]

<table>
<thead>
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### [B]

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Figure F.2: hGF proliferation after stimulation with leptin, pam2CSK4 and *E. coli* LPS

hGFs were stimulated with [A] leptin (10 μg/ml), pam2CSK4 (50 or 100 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 or 100 ng/ml), [B] leptin (10 μg/ml), *E.coli* LPS (10 or 1000 ng/ml) or leptin+*E. coli* LPS (leptin 10 μg/ml, LPS 10 or 1000 ng/ml) for 24 h. hGFs cultured in 10 % FBS-containing medium were used as a positive control. Proliferation was assessed using a tetrazolium compound-based assay. Data (mean+SD, n=9-12) are shown relative to the unstimulated control (% control) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected): *=p<0.05, **=p<0.01, ***=p<0.001 compared to unstimulated control. Only selected comparison are shown for clarity.
G. Appendix G. Time course of STAT3 and ERK phosphorylation in hGFs after stimulation with leptin.

<table>
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<tr>
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<th>0</th>
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</tr>
<tr>
<td>STAT3</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pERK</td>
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</table>

**Figure G.1: Phosphorylation status of STAT3 and ERK in hGFs after stimulation with leptin.**

hGFs were stimulated with leptin (10 μg/ml) for 0, 10, 30, 60, 120 and 240 min. Cells were lysed and prepared for SDS-PAGE. Extracts were immunoblotted with antibodies against phospho-pSTAT3 (Y705), STAT3, phospho-ERK (p42/44) (T202/Y204) and GAPDH as a loading control.
Appendix H. MMP-1 gene expression in hGFs in the presence of DMSO.

**Figure H.1: DMSO does not affect MMP-1 gene expression in stimulated hGFs.**

hGFs were either pre-treated with 0.1 % v/v DMSO or SFM for 30 min and then stimulated with leptin (10 μg/ml), IL-1α (0.05 ng/ml), leptin+IL-1α (leptin 10 μg/ml, IL-1α 0.05 ng/ml), pam2CSK4 (50 ng/ml) or leptin+pam2CSK4 (leptin 10 μg/ml, pam2CSK4 50 ng/ml) for 24 h. Cells were lysed and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 mRNA expression was determined using the 2-ddCt method by using RNAP as the reference gene. Data (fold unstimulated, no DMSO control) are shown as mean+SD (n=12) from three donors stimulated in independent experiments. Statistics: T tests for independent samples (Bonferroni-corrected).
I. Appendix I. Stimulation of hGFs with leptin and a leptin antagonist

**Figure I.1:** MMP-1 production and STAT3 tyrosine phosphorylation in hGFs after stimulation with leptin and a leptin antagonist.

hGFs were stimulated with leptin (10 µg/ml) and/or a leptin antagonist (10 µg/ml unless indicated otherwise). [A] After 24 h supernatants were collected and assessed for MMP-1 concentration by ELISA. Data are presented as mean±SD (ng/ml, n=2, one donor). [B] After 20 min lysates were prepared from hGFs and STAT3, pSTAT3Y705 and GAPDH were resolved by SDS-PAGE and immunoblotting. GAPDH was used as a loading control.
Figure J.1: hGF proliferation after stimulation with IL-1α or IL-1β

hGFS were stimulated with IL-1α (0.05 ng/ml) or IL-1β (0.05 ng/ml) for 24 h. hGFS cultured in 5% FBS-containing DMEM were used as a positive control. Proliferation was assessed using a tetrazolium compound-based assay. Data (mean±SD, n=19-20) are shown relative to the unstimulated control (% control) from two donors stimulated in independent experiments. Statistics: T tests or Mann–Whitney U test for independent samples as required (Bonferroni-corrected): **=p<0.01 compared to unstimulated control.
hGFs from three donors were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed, RNA extracted and cDNA was prepared by reverse transcription. Real-time RT-PCR was used to assess MMP-1 and RNAP gene expression. Relative MMP-1 gene expression was determined using the $2^{-\Delta\Delta Ct}$ method by using RNAP as the reference gene. Data (fold unstimulated control) on each graph are shown as mean+SD (n=2) from one donor as indicated.
Table J.1: hGF RNA concentration and quality assessment for the genome-wide expression microarray

hGFs from three donors (donors 2-4 as indicated) were stimulated with leptin (10 μg/ml), IL-1β (0.05 ng/ml) or leptin+IL-1β (leptin 10 μg/ml, IL-1β 0.05 ng/ml) for 24 h in independent experiments (6-well format). Cells were lysed and RNA extracted. RNA yield (concentration - ng/μl) and quality (260/280 and 260/230 ratios) were assessed using a NanoDrop spectrophotometer. Treatment groups: NT – unstimulated control, L – leptin, I – IL-1β, LI – leptin+IL-1β.

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K. Appendix K. ELISA standard curves

A

MMP-1 Standard curve

B

MMP-3 Standard curve
Figure K.1: Representative standard curves for the ELISAs used in this study.

Mean±SD absorbance (450 nm-550 nm/deltaOD) was plotted against standard concentrations (0 – 10000 pg/ml for MMP-1 [A], 0 – 2000 pg/ml for MMP-3 [B], 0 – 300 pg/ml IL-6 [C], 0 – 4000 pg/ml IL-24 [D]). A 4PL curve was fitted and used to determine the unknown concentration of these proteins in samples.
L. List of presentations and manuscripts

Manuscripts in preparation:

Williams, R.C., Rowan, A.D., Preshaw, P.M. and Taylor, J.J. ‘The role of gingival fibroblasts in inflammation’.

Williams, R.C., Todryk, S., Rowan, A.D., Preshaw, P.M. and Taylor, J.J. ‘Leptin synergises with IL-1, LPS and the TLR-2 agonist pam2CSK4 to enhance MMP production in human gingival fibroblasts via the MAPK and JAK-STAT pathways’

Presentations:

**Jun. 2014:** International Association for Dental Research General Session, Cape Town, South Africa (Poster presentations)

*Leptin-mediated MMP-1 production in gingival fibroblasts requires MAPK signalling*

*The regulation of gingival fibroblast MMPs by pro-inflammatory ligands*

**May 2014:** Gordon Research Seminar and Conference in Immunochemistry and Immunobiology, Maine, USA (Poster presentation)

*Interleukin-1 and leptin require ERK signalling to synergistically increase MMP-1 production in gingival fibroblasts*

**Apr. 2014:** FameLab UK Grand Final, London (Oral Presentation)

*Inflammation: what, why and ow!*

https://www.youtube.com/watch?v=d76c3FCvYi0

**Nov. 2014:** FameLab NorthEast regional heats and final, Newcastle (Oral presentations) – Regional winner

*What’s hiding in your mouth?!*

https://www.youtube.com/watch?v=vOn6TXTXnF0

*Collagen* https://www.youtube.com/watch?v=Kq9tOy28vhk

**Sep. 2014:** British Science Festival, Newcastle (Interactive workshop)

*What’s hiding in your mouth?!*

**Sep. 2014:** British Society for Oral and Dental Research Annual meeting, Bath (Oral presentation) - Senior Colgate Prize runner up

*Leptin synergistically enhances a matrix-degrading gingival fibroblast phenotype*

**Jul. 2013:** British Society for Immunology Summer School, Newcastle (Poster presentation)

*Leptin synergistically elevates matrix metalloproteinase-1 and -3 production by human gingival fibroblasts*
Jan. 2013: British Society for Oral and Dental Research Oral Microbiology and Immunology Group postgraduate meeting, UCL (Oral presentation) – first prize

*Leptin synergistically upregulates matrix metalloproteinase-1 in human gingival fibroblasts*

Dec. 2012: European Congress of Immunology, Glasgow (Poster presentation)

*Interleukin-1 and leptin synergistically enhance matrix metalloproteinase expression in human gingival fibroblasts*

Apr. 2012: British Society for Matrix Biology Annual Meeting, Oxford (Poster presentation)

*Leptin regulation of matrix metalloproteinase expression in human gingival fibroblasts*
9 References


Enhancement of LPS


to monopolar electrocautery at standard power level settings in a pig model', *BMC Surg*, 12, p. 3.


Montreekachon, P., Chotjumlong, P., Bolscher, J.G., Nazmi, K., Reutrakul, V. and Krisanaprapornkit, S. (2011) 'Involvement of P2X(7) purinergic receptor and MEK1/2 in...
function of STAT1beta in B cells: induction of cell death by a mechanism different from that of STAT1alpha', *J Leukoc Biol*, 84(6), pp. 1604-12.


Zhou, W., Guo, S. and Gonzalez-Perez, R.R. (2011) 'Leptin pro-angiogenic signature in breast cancer is linked to IL-1 signalling', Br J Cancer, 104(1), pp. 128-137.


