The role of cytokines and the suppressors of cytokine signalling (SOCS) in human osteoblastic cell survival and bone remodelling.

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by

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Abstract

A number of growth factors and cytokines involved in the local regulation of bone remodelling are either synthesised by osteoblasts or have osteoblasts as their target. These include the RANK-L/OPG system, the gp130 cytokine family, including IL-6, and insulin like growth factors. In addition, aberrant cytokine signalling is strongly linked with pathological states characterised by increased bone resorption, including osteoporosis and renal osteodystrophy. The range of action and potency of these osteotropic cytokines requires that their actions are tightly regulated. Amongst such potential control mechanisms are the suppressors of cytokine signalling (SOCS), the presence and role of which in bone has not been studied in detail.

The aim of this thesis was (i) to examine the direct effect of uraemia on cytokine release in human osteoblastic cells; (ii) to determine if the regulatory SOCS genes are expressed in these cells and, if so, (iii) to characterise their functional significance.

In initial studies, osteoblastic cells were cultured in media containing sera from either healthy volunteers or haemodialysis treated chronic kidney disease patients. Concentrations of OPG and IL-6 were then measured in harvested supernatants. Additionally, individual serum samples collected prior to, and during, a haemodialysis (HD) session were assayed for IL-6, IL-1β and soluble IL-6 receptor (sIL-6R). HD patients had significantly higher concentrations of IL-6 than normal subjects, but there were no significant differences in either IL-1β or sIL-6R. These concentrations did not change significantly during HD. There were no differences in OPG production by osteoblastic cells after exposure to either normal or uraemic serum. Incubation with untreated sera from normal subjects increased IL-6 production by ~6-fold above control, whereas sera from uraemic subjects increased it only ~2-3-fold. HD did not restore the capacity of uraemic serum to augment IL-6 release to the same degree as normal serum.

Further work examined a variety of osteotropic stimuli for their ability to induce SOCS1-3 and CIS expression in human osteoblastic cells. The utility of both conventional RT-PCR and fluorescence-based kinetic real time PCR for this purpose are compared. These SOCS were found to be expressed constitutively and could be induced to a variable degree by relevant growth factors. In general, the temporal pattern of SOCS expression was consistent with a negative feedback function. Potential functionality was explored following transfection with SOCS1 and SOCS3 plasmid DNA. Significantly enhanced IL-6 secretion was found in both the basal and stimulated state, whilst OPG production was enhanced only in the latter. Function was also studied in the context of osteoblastic apoptosis, the regulation of which is highly relevant to skeletal disease. Initial experiments developed a framework for subsequent studies: serum starvation for 24h produced reproducible cell death that could be attenuated in a dose dependent manner by IGF-I. SOCS1 and SOCS3 overexpression had limited influence on osteoblast survival, whereas gene knock down experiments using siRNA indicated that IL-1β-induced cell death is mediated differentially, depending on the type of cell death involved. SOCS1 and SOCS3 are involved in the apoptotic cascade, while IL-1β-induced necrosis appears to be independent of SOCS3.

Collectively these studies demonstrate that the augmentation of IL-6 production by osteoblastic cells after exposure to normal serum is greater than after uraemic serum. HD does not correct this disparity; perhaps indicating a non-dialysable inhibitor of IL-6 release is involved in the dysregulated bone turnover of uraemic patients. Further work establishes the constitutive presence of the SOCS family in human osteoblastic cells, as well as their transient inducibility by key osteotropic stimuli. Several novel aspects of SOCS function, including influence on IL-6 and OPG production and involvement within apoptotic pathways are demonstrated.

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List of abbreviations

ABD	adynamic bone disease
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ALP	alkaline phosphatase
ANKH	ankylosis family human homologue
AP24	apoptosis protease 24
ApoE	apolipoprotein E
ßME	ß mercaptoethanol
BCA	bicinchoninic acid
Bcl	B-cell lymphoma family proteins
BCIP	5-bromo-4-chloro-3-indoyl phosphate
β-ME	beta-mercaptoethanol
BMD	bone mineral density
BMP	bone morphogenetic protein
BMU	basic multicellular unit
bp	base pair
BRC	bone remodelling compartment
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAPS	3-cyclohexylamino-1 propane sulfonic acid
CaR	calcium sensing receptor
Cbfa1	core binding factor one
CD	cluster of differentiation
cDNA	complementary DNA
CIS	cytokine inducible SH2 domain containing protein
CKD	chronic kidney disease
CNS	central nervous system
CNTF	ciliary neurotropic factor
CRP	c reactive protein
C_t	threshold cycle
DBP	vitamin D binding protein
DD	death domains
DEPC	diethyl pyrocarbonate
DMP-1	dentix matrix protein 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxy-nucleotide triphosphates

DTT	dithiothreitol
EDTA	ethanolamine diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EPO	erythropoietin
ERK	extracellular signal regulated kinases
ESRD	end stage renal disease
EV	empty vector
FADD	fas associated death domain
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fz	frizzled
GAPDH	glyceraldehydes-3-phosphate-dehydrogenase
GFR	glomerular filtration rate
GH	growth hormone
GHS-R	growth hormone secretagogue receptor
GITC	guanidine isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
HCL	hydrochloric acid
HD	haemodialysis
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IFNγ	interferonγ
IL	interleukin
IL-1RA	IL-1 receptor antagonist
IL-6R	interleukin-6 receptor
IAPS	inhibitor of apoptosis proteins
IRS	insulin receptor substrates
JAB	JAK-binding protein
JAK	janus kinase
KD	knock down
kDa	dalton
KDIGO	kidney disease: improving global outcomes
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LIF	leukaemia inhibitory factor

LPS	lipopolysaccharide
L-TBP	latent TGF-ß binding protein
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
mRNA	messenger RNA
NBT	nitro-blue tetrazolium
NGF	nerve growth factor
NK	natural killer
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
NPP	nucleotide pyrophosphatase phosphodiesterase
OSM	oncostatin M
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
Phex	phosphate-regulating gene with homologies to endopeptidases on the X chromosome
PICP	pro-collagen type 1 c-terminal peptide
PTH	parathyroid hormone
PTH-1R	PTH receptor type 1
PTHrP	PTH related peptide
PDGF	platelet derived growth factor
PBS	phosphate buffered saline
PI3-kinase	phosphatidylinositide 3-kinase
PMSF	phenylmethanesulfonylfluoride
PO_4	phosphate
PPARγ1	peroxisome proliferator-activated receptor-1
PVDF	polyvinyldifluoride
RANK	receptor activator of NF-κB
RANK-L	receptor activator of NF-κB Ligand
RAS	rat sarcoma
rhGH	recombinant human growth hormone
RIP-1	receptor interacting protein 1
RNA	ribonucleic acid
ROCK-1	rhokinase isoform-1
Runx2	runt-related transcription factor 2

RRT	renal replacement therapy
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SERM	selective oestrogen receptor modulator
SHP	Src homology domain 2 -containing protein tyrosine phosphatase-1
SHPT	secondary hyperparathroidism
sIL-6R	soluble interleukin-6 receptor
SOCS	suppressors of cytokine signalling
SSi	STAT inducible STAT inhibitor
STAT	signal transducer and activator of transcription
Taq	*Thermus aquaticus*
TBE	tris borate EDTA
TBS-T	Tris-Buffered Saline and Tween 20
TGF-ß	transforming growth factor – ß
TNF	tumour necrosis factor
TLR	Toll-like receptor group
TRAF	TNF receptor associated factor
TRAIL	TNF-related-apoptosis-inducing-peptide
TRAP	tartrate resistant acid phosphatase
VDR	vitamin D receptor
VDRE	vitamin D response elements
VEGF	vascular endothelial derived growth factor
UV	ultraviolet
Wnt	wingless and Int-1

Publications associated with this work

Peer reviewed papers

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Papers in preparation

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Presentations at national and international meetings

STEDDON, S.J., ALLEN, D.A., BURRIN, J.M., CUNNINGHAM, J., and YAQOOB, M.M. *Expression of the suppressors of cytokine signalling (SOCS) in human osteoblastic cells*.
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STEDDON, S.J., ALLEN, D.A., BURRIN, J.M., CUNNINGHAM, J., and YAQOOB, M.M. *Calpain activation is concurrent with caspase-3 suppression in IGF-I treated human osteoblastic cells.*
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STEDDON, S.J., ALLEN, D.A., BURRIN, J.M., CUNNINGHAM, J., and YAQOOB, M.M. *Overexpression of SOCS1 and SOCS3 Enhances Interleukin-6 Production in Human Osteoblastic Cells: Potential Implications for Skeletal Remodelling.*
American Society of Nephrology, St Louis, October 2004 (oral) and Renal Association Autumn Meeting 2004

STEDDON, S.J., HARWOOD, S.M., ALLEN, D.A., BURRIN, J.M., CUNNINGHAM, J., and YAQOOB, M.M *SOCS Knockdown differentially attenuates IL-1β induced cell death cascades in human osteoblastic cells*
Renal Association Meeting 2009

Chapter 1
Introduction

1.0 Introduction

1.1 Bone

Bone tissue is not static, it is continually being remodelled according to mechanical and metabolic requirements. This process is complex, involving the concerted action of two important cell populations, bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts), and proceeds under the regulatory influence of both systemic osteotropic hormones and local paracrine factors. Remodelling serves several important physiological purposes, functioning not just to renew bone as it deteriorates, but to redistribute bone matrix along lines of mechanical stress, allow healing of injured/fractured bone and permit the skeleton to act as a reservoir for calcium and phosphate. During normal remodelling, the development, longevity and interactions between cells within skeletal tissue are all critical steps (Coe and Favus, 2002).

Bone is a specialised connective tissue that makes up, together with cartilage, the skeletal system. It consists of several dedicated cell types and a distinctive extracellular matrix composed of collagen fibres and non-collagenous proteins. The ability of this matrix to undergo hydroxyapatite mineralization endows bone with many of its unique characteristics (Favus and Christakos, 1999).

Bone serves three important functions: -

- Mechanical - it is the site of muscle attachment for locomotion.
- Protection - for vital organs and the bone marrow.
- Metabolic - it acts an ion reservoir for mineral metabolism.

1.1.1 Macroscopic organisation

Two types of bone can be distinguished in the skeleton: flat bones (e.g. skull bones, scapula, and mandible) and long bones (e.g. tibia, femur and humerus). Long bones are composed of two wider extremities (the epiphyses), separated from a cylindrical central tube (the midshaft or diaphysis) by a developmental zone (the metaphysis).

This is shown schematically in figure 1. In a growing long bone, the epiphysis and the metaphysis are separated by a layer of cartilage called the growth plate (epiphyseal cartilage). This area of cell proliferation and expanding matrix is the site of longitudinal growth. Once growth is completed the epiphyseal cartilage is destined to become calcified itself. The outer region of bone is formed by an extremely dense layer of tissue, known as cortical or compact bone. This strong layer encloses the medullary cavity, which houses the haematopoietic bone marrow. In areas close to the metaphysis this internal space is honeycombed by a lattice work of plates called trabeculae. These create cavities that can be filled with either haematopoietic marrow or adipose tissue. In certain cases, as in the sinuses of the skull, the spaces are filled with air. These trabeculated areas constitute cancellous, also termed spongy or trabecular, bone. Cortical and trabecular bone are populated by the same cell types and have identical matrix elements, though they exhibit important differences. Structurally the most significant difference is that 80 to 90% of cortical bone is calcified, in contrast to only 15 to 25% of trabecular bone. Functionally, cortical bone has a primarily mechanical or protective purpose, while trabecular bone is more actively engaged in metabolic processes.

1.1.2 Microscopic organisation

Bone is formed by collagen fibres, 90% of which are type I collagen, and non-collagenous proteins. Once secreted, the collagen fibres undergo extracellular processing to produce mature three chain type I collagen molecules that are able to assemble into fibrils. Individual collagen molecules are interconnected via unique pyridinoline cross-links. The orientation of the fibres alternates from layer to layer, giving bone a typical lamellar structure. This arrangement permits the highest density of collagen per unit volume of tissue. The lamellae form either continuous layers at the surface (circumferential lamellae), or concentric cylinders around neurovascular channels (Haversian canals). In cases where bone is being formed rapidly, as is the case during development, fracture healing and some metabolic bone diseases,

including those associated with kidney disease, the preferential organisation of the collagen fibres is disturbed, resulting in poorer quality, woven, bone.

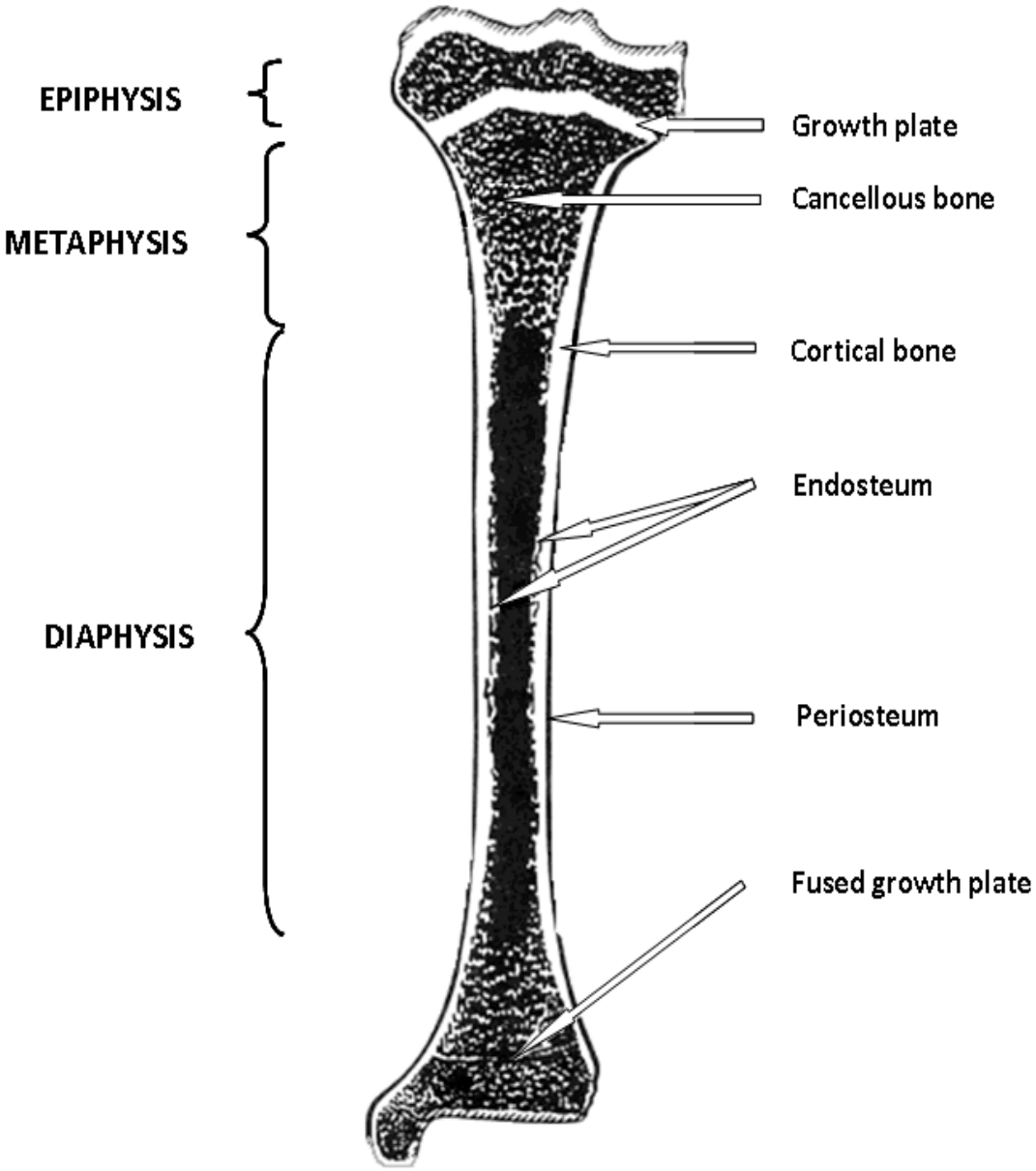


Figure 1.1: Schematic view of a longitudinal section through a growing long bone. (Adapted from Jee W. The skeletal tissues. In Weiss L, ed. Histology, cell and tissue biology. New York: Elsevier Biomedical, 1983: 200-255, reproduced in Favus 1999)

Osteocalcin and osteonectin constitute 40 to 50% of the non-collagenous matrix proteins. Mice deficient in osteonectin exhibit decreased osteoclast and osteoblast numbers and profound osteopenia (Delany et al., 2000), suggesting an active role in the life cycle of these cells. A number of other proteins such as osteopontin, bone sialoprotein, fibronectin, vitronectin, and thrombospondin are present and function as attachment factors that interact with integrins. The majority of the non-collagenous proteins present in the matrix are synthesised by fully differentiated osteoblasts, though some are synthesised elsewhere and preferentially imported; e.g. fetuin (α_2-heremans schmid glycoprotein), synthesised in the liver. The remainder of the extracellular matrix consists mainly of highly anionic glycoproteins and proteoglycans. These have a high ion-binding capacity and play an important part in the calcification process. Mineralisation occurs via the deposition of hydroxyapatite crystals [$3Ca_3(PO_4)_2]\cdot(OH)_2$] throughout the collagen fibres. Osteoblasts are thought to regulate local concentrations of calcium and phosphate in such a way as to promote the formation of hydroxyapatite (Boskey, 1998). Prior to undergoing mineralisation, bone matrix is known as osteoid.

1.2 Osteoblasts

Osteoblasts, the cell type at the centre of this thesis, are cuboidal, basophilic, mononuclear cells found on the surface of growing or remodelling bone. They occur in clusters and never function in isolation. Osteoblasts occupy a central position in the homeostasis of skeletal tissue. Their functions include:-

- Synthesis and secretion of matrix.
- Mineralisation of the matrix.
- Regulation of bone turnover - osteoblasts are the major target of the 'classical' osteotropic hormones parathyroid hormone (PTH) and 1,25 – dihydroxyvitamin D3 ($1,25(OH)_2D_3$ or calcitriol) as well as the source of many local growth factors and cytokines.
- Control of osteoclastogenesis.

1.2.1 Origin and lineage

The precursors of osteoblasts are pluripotent mesenchymal stem cells. These may also give rise to chondrocytes, myocytes, fibroblasts, adipocytes and a variety of haematopoiesis support cells (Aubin, 2001). Osteoblast precursors most likely reach bone by migration from neighbouring connective tissues. Commitment of mesenchymal precursors to the osteoblastic lineage is initiated by bone morphogenetic proteins (BMPs), particularly BMP -2 and - 4, members of the transforming growth factor ß (TGF-ß) superfamily (Abe et al., 2000). BMPs stimulate transcription of the gene encoding Cbfa1 (core binding factor a1), a transcription factor that induces osteoblast differentiation via the activation of a range of osteoblast specific genes including those for alkaline phosphatase, osteopontin, bone sialoprotein, type I pro-collagen and osteocalcin (Ducy et al., 1997, Ducy, 2000). Lack of Cbfa1 prevents osteoblast development and, because of the dependence of osteoclastogenesis on mesenchymal cell differentiation, also reduces the population of osteoclasts (Gao et al., 1998, Komori et al., 1997). Other factors such as TGF-ß, platelet derived growth factor (PDGF), fibroblast growth factors (FGFs), and insulin like growth factors (IGFs) are able to influence the replication and differentiation of committed osteoblast precursors toward the osteoblast lineage, though they are unable to induce osteoblast differentiation from uncommitted progenitors (Marcus et al., 2001). The relationship between osteoblastogenesis and adipogenesis is a particularly intimate one, with peroxisome proliferator activated receptor γ1 (PPARγ1) appearing, through its ability to inhibit the expression of other lineage specific transcription factors, to act as an important early determinant of mesenchymal progenitor cell destiny (Shao and Lazar, 1997, Lecka-Czernik et al., 1999). In fact, the discovery of communication between cells of osteoblast and adipocytes lineage (via leptin and other hormones) and therefore between bone remodelling and energy metabolism is an area of increasing interest. It may have particular relevance in diabetes mellitus, where disorders of bone remodelling are common (Kawai et al., 2009).

The isolated mature osteoblast cannot simultaneously perform all the functions needed for the initiation and maintenance of bone formation. This is achieved by maintaining a pool of osteoblasts populated by cells at various differentiation stages. This phenotypic heterogeneity, despite common lineage, provides the diverse functionality required for bone generation. The principal forms that mediate bone-forming processes are (i) stromal osteoprogenitor cells (contribute to the maintenance of the osteoblast population and bone mass); (ii) mature osteoblasts (synthesise bone matrix at the bone forming surfaces); and (iii) osteocytes (terminally differentiated cells that support bone structure). As a general rule, osteoblastic maturity increases in relation to the proximity to the bone surface.

1.2.2 Ultrastructure and properties of the osteoblast

The osteoblast is a highly metabolically active cell, with a large proportion of this activity channelled into the production of type I collagen (Bilezikian et al., 2002). It has an abundant rough endoplasmic reticulum committed to the production of extracellular matrix proteins, including osteocalcin and osteopontin. A number of the growth factors and cytokines that are intimately involved in the local regulation of the remodelling cycle are also synthesised by osteoblasts. The most important of these are the BMPs, the transforming growth factor ßs (TGF-ßs), the insulin–like growth factors (IGFs), the gp130 family of cytokines (chiefly IL-6 and IL-11), platelet derived growth factors (PDGFs) and osteoprotegerin (OPG) (Heymann and Rousselle, 2000, Hofbauer et al., 2000).

The cell membrane of the osteoblast is characteristically rich in a specific isoform of alkaline phosphatase (ALP), the precise role of which remains unclear. However, relevance has always been inferred from the observation that ALP deficiency in hypophosphatasia, an inherited systemic disorder, results in defective mineralisation and poor dentition (Whyte, 1994). It seems likely that local inorganic pyrophosphate,

generated by both the nucleotide pyrophosphatase-phosphodiesterase designated NPP1, as well as the transporter ANKH, can inhibit hydroxyapatite formation unless hydrolysed by ALP – a process that will also provide free inorganic phosphate for mineralisation (Orimo, 2010). The serum concentration of bone specific ALP is used clinically as a useful marker of bone turnover.

1.2.3 Control of osteoblast function

Osteoblastic function is controlled by endocrine, paracrine and autocrine influences. Expression of receptors for PTH and active vitamin D means that these important systemic hormones can exert influence. Furthermore, it is through the mediation of the osteoblast that these stimuli are processed and passed on to the osteoclast prior to the initiation of bone resorption.

1.2.4 Relationship with osteoclasts: the RANK-L/OPG system

It has long been recognised that, within the bone microenvironment, osteoblasts exert an important influence over neighbouring osteoclasts. While the paracrine release of cytokines (including IL-1, IL-6, IL-11, TNF and TGF-ß) ostensibly explained this cellular cross-talk, it was noticeable that spatial proximity of osteoblast to osteoclast was a prerequisite for behavioural change in the latter (Suda et al., 1992, Martin and Ng, 1994, Martin et al., 1998). Growth factors in isolation provided an inadequate stimulus. There seemed to be a concealed line of communication that enabled the osteoblast to orchestrate bone homeostasis so effectively. The discovery of two glycoproteins, both from the TNF receptor superfamily, termed (after several early nomenclature changes) osteoprotegerin (OPG) and the ligand for receptor activator of NF-κB (RANK-L) provided the missing link (Simonet et al., 1997a, Yasuda et al., 1998a, Lacey et al., 1998). These constitute a system that acts as the final effector pathway for a host of osteotropic stimuli. RANK-L is expressed in committed pre-osteoblastic cells (as well as T lymphocytes), while its receptor (RANK) is found on osteoclastic progenitors. The

activation of RANK in osteoclasts results in activation of several intracellular signal transduction pathways, particularly TRAF 6, which bind the nuclear factor *κ*B (NF-*κ*B). This translocates to the nucleus, where it upregulates cofactors that induce osteoclastogenic gene transcription. These promote osteoclastic differentiation, activation and survival, thereby increasing the pool of mature, functioning osteoclasts able to undertake bone resorption (Suda et al., 1999). RANK-L and macrophage colony stimulating factor (M-CSF) alone are sufficient to invoke osteoclastogenesis independent of other growth factors. This is summarised in figure 1.2. OPG provides an ingenious regulatory mechanism; like RANK-L it is an osteoblastic product, but it differs importantly in being secreted rather than membrane bound. Once released it acts as a decoy receptor for RANK-L, preventing its interaction with RANK and opposing the stimulation of osteoclasts. It is a very potent inhibitor of osteoclastogenesis and bone resorption both *in vitro* and *in vivo*. In this way, bone remodelling becomes intimately related to changes in expression of OPG and RANK-L (Hofbauer et al., 2000). When OPG levels decrease, more RANK-L is available to bind RANK and induce bone resorption. Conversely, a rise in OPG tips the balance in favour of bone formation. Factors that decrease the OPG/RANK-L ratio, thereby favouring bone resorption with higher rates of bone turnover, include TNF-α (Hofbauer et al., 1999c), PTH (Yasuda et al., 1998b), PTH related peptide (PTHrP), calcitriol (Yasuda et al., 1998b), glucocorticoids (Hofbauer et al., 1999a), gp130 activating cytokines (e.g. IL-6, IL-11) (Yasuda et al., 1998b), IL-1 (Hofbauer et al., 1998, Hofbauer et al., 1999c) and the immune suppressant drug cyclosporine (Hofbauer et al., 2001). This latter observation may provide a possible explanation for the more marked effects on osteoblastic proliferation and differentiation of cyclosporine than the related immune suppressant tacrolimus (Moreira et al., 2009). Those that increase the OPG/RANK-L ratio, thereby decreasing bone resorption and slowing bone turnover include oestrogens (Hofbauer et al., 1999b), androgens (Hofbauer et al., 2002), BMP-2 (Hofbauer et al., 1998) and possibly IGF-I (Rubin et al., 2002).

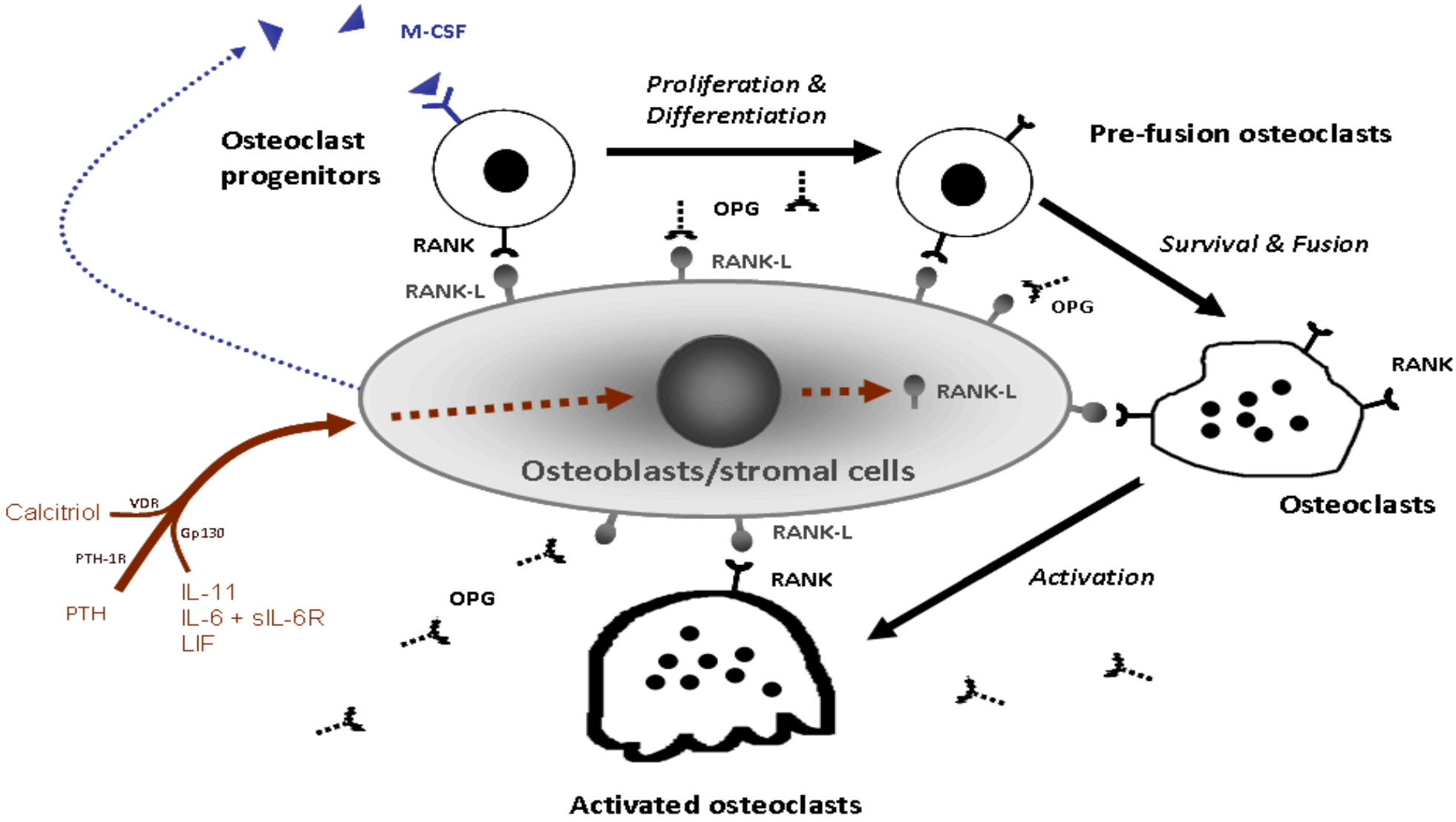


Figure 1.2: A schematic representation of osteoclast differentiation and function as supported by osteoblasts/stromal cells.

Adapted from (Suda et al., 1999, Harada and Rodan, 2003)

The crucial role of the OPG/RANK-L system in bone remodelling is emphasised by observations in transgenic mice, where manipulation of the relevant genes produces extremes of skeletal phenotype. OPG over-expression and both RANK-L and RANK gene deletion all result in osteopetrosis (characterised by deficient osteoclastic resorption, increased bone mass and marked skeletal fragility), while OPG knockout leads to severe osteoporosis (Simonet et al., 1997b, Bucay et al., 1998, Mizuno et al., 1998, Kong et al., 1999). These observations, coupled with the finding that OPG administration prevented bone loss in ovariectomised mice (Kostenuik et al., 2001), stimulated huge interest in the potential for manipulation of the OPG/RANK-L system in the treatment of osteoporosis and/or other pathological conditions involving bone loss. Recently, a full year of transgenic overexpression of OPG has demonstrably suppressed bone resorption, while increasing vertebral strength, in rats (Ominsky et al., 2009) and inhibition of RANK-L has improved bone mass in both healthy rodents (Rosen, 1994) and in several animal models of human disease (Kostenuik et al., 2001, Samadfam et al., 2007). Encouraging clinical data is also emerging - with the demonstration of reduced bone turnover, as inferred from biochemical data, in post-menopausal women following a single OPG dose (Bekker et al., 2001) and recent preliminary clinical studies involving a fully human monoclonal antibody to RANK-L. The latter possesses high specificity and affinity for its antigen and acts to neutralise it in a similar manner to OPG. (McClung et al., 2006, Cummings et al., 2009, Lewiecki et al., 2007) However, it is worth mentioning that the correlation between serum OPG levels and fractures or BMD is inconsistent and clinically unhelpful (Browner et al., 2001).

In addition to skeletal metabolism, the RANK/OPG system may play a significant regulatory role in several other biological systems. OPG is produced by many tissues in addition to bone, including skin, liver, stomach, intestine, lung, heart, kidney, placenta and haematopoietic/immune cells. Of particular note is the observation that mice deficient in RANK-L lack lymph nodes as well as osteoclasts (Kong et al., 1999).

RANK/OPG is also credited with a role in the regulation of antigen presentation and T cell activation (Emery et al., 1998). In addition, OPG also binds with high affinity to the cytotoxic ligand TRAIL (TNF-related apoptosis-inducing ligand) and inhibits TRAIL-mediated apoptosis in lymphocytes (Degli-Esposti, 1999). This, and the realisation that antibodies to exogenous OPG could be raised, is one of the principle reasons for the deceleration in its development as a therapeutic agent - inhibition of TRAIL-mediated apoptosis could conceivably interfere with tumour surveillance.

An additional, unexpected, finding of profound interest was that OPG knockout animals developed widespread medial vascular calcification (Bucay et al., 1998), preventable by transgenic overexpression of soluble OPG (Min et al., 2000). Recombinant OPG has also been shown to prevent the vascular calcification induced by both warfarin and vitamin D in rats (Price et al., 2001). The subsequent accumulation of evidence has suggested that vascular calcification is intimately related to bone resorption. For example, if OPG knockout mice are cross bred with Apolipoprotein E (ApoE) knockouts, the latter predisposing to atherosclerosis and vascular calcification, bone turnover demonstrably increases in association with accelerated calcification and plaque progression (Bennett et al., 2006). Evidence from human studies also supports the relationship. An observational study in postmenopausal women has shown that aortic calcification is inversely related to bone density and positively associated with fractures (Schulz et al., 2004). It therefore seems somewhat counterintuitive that several observational studies have demonstrated a positive correlation between serum OPG concentration and vascular disease (Ziegler et al., 2005, Browner et al., 2001, Jono et al., 2002, Schoppet et al., 2003, Kiechl et al., 2004, Nitta et al., 2003). Possible interpretations of this include OPG playing an active role in disease progression, or functioning as part of a compensatory mechanism to retard it (Kearns et al., 2008). It is possible that while OPG reduces vascular calcification through suppression of bone turnover, it may have a neutral effect on atherosclerosis. To support this, LDL receptor knockout mice fed on a high-fat diet have a significant reduction in calcified aortic

lesions, but not atherosclerotic ones, following treatment with OPG (Morony et al., 2008).

1.2.5 Osteocytes

The final fully differentiated cells of the osteoblast lineage are known as osteocytes. They are the most abundant cell type in mature bone (about ten times more common than osteoblasts) and are essentially osteoblasts that have been engulfed by mineralised matrix. Despite this incarceration, they are not metabolically inactive and continue to synthesise several matrix components. Osteocytes are characterised by a striking stellate morphology, reminiscent of the dendritic network of the nervous system (Bilezikian et al., 2002). Their contact with other cells, including bone forming osteoblasts, through these cellular processes has led to suggestions that they act as local sensors, or barometers, of prevailing mechanical and metabolic circumstances (Aarden et al., 1994).

1.2.6 Lining cells

The surface of normal quiescent bone (i.e. bone that is not undergoing remodelling) is covered by a layer of unmineralised collagen matrix on top of which there is a layer of flat and elongated cells. These 'lining cells' are the descendents of osteoblasts (Parfitt, 1994). Osteoclasts cannot attach to the unmineralised collagenous lining layer and it seems to be the responsibility of lining cells to remove it, while sending a signal of attraction to the resorbing cells. This is the first step in remodelling and probably proceeds under the coordination of osteocytes (Parfitt et al., 1996).

1.2.7 Wnt signalling

Mention should be made of the emerging importance of the Wnt (Wingless and Int-1) signalling pathways for bone over the last decade. Wnt are a family of secreted glycoproteins with shared cysteine residues, that signal through members of the

Frizzled (Fz) receptor family. Ligand binding induces one of three known downstream pathways, but the canonical (Wnt/β-catenin) pathway appears the most relevant in a skeletal context (Westendorf et al., 2004). Evidence from several rare hereditary bone disorders suggests a role in bone formation (Krishnan et al., 2006) and the pathway appears active in many aspects of pre-osteoblast and osteocyte function, including differentiation, proliferation and apoptosis (Piters et al., 2008). It also presents an attractive target for drugs as it offers several potential targets for intervention (Rawadi and Roman-Roman, 2005).

In summary, cells of osteoblastic lineage perform two crucial tasks. Firstly they are responsible for the production and subsequent mineralisation of the bone matrix. Secondly, they have a pivotal role in the regulation of the bone remodelling cycle through (i) their response to endocrine influences such as PTH and active vitamin D and (ii) their influence over osteoclastic development and function through the RANK-L/OPG system.

1.3 Osteoclasts

The osteoclast is the only cell capable of resorbing bone directly. Unlike osteoblasts, they are derived from the monocyte/macrophage lineage. Although the exact stem cell is still uncertain, the colony forming unit for the granulocyte-macrophage series is considered the most likely (Roodman, 1999). Osteoclasts are giant multi-nucleated cells, containing four to twenty nuclei. Their cytoplasm contains abundant Golgi complexes and many vacuoles containing lysosomal enzymes. They are usually found in close proximity to the bone matrix. The zone of contact between cell and matrix is recognisable by a ruffled cellular border. Here the cell membrane is highly folded and flanked by a specialised area, known as the clear or sealing zone, containing a ring of contractile proteins. These, with the help of specific integrin receptors for matrix proteins, serve to attach the cell to the bone surface and provide a tight seal around the bone resorbing compartment. This allows secreted enzymes to be contained and

controlled within an optimised microenvironment and minimises unwanted effects on surrounding cells. The area of the ruffled membrane depends on the state of bone resorption, increasing when bone resorption is stimulated; for example by PTH, and decreasing (often disappearing) when resorption is inhibited; for example by oestrogen (Roodman, 1996). Osteoclasts are motile cells capable of resorbing more than just the cavity within which they are identified. After an osteoclast digs a cavity, it can detach from bone and move on to a new resorption site. A further characteristic feature of mature osteoclasts is their high content of the phosphohydrolase enzyme, tartrate-resistant acid phosphatase (TRAPase). Measurement of TRAP is commonly exploited for the detection of osteoclasts in bone specimens (Udagawa et al., 1990).

1.3.1 Mechanisms of bone resorption

The proteolytic enzymes that digest bone (chiefly matrix metalloproteinases and cathepsins K, B and L) work best at acidic pH (Bossard et al., 1996). Protons are generated by carbonic anhydrase type II and pumped across the ruffled border into the sealed space between cell and bone. The maintenance of the pH and electrochemical balance in this space is dependent on a variety of ion channels, pumps and exchange mechanisms, including a Ca^{2+} ATPase, K^{+} channel and HCO_3/Cl^{-} exchanger (Blair et al., 1989, Blair et al., 2002). These features put the osteoclast in the same functional category as both gastric oxyntic cells and intercalated cells of the renal tubule, both of which are also of epithelial origin and secrete acid in a polarized fashion. The degraded bone matrix components are endocytosed along the ruffled border and trancytosed to the opposite side of the cell for release (Nesbitt and Horton, 1997, Salo et al., 1997).

1.3.2 Osteoclastogenesis

In addition to the RANK/OPG system (as shown in figure 1.2) a complex network of cytokines is involved in the fine tuning of osteoclastogenesis (Manolagas, 1995). The early stages are very similar to haematopoiesis and proceeds under parallel influences. These include the interleukins IL-1, IL-3, IL-6, IL-11, leukaemia inhibitory factor (LIF),

oncostatin M (OSM), ciliary neurotropic factor (CNTF), tumour necrosis factor (TNF), granulocyte macrophage-colony stimulating factor (GM-CSF), M-CSF, and c-kit ligand (Manolagas, 1998). IL-4, IL-10, IL-18 and interferon γ all exert a negative influence. In the case of IL-18 this effect is mediated through GM-CSF (Udagawa et al., 1997).

Stimulate osteoclast development	Inhibit osteoclast development
IL-1, IL-3, IL-6, IL-11 Leukaemia Inhibitory factor (LIF) Oncostatin M (OSM) Ciliary neurotropic factor (CNTF) Tumour necrosis factor (TNF) Granulocyte macrophage-colony stimulating factor (GM-CSF), M-CSF and c-kit ligand.	IL-4, IL-10, IL-18, IFN-γ

Table 1.1: The positive and negative regulatory influences on osteoclast development in addition to the RANK-L/OPG system

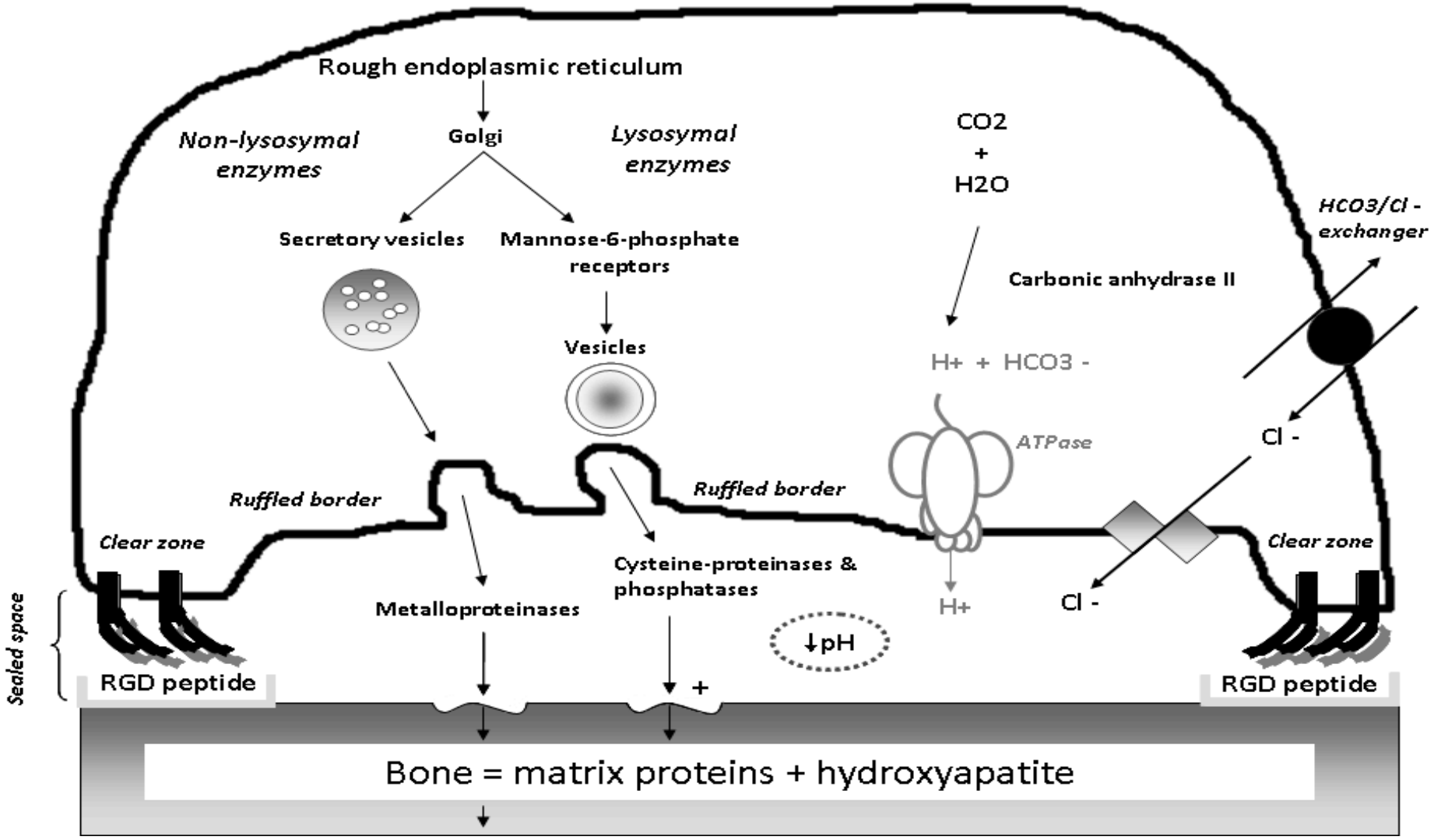


Figure 1.3:.Schematic representation of enzyme secretion and ion transport in an osteoclast during the initiation phase of bone remodelling. Adapted from Noda 1993

1.4 Bone remodelling cycle

The remodelling cycle is a complex multi-step process consisting of coordinated resorption and replacement of bone (figure 1.4) (Marcus et al., 2001). Bone turnover begins by conversion of an inactive or quiescent skeletal surface to a remodelling site, a process referred to as activation. Activation involves proliferation of new blood vessels needed to bring recruited resorbing cells to the site and retraction of the flat, pavement-like cells that cover quiescent surfaces to expose the mineralised bone surface. New osteoblasts assemble only at sites where osteoclasts have recently been active, a process known as coupling. The aim is the maintenance of an ordered mineralised matrix under the control of systemic endocrine factors and locally released paracrine factors. It occurs as a consequence of the interactions between small groups of osteoblasts and osteoclasts grouped into a 'basic multicellular unit' (BMU) by virtue of close anatomical proximity. In healthy human adults, 3 to 4 million BMUs are initiated every year and approximately one million are active at any moment (Parfitt, 1994). Once activated, the BMU moves over the surface of bone as a resorptive tide. The leading edge of the BMU consists of recently recruited and activated osteoclasts, and is followed by cells of the osteoblast lineage, reforming fresh matrix as old is removed. The process is driven by osteoblastic processing of local and systemic resorptive stimuli with subsequent recruitment of osteoclasts from the haematopoietic precursor pool. Further regulation occurs as bone dissolution releases mineral ions and other factors that feed back directly on the osteoblast and the osteoclast. During the six to nine month lifespan of a BMU, the shorter-lived osteoclasts and osteoblasts are continually replaced while the same discrete spatial and temporal relationships are maintained (Manolagas, 2000).

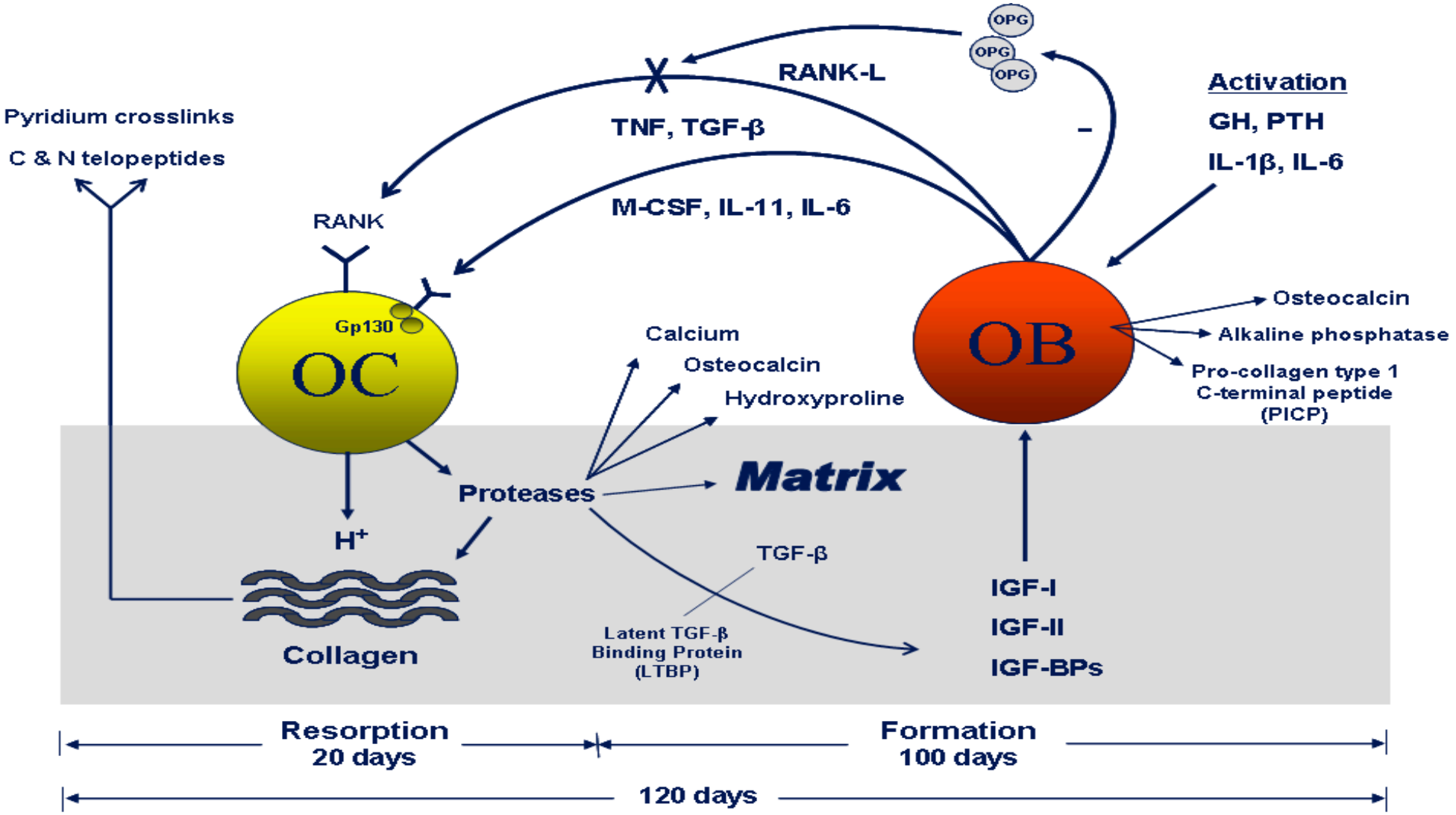


Figure 1.4 Schematic representation of the bone remodelling cycle

1.5 Endocrine influences on the bone remodelling process

1.5.1 Parathyroid hormone

Parathyroid hormone (PTH) is a single chain polypeptide of 84 amino acid residues (molecular weight 9500 D) synthesised in the parathyroid glands in the neck. Biological activity resides in the N-terminal 1 to 34 region. It is a crucial hormone for the maintenance of bone turnover and calcium homeostasis in man (Schrier, 1997). It acts on a wide variety of target cells through a G protein coupled PTH receptor (PTH-1R) (Juppner et al., 1991). The effects of PTH on bone, and in the osteoblast in particular, are complex and vary widely between *in vitro* and *in vivo* systems (Jilka et al., 1999). PTH is capable of both stimulation and inhibition of matrix synthesis, though the former appears the predominant effect *in vivo* (Dempster et al., 1993). The response is dependent to a degree on whether it is administered continuously or intermittently (Tam et al., 1982, Ma et al., 2001). Intermittent administration of PTH stimulates bone formation by increasing osteoblast number. PTH directly activates survival signals in osteoblasts and induces a delay in osteoblast apoptosis (Jilka, 2007). PTH may also cause replicating progenitors to drop out of the cell cycle through decreased expression of cyclin D and increasing expression of several cyclin dependent kinase inhibitors (Qin et al., 2005). This drop out may allow response to the pro-differentiation and survival effects of locally produced growth factors and cytokines (Partridge et al., 2006). The anabolic effect of PTH appears, in part, to be mediated through the production and release of local factors, such as IGF-I and fibroblast growth factor-2, and is linked to an increase in cAMP - the classical intracellular messenger for PTH (Canalis et al., 1989, McCarthy et al., 1990, Hurley et al., 2006). PTH also stimulates the production of RANK-L and the gp130 cytokines IL-6 and IL-11 by stromal and osteoblastic cells (Yasuda et al., 1998b, Girasole et al., 1994, Greenfield et al., 1995). PTH fragments are also present in the circulation. These include a large C-terminal fragment called PTH (7-84). This has a longer half-life and may exert an inhibitory

influence on osteoclastic bone resorption (probably via a distinct receptor) (Martin et al., 1979, Slatopolsky et al., 2000).

1.5.2 Vitamin D

Vitamin D and its metabolites are transported in the circulation by a specific binding protein, vitamin D binding protein (DBP), which is normally present in large excess. Active vitamin D, 1α,25-dihydroxyvitaminD3 (1,25$(OH)_2D_3$ or calcitriol), is generated by hepatic 25-hydroxylation and renal 1α-hydroxylation of inactive precursors. The 25-hydroxyvitamin D-DBP complex gains access to a 25$(OH)D_3$-1α-hydroxylase in renal proximal tubular cells by binding to megalin located on their apical membrane (Nykjaer et al., 1999). In target cells, calcitriol alters gene expression by binding with high affinity to its intracellular receptor, the vitamin D receptor (VDR), which acts as a nuclear transcription factor. Liganded VDR undergoes conformational change and forms a heterodimer with a second protein, the retinoid X receptor (RXR). This, in turn, binds to DNA elements in the promoter regions of target genes described as vitamin D response elements (VDREs). Binding to the VDREs may promote transcription, as is the case with osteocalcin in the osteoblast, or inhibit transcription, as for parathyroid hormone in the parathyroids, by either enhancing or repressing activity of transcription machinery (Darwish and DeLuca, 1996). Calcitriol has complicated effects on osteoblast function (Brown et al., 1999), though the predominant influence is to increase bone resorption. It is also required for matrix mineralisation, though this does not appear to be mediated through the osteoblast, but rather through calcium and phosphate homeostasis (van Leeuwen et al., 2001). Indeed, it is interesting to note that normalisation of serum levels of calcium and phosphate in VDR knockout mice results in normalisation of bone histology (Amling et al., 1999). Additionally, 1,25$(OH)_2D_3$ appears to bind to one or more additional cell surface receptors that, through second messenger pathways, mediate certain non-genomic effects (Baran et al., 2000). In addition to its ‘classical’ role in mineral metabolism, vitamin D exerts various influences

on differentiation, proliferation, and apoptosis across a variety of cell types (Baeke et al., 2010). Within bone, physiological doses of $1,25(OH)_2D_3$ protect against osteoblastic apoptosis by mechanisms involving non-genomic activation of a VDR/PI3K/Akt survival pathway that can generate phosphorylation of multiple p-Akt substrates as well as a reduction in caspase activity (Zhang and Zanello, 2008). Vitamin D insufficiency has been implicated (based on varying degrees of epidemiological and biological evidence) in the pathogenesis of several non-osseous disorders, including cancer (Freedman et al., 2007), diabetes mellitus (Mathieu et al., 2005), hypertension (Rostand, 2010) cardiovascular disease (Swales and Wang, 2010) as well as increased overall mortality in patients with kidney disease undergoing dialysis (Wolf et al., 2007).

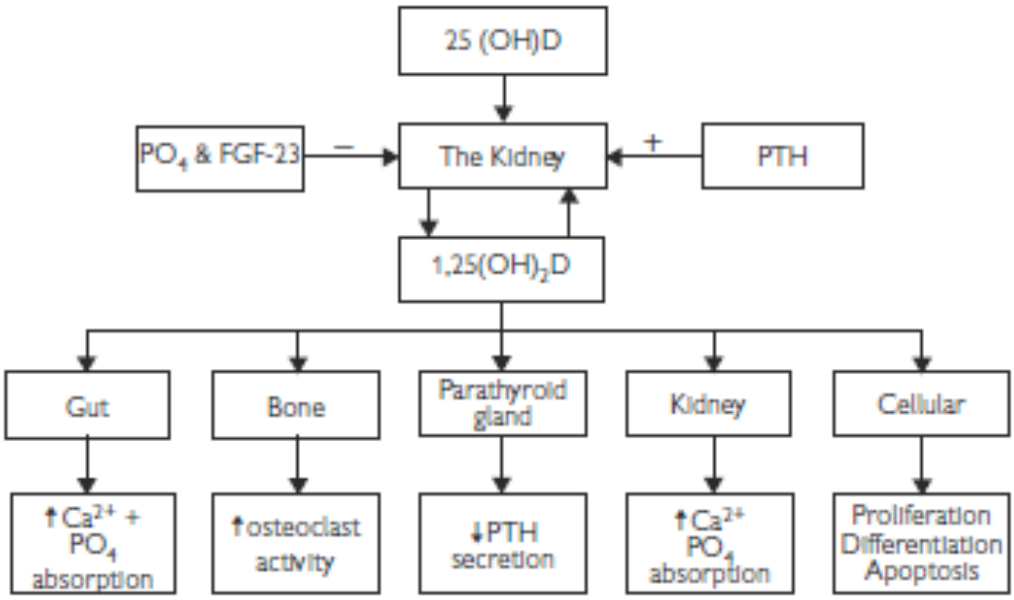


Figure 1.5. An overview of vitamin D function (PO_4 = serum phosphate, Ca^{2+} = serum calcium, FGF-23 = fibroblast growth factor 23). Reproduced from Steddon et al (Steddon, 2006)

1.5.3 Oestrogens

After the menopause, the rate of bone remodelling increases sharply. This increase is mediated through enhanced local cytokine production with consequent augmentation of osteoclastogenesis and osteoblastogenesis (Manolagas et al., 1995). When present, oestrogen suppresses the osteoblastic production of IL-6, as well as of its two receptor

subunits IL-6Rα and gp130 (Jilka, 1998, Lin et al., 1997). Accordingly, concentrations of these are demonstrably higher in oestrogen deficient mice, rats and humans (Miyaura et al., 1995, Bismar et al., 1995, Cheleuitte et al., 1998). Oestrogen deficiency may also increase osteoblastic sensitivity to IL-6 and other gp130 type cytokines (Manolagas, 2000). The production of TNF and M-CSF are also suppressed by oestrogen (Srivastava et al., 1999, Srivastava et al., 1998), while the production of OPG is enhanced (Hofbauer et al., 1999b). Oestrogen lack also increases osteoclast longevity, giving them more time to resorb and erode bone (Hughes et al., 1996, Parfitt et al., 1996, Eriksen et al., 1999).

1.6 Local regulatory factors acting on the bone remodelling process

These factors consist of cytokines and growth factors secreted by skeletal cells (mainly osteoblasts, with a small contribution from other marrow/stromal cells) and other factors embedded in bone at the time of production and released as a result of osteoclastic bone breakdown. As discussed above, modulation of osteoclastic activity is via the osteoblastic processing of systemic (e.g. PTH), or local (e.g. IL-1) signals, predominantly through the RANK/OPG system. Osteoclastic activity can be regulated in several ways; (i) an increase in the recruitment and differentiation of pre-osteoclasts to expand the active osteoclast pool; (ii) modification of the highly specialised cell structure required for active resorption (iii) through the presence, or absence, of chemotactic factors required for osteoclastic movement to areas of bone resorption.

1.6.1 Insulin-like growth factors (IGFs)

The IGFs are a small family of anabolic polypeptides that are structurally related to pro-insulin. Two have been characterized, IGF-I and IGF-II. They are predominantly synthesised in the liver (under the regulation of growth hormone), but are also produced locally within bone (Jones and Clemmons, 1995, Middleton et al., 1995). The two IGFs vary little in their action on *in vitro* and *in vivo* systems, but do vary in their

potency, with IGF-I around five times more potent than IGF II (Middleton et al., 1995). In utero, linear bone growth appears independent of growth hormone and regulated mainly by IGF-I and IGF–II. After birth, however, it is reliant on growth hormone and IGF-I (van der Eerden et al., 2003). IGF-I can act as a systemic hormone or as an autocrine/paracrine growth factor in the skeleton (Slootweg et al., 1990). *In vitro* studies have demonstrated that IGF-I modestly stimulates bone cell proliferation, markedly accelerates osteoblast differentiation, and increases production of several components of bone matrix (Canalis, 1993, Birnbaum et al., 1995). It also increases transcription of the type I collagen gene (Hock et al., 1988) and inhibits the synthesis and release of collagen degradatory enzymes (Canalis et al., 1995). In addition, it may also have a role in the prevention of apoptosis (Hill et al., 1997, Neuberg et al., 1997). *In vivo*, IGF-I deficiency, as noted in IGF-I null mice (and in a single human case with a point mutation in exon 5 of the IGF-I gene), results in a significant growth retardation and extremely low bone mineral density (Bikle et al., 2001, Miyakoshi et al., 2001). Furthermore, osteoblast specific deletion of the IGF-I receptor gene in mice leads to is a marked decrease in both cancellous bone volume and trabecular number along with increased trabecular spacing and decreased matrix mineralisation (Zhang et al., 2002). Conversely, genetically manipulated mice with targeted overexpression of IGF-I in mature osteoblasts, exhibit increased bone formation and enhanced trabecular and cortical bone volume (Zhao et al., 2000). In a large human cohort study of males between the ages of 19 and 85 there was a positive correlation between serum IGF-I levels and bone mineral density (Szulc et al., 2004). These findings implicate IGF-I as a major anabolic factor in the growth and maintenance of skeletal tissue and have engendered much interest in a possible role for IGF-I in the treatment of disorders characterised by bone loss, including osteoporosis. This interest has been qualified, however, by the finding that IGF-I may also stimulate bone resorption. For example, mice with targeted overexpression of IGF-I directed by the osteocalcin promoter have a demonstrable increase in their number of osteoclasts by age 6 weeks (Zhao et al., 2000). The major substrates of IGF-I receptor tyrosine kinases are known to be the

closely related proteins called insulin receptor substrates -1 and -2 (IRS-1 and IRS-2), which become phosphorylated on multiple tyrosine residues following ligand binding (Kadowaki et al., 1996). They have significant structural differences which endow them with distinct signalling properties. Both are expressed in bone (Yamaguchi et al., 2005). It has been proposed that IRS-2 maintains the predominance of bone formation over bone resorption, whereas IRS-1 preserves bone turnover – while the fusion of these two signals can orchestrate the skeletal anabolic response to IGF-I (Akune et al., 2002, Ogata et al., 2000)

Osteoclasts express mRNA for IGF-I, IGF-II, and type I IGF receptor (Middleton et al., 1995). *In vitro*, IGF-I promotes formation of osteoclasts from mononuclear precursors, stimulates activity of pre-existing osteoclasts and increases resorption activity in bone cell cultures (Slootweg et al., 1990, Mochizuki et al., 1992). In osteoblasts from osteoporotic subjects, the IGF-I receptor shows increased basal tyrosine phosphorylation and a blunted response to stimulation by IGF-I (Perrini et al., 2008). Such abnormalities may be responsible for the observed reduction in cell proliferation and impaired bone formation that occur in human osteoporosis. *In vivo* studies with recombinant human growth hormone (rhGH) and/or recombinant IGF-I in elderly subjects or those with GH deficiency have demonstrated a significant increase in both bone formation *and* resorption, especially within the first twelve months (Johannsson et al., 1996, Brixen et al., 1995, Ghiron et al., 1995). More recently it was noted that low dose recombinant IGF-I given for one year in older postmenopausal women, was not associated with an increase in spine or hip bone mineral density, but was associated with higher rates of bone formation than in women receiving both IGF-I and concomitant oestrogen replacement therapy (Friedlander et al., 2001). It has been shown that IGF-I regulates OPG and RANK-L expression both *in vitro* and *in vivo* (Rubin et al., 2002). This may explain the dual activation of both bone formation and bone resorption; the latter effect mediated through the OPG/RANK-L system. The major osteotropic hormones appear to be important in regulating the effects of IGF-I

(and vice versa). Both *in vitro* and *in vivo* studies suggest that the anabolic effects of intermittent PTH are, at least in part, mediated through local increased IGF-I expression (Canalis et al., 1989, McCarthy et al., 1989). Osteoblasts produce several IGF-binding proteins (IGFBPs). Of these, IGFBP-4 binds to IGF and blocks its action, whereas IGFBP-5 promotes IGFs stimulatory effects (Nasu et al., 2000, Marcus et al., 2001).

Another factor in the growth hormone axis that appears to play a role in skeletal metabolism is ghrelin. This 28 amino acid peptide, first identified in the submucosal layer of the stomach in 1999, has been found to strongly stimulate growth hormone release via the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). Ghrelin is widely expressed and has a variety of roles within glucose metabolism and energy homeostasis (Pusztai et al., 2008, Nikolopoulos et al., 2010). It also appears to influence bone metabolism independently of both growth hormone and IGF-I (Cocchi et al., 2005). Expression has been demonstrated in osteoblasts, where it functions to stimulate proliferation (Fukushima et al., 2005) and inhibit apoptosis (Kim et al., 2005, Maccarinelli et al., 2005).

1.6.2 Interleukin 1 (IL-1)

Two forms of IL-1 exist, alpha and beta, with broadly similar biological actions. Locally produced IL-1 is involved in the initiation of bone resorption (Gowen et al., 1983) (Kimble et al., 1994) and like other factors that control resorption, its signal to the osteoclast appears to be processed via the osteoblast and the RANK-L/OPG system, specifically RANKL expression (Hofbauer et al., 1999c). The bone resorbing activity of IL-1β has been associated with osteoporosis, myeloma and rheumatoid arthritis (Hofbauer and Schoppet, 2004, Strand and Kavanaugh, 2004, Roodman, 2001). In inflammatory situations such as the latter, IL-1β, in combination with the potent inflammatory cytokine TNF-α, may play a particularly key role (Kobayashi et al., 2000). What has been more difficult to understand is its stimulatory effect on OPG mRNA

levels and protein release (Hofbauer et al., 1998, Pantouli et al., 2005), which appears to be dependent on the phosphorylation of p38 and ERK, but not the classical NF-κB pathway (Lambert et al., 2007). This production of OPG transcripts may be some form of counter-regulatory process, or the OPG may act as a survival factor via another pathway such as TRAIL. Both remain to be demonstrated, however.

1.6.3 Interleukin-6 (IL-6)

IL-6 is a multifunctional cytokine of around 20 kD. It consists (in man) of 212 amino acids in the precursor form and 183 in the mature form. IL-6 is produced by a wide variety of cells including T and B lymphocytes, macrophages, fibroblasts, mesangial cells, astrocytes, endothelial cells as well as bone marrow stromal cells and osteoblasts. In general it is considered a pro-inflammatory cytokine (Naka et al., 2002, Kishimoto, 2005). It exerts its biological effects via a cell surface receptor consisting of two components: a ligand binding 80 kD glycoprotein (IL-6 receptor, IL-6R) and a non-ligand binding, but signal transducing, gp130 protein-coupled component. Binding of IL-6 induces dimerisation of the two components and tyrosine phosphorylation of the cytoplasmic tail of gp130 (Stahl and Yancopoulos, 1993, Darnell, 1997). This leads (with a degree of interdependence) either to activation of the Ras/MAPK cascade or the recruitment of STAT1 and STAT3 (Ernst and Jenkins, 2004, Kishimoto, 2005). Further regulation is provided by the suppressors of cytokine signalling (SOCS), as discussed later. The α subunit of the IL-6 receptor also exists in a soluble form (sIL-6R), but unlike other soluble cytokine receptors, it functions as an agonist - binding to IL-6 and stimulating JAK/STAT signalling via the gp130 subunit (Stahl and Yancopoulos, 1993). In this way, it bestows IL-6 responsiveness to cells expressing gp130, but missing IL-6Rα. Conversely, the soluble form of gp130 blocks IL-6 action (Narazaki et al., 1993). The source of sIL-6R in bone may be non-skeletal; e.g. liver derived, but it may also be locally released by osteoblasts (Jones et al., 2001, Franchimont et al., 2005a). Many *in vitro* studies have reported that IL-6 exerts minimal influence on osteoblasts unless sIL-6R is present (Franchimont et al., 1997a,

Nishimura et al., 1998, Erices et al., 2002, Franchimont et al., 2005b), although osteoblastic IL-6 receptor expression has been demonstrated *in vivo* (Hoyland et al., 1994). Ligand binding induces progression toward a more mature phenotype and it is possible that IL-6 functions to regulate differentiation of osteoblastic cells as well as to initiate apoptosis in more mature cells (Bellido et al., 1996, Bellido et al., 1997, Li et al., 2008).

IL-6 is produced at high levels by cells of osteoblastic lineage in response to a plethora of cytokines and growth factors, including IL-1, TNF, TGF-ß, PDGF and IGF-II (Girasole et al., 1992, Franchimont and Canalis, 1995, Franchimont et al., 2000, Manolagas, 1998). In addition IL-6 is an important (Grey et al., 1999), although possibly not essential (O'Brien et al., 2005), downstream mediator of PTH action, the effects of which can be antagonised by neutralising IL-6 antibody (Greenfield et al., 1995). Activation of the gp130/STAT3 pathway by IL-6 can stimulate RANKL expression and osteoclastogenesis *in vitro* (Udagawa et al., 1995, O'Brien et al., 1999). However, IL-6 in isolation is insufficient to stimulate osteoclast formation *in vivo* (de la Mata et al., 1995, O'Brien et al., 2005). Furthermore, blockade of either osteoblastic gp130/STAT3 signalling or IL-6 action abolishes osteoclastogenesis stimulated by IL-6, but not by PTH (Devlin et al., 1998, O'Brien et al., 1999). Transgenic mice overexpressing IL-6 demonstrate significant skeletal abnormalities including osteopenia and defective ossification (De Benedetti et al., 2006) and inappropriate expression of IL-6 appears to play an important role in the pathogenesis of skeletal disorders characterised by accelerated bone remodelling and resorption - including lytic metastases (Roodman, 2001), malignant hypercalcaemia (Yoneda et al., 1993), multiple myeloma (Callander and Roodman, 2001), renal osteodystrophy (Langub et al., 1996), hyperthyroidism (Siddiqi et al., 1998, Siddiqi et al., 1999) and osteoporosis (Manolagas and Jilka, 1995). However, IL-6 knockout mice have a surprisingly normal skeletal phenotype, although they do demonstrate cortical microstructural abnormalities, delayed fracture healing and reduced bone resorption following

oestrogen depletion (Poli et al., 1994, Yang et al., 2007). They are also protected against joint inflammation in both collagen and antigen induced arthritis (Alonzi et al., 1998, Ohshima et al., 1998). Moreover the number of osteoclasts was found to be unexpectedly increased in foetuses where gp130 had been knocked out (Kawasaki et al., 1997). It is therefore possible that while IL-6 may not be mandatory for physiological bone remodelling under normal circumstances, it may undertake a more dominant role when prevailing turnover is high (Franchimont et al., 2005b). This may be mediated through regulation of both its receptor and downstream signalling biology. For example, mice carrying a point mutation that favours JAK/STAT signalling by selectively blocking the Ras/MAPK pathway display increased bone turnover and decreased trabecular bone volume. When crossed with IL-6 knockouts, the level of bone formation returned to the level of wild type mice (Sims et al., 2004, Sims, 2009).

1.7 Renal bone disease

Renal bone disease, or osteodystrophy, is one facet of chronic kidney disease – mineral and bone disorder (CKD-MBD). This relatively new term aims to encompass all the mineral, skeletal, and related cardiovascular consequences of chronic kidney diseases, including bony pathology and vascular calcification (Moe et al., 2006). This expanded focus has been driven by observational studies suggesting that CKD-MBD (and its treatment) may influence morbidity and mortality in patients with kidney disease (Block et al., 1998, Tentori et al., 2008, Covic et al., 2009). Vascular disease is common and progresses rapidly in CKD - typically occlusive lesions due to atheromatous plaques. However, the full spectrum of arterial disease in these patients is broader, including vessel wall calcification with resulting impaired vascular function (Moe and Chen, 2008). The prevalence of both soft tissue and vascular calcification far exceeds the general population and progression is vastly accelerated. Increasingly, the phenomenon appears intimately linked to the well described disturbances in mineral and bone metabolism (Moe, 2006a). In fact, a poorly understood association between arterial calcification and bone disease is long established in the context of osteoporosis (Hofbauer et al., 2007) and the evidence trail in this context has rapidly expanded. It includes the observation that calcium deposited in vessels is in the form of hydroxyapatite crystals - the same as in the skeleton (Zaheer et al., 2006), and the demonstration that several stimuli, including inorganic phosphate and uraemic serum, can promote differentiation of vascular smooth muscle cells to an osteoblastic phenotype via upregulation of the sodium-dependent co-transporter, Pit-1 (Chen et al., 2002, Moe, 2006b, Lau et al.). The term renal bone disease (osteodystrophy) is now reserved to describe the specific bone pathologies associated with CKD.

The pathophysiology of renal bone disease is extremely complex, but, essentially, a reduction in functioning renal mass causes the accumulation of phosphate and a deficiency of $1,25(OH)_2D$. These, in turn, generate a reduction in the serum calcium

concentration and all three, will if uncorrected, relentlessly drive the development of secondary hyperparathyroidism, with eventual clonal proliferation of parathyroid cells (Mondry et al., 2005, Hruska et al., 2007)

KDIGO classification of CKD-MBD and renal osteodystrophy
Definition of CKD-MBD
A systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following • Abnormalities of calcium, phosphorus, PTH or vitamin D metabolism • Abnormalities of bone turnover, mineralisation, volume, linear growth or strength • Vascular or other soft tissue calcification
Definition of renal osteodystrophy
• Renal osteodystrophy is an alteration of bone morphology in patients with CKD • It is one measure of the skeletal component of the systemic disorder that is quantifiable by histomorphometry of bone biopsy material

Table 1.2 Classification of mineral and bone disorders in chronic kidney disease. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group (Moe, 2009).

These clones express less calcium sensing and vitamin D receptors, further constraining the effects of therapy (Steddon and Cunningham, 2005). This later situation is termed tertiary or autonomous hyperparathyroidism. In addition there is a relative 'skeletal resistance' to the effects of PTH, the cause of which is unclear (Friedman and Goodman, 2006). The end product is an abnormal bone remodelling cycle and, ultimately, diminished bone strength. Within this framework, fibroblast growth factor 23 (FGF-23) has recently emerged as an important pathophysiological driver (Juppner et al.). FGF-23 is produced in bone and its concentration increases as renal function falls. This elevation causes potent suppression of 1α-hydroxylase and therefore $1,25(OH)_2D$ production. FGF-23 excess is now known to underlie autosomal dominant hypophosphataemic rickets, x-linked hypophosphataemia and tumour

induced osteomalacia (all characterized by phosphate wasting, reduced $1,25(OH)_2D$ and skeletal abnormalities) (Amatschek et al.). In addition, FGF-23 is also emerging as a very powerful biomarker of mortality (Gutierrez et al., 2008).

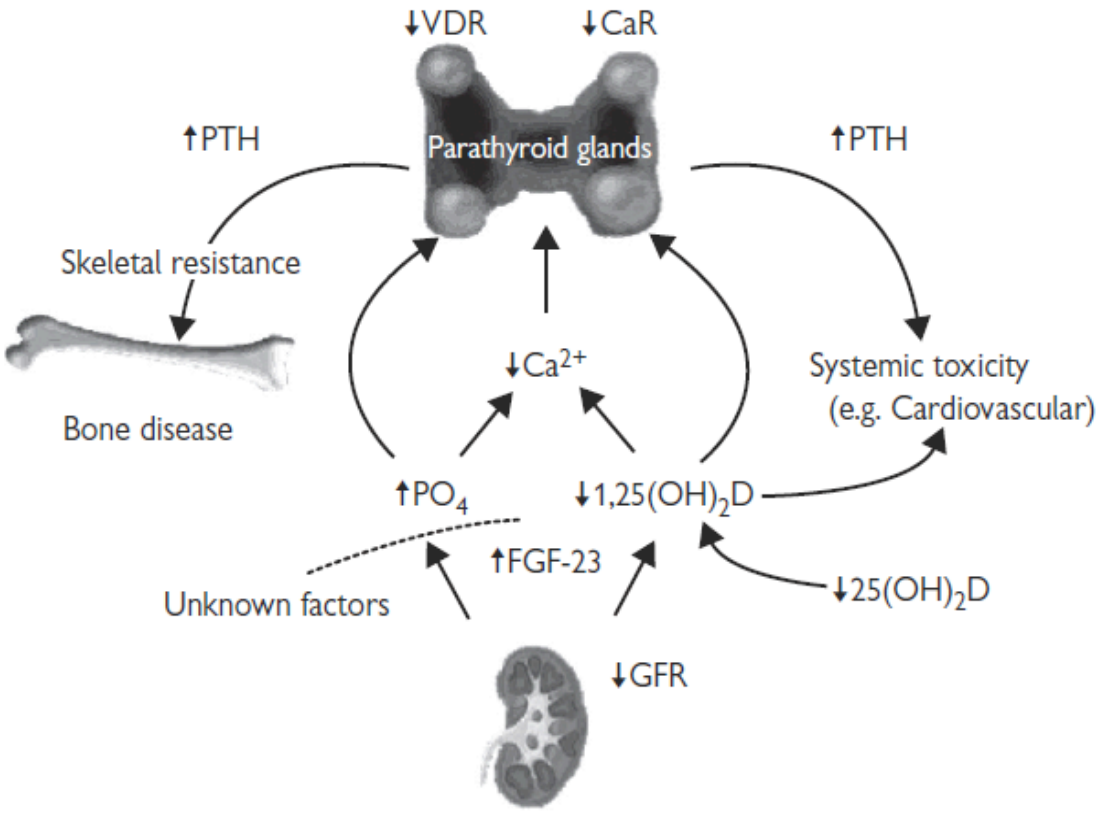


Figure 1.6: The pathogenesis of secondary hyperparathyroidism. Modified from Steddon et al (Steddon, 2006)

Renal osteodystrophy is now defined and considered in terms of bone turnover, mineralisation and volume (Malluche and Monier-Faugere, 2006). Strictly speaking a bone biopsy, usually from the iliac bone, with both histological and histomorphometric assessment is required for accurate diagnosis and classification, but, in practice, these are very rarely performed (as they are invasive and require considerable – and scarce - expertise for their interpretation), so surrogate markers of bone turnover, including serum PTH and alkaline phosphatase, are generally utilised. Osteodystrophy represents a histological continuum, with high and low turnover disease at opposing ends (Favus and Christakos, 1999). High turnover disease corresponds to the clinical

entity of secondary hyperparathyroidism. It is characterised by increased numbers of osteoblasts and osteoclasts, abnormally high rates of bone formation and a high serum PTH (Slatopolsky et al., 1999). Haphazardly organized, weakened bone results, which is classically described as osteitis fibrosa cystica. Low turnover disease, or adynamic bone disease (ABD), is typified by reduced numbers of osteoblasts and osteoclasts, very low rates of bone formation and a serum PTH that is within (or just above) the normal range (Malluche and Monier-Faugere, 1992). However, these high and low bone turnover states are not mutually exclusive entities; they can, and often do, co-exist in bone biopsy specimens (Malluche and Monier-Faugere, 1994). This can be difficult to conceptualise because they are usually categorised clinically in terms of an absolute value of serum PTH, rather than within a histological framework. Osteomalacia generally refers to a defect in mineralisation; however, in the context of chronic kidney disease, this is a relatively uncommon isolated finding and therefore not a particularly helpful clinical description. It is generally related to a deficiency of 1,25(OH)2D (calcitriol), but aluminium intoxication and uraemic acidosis are also important risk factors (Steddon, 2006).

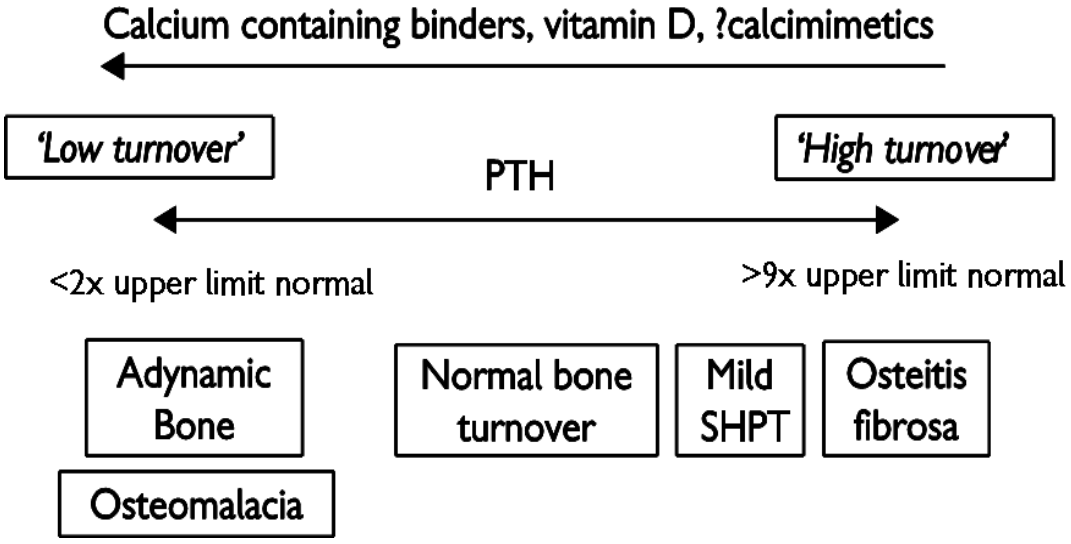


Figure 1.7: The spectrum of renal osteodystrophy, including the potential influence of common treatments. Reproduced, with modifications, from (Steddon, 2006). SHPT = secondary hyperparathyroidism.

Adynamic bone disease is an increasingly common lesion in CKD (Salusky and Goodman, 2001, Frazao and Martins, 2009). Histologically, ABD is characterised by a reduction in the numbers of osteoblasts and osteoclasts and a paucity of bone formation (Malluche et al., 1997). The appearance of ABD is often viewed as the cost of striving to normalise bone turnover through suppression of PTH, though it almost certainly has a more complex pathophysiological basis (Salusky and Goodman, 2001, Ferreira, 2006). Although historically linked with aluminium toxicity, this association has depreciated in contemporary relevance. The major associations of ABD are treatment with active vitamin D, calcium overload and, possibly, choice of peritoneal dialysis as modality of renal replacement therapy (RRT) (Frazao and Martins, 2009). In addition, in a significant proportion of the CKD population, the coexistence of other low turnover states, including age related, or post-menopausal, osteoporosis and diabetes mellitus, may predispose to the adynamic lesion (Rigalleau et al., 2007, Miller, 2009). As discussed, a low serum PTH, often in combination with a high serum calcium, is used clinically as a surrogate marker for ABD. Though useful, this may serve to exaggerate the importance of PTH in the evolution of the disorder. The occurrence of ABD in those with persistent elevations of PTH despite administration of active vitamin D implies a degree of PTH independence (Qi et al., 1995, Wang et al., 1995). Calcitriol itself may be culpable through an inhibitory influence on osteoblast function (Brown et al., 1999). Support for this comes from the differential properties, at the level of bone, of the newer analogues of vitamin D (or so called vitamin D receptor analogues, VDRAs). These have demonstrable differences in their ability to stimulate the production of IL-6 (McIntyre et al., 1999). In addition, the analogue 22-oxacalcitriol has been shown to suppress PTH in the uraemic dog without increasing the risk of adynamic bone (Monier-Faugere et al., 1999). Despite these, and other, findings, ABD cannot be regarded solely as a by-product of calcitriol therapy; the appearance of ABD in biopsy specimens prior to calcitriol administration militates strongly against this (Malluche et al., 2008). PTH and calcitriol coordinate the latter steps in osteoblast and osteoclast differentiation, while other growth factors and cytokines coordinate the early stages. It

may be that anomalies in the synthesis and secretion of the latter may be more relevant to the development of ABD.

1.7.1 Effects of chronic kidney disease on skeletal biology

Given the preceding discussion concerning the relatively recent expansion of our understanding of the complex physiology within bone – and, in particular, the recognition that a multitude of growth factors and cytokines occupy important roles in the remodelling cycle – it seems almost certain that the heterogeneous manifestations of renal bone disease will not be explained exclusively within a framework of aberrant PTH and vitamin D metabolism. Abnormalities in several other pathways are likely to occur during the progression of CKD and require exploration for their potential contribution to the abnormal bone biology found in the uraemic state. In general, it has been inferred from abnormal serum concentrations of cytokines that their coordination of the remodelling cycle may be disturbed in CKD (Hory and Drueke, 1997) (Gonzalez, 2000). For example, high levels of IL-1β have been consistently demonstrated in dialysis patients (Herbelin et al., 1990, Ferreira et al., 1996), as have high levels of the IL-1 receptor antagonist (IL-1Ra), which opposes IL-1β's cellular effect (i.e. a high serum concentration of the IL-1Ra makes the skeleton relatively insensitive to circulating IL-1β) (Moutabarrik et al., 1995). In addition, concentrations of TNF-α, another promoter of remodelling, are demonstrably higher in the circulation of uraemic patients (Herbelin et al., 1990). Elevated levels of IL-6 and the sIL-6R have been reported in both pre-dialysis CKD patients and patients on dialysis (Herbelin et al., 1991, Le Meur et al., 1999), as has a relationship between IL-6 concentration and other markers of bone turnover (Montalban et al., 1999). There also appears to be an inverse correlation between the sIL-6R:IL-6 ratio and the surface area of osteoclast activity (Ferreira et al., 1996). IL-6 receptor mRNA is present in osteoclasts of CKD patients, with the level of expression corresponding to their bone resorbing activity (Langub et al., 1996). Recently, several cytokines, including IL-1, TNF-α and IL-6 and were shown

to be positively correlated with bone formation rate in prevalent dialysis patients by histomorphometric criteria (Ferreira et al., 2009). This association was particularly strong for IL-6.

Increased circulating levels of IGF-I have also been reported, and there is evidence to suggest that bone formation rate correlates with IGF-I concentration in these patients (Andress et al., 1989). There is also robust evidence that individuals with kidney failure have relative IGF-I resistance. This resistance appears to be multilayered; there is an elevation of IGF binding protein-1 which in itself is likely to reduce IGF-I activity (Jain et al., 1998). There may also be an IGF-I receptor defect (Feld and Hirschberg, 1996). In addition, IGF-I production by a variety of tissues appears to be reduced in uraemic animals, with demonstrably less mRNA levels in the steady state in liver (Tonshoff et al., 1997), skeletal muscle (Ding et al., 1996) and growth plate cartilage (Hanna et al., 1995). Studies utilising in situ hybridisation have analysed IGF-I expression in uraemic bone (Picton et al., 2000, Hoyland and Picton, 1999). Expression was found predominantly in osteoblasts with semi-quantitative analysis showing a decrease in mRNA in renal bone compared to normal and non-uraemic high turnover bone (healing fracture callus and bone from patients with Paget's disease). Levels of expression were significantly lower in adynamic bone that in high turnover hyperparathyroid bone. This implies that there is a downregulation of osteoblast IGF-I in renal bone. Comparison of IGF-I mRNA signal density with serum biochemical markers showed a significant correlation with intact PTH levels in patients with adynamic bone. This is consistent with studies demonstrating lower levels of osteoblastic IGF-I mRNA expression in older bone where similarities with adynamic bone might be expected (Tanaka et al., 1996, Wakisaka et al., 1998).

Although the OPG/RANKL system has only come under scrutiny in the context of renal bone disease relatively recently, aberrations might have been expected as it is, at least in part, regulated by osteotropic hormones known to be affected in kidney failure. High

circulating levels of OPG are now well documented in CKD patients (Kazama et al., 2002, Gonnelli et al., 2005, Wittersheim et al., 2006), and it seems likely they will have some impact on remodelling (Padagas et al., 2006, Fahrleitner-Pammer et al., 2009). They also act as biomarkers of cardiovascular risk (Mikami et al., 2008, Sigrist et al., 2009).

Epidermal growth factor is also known to affect osteoblast development, and the uraemic state may affect the actions of EGF on bone by regulating the expression of its receptor (Drake et al., 1994). A member of the TGF-β family, bone morphogenetic protein-1 (BMP-1), crucial for osteoblast differentiation, is normally manufactured in the kidney, therefore a deficiency as renal mass declines could potentially influence osteoblast development and contribute to the low turnover bone (Ozkaynak et al., 1991). Furthermore, deficiency of other bone morphogenetic proteins in CKD may contribute in additional ways to disordered remodelling. Of particular note, treatment with BMP-7 in the relevant animal models results in normalisation of both high and low turnover states (Hruska et al., 2004)

Metabolic bone disorder	Cellular changes	Probable mechanism
Sex steroid deficiency (Jilka, 1998, Pacifici, 1988, Manolagas et al., 2002	↑Osteoblastogenesis ↑Osteoclastogenesis ↑Lifespan of osteoclasts ↓Lifespan of osteoblasts ↓Lifespan of osteocytes	Increased IL-6; TNF; IL-1; MCSF Decreased TGF-β; OPG Oestrogen deficiency extends the working life of osteoclasts while shortening that of osteoblasts through pro- and anti-apoptotic effects.
Old age Garnero et al., 1996, Nuttal et al., 1998, Manolagas and Parfitt, 2010)	↓Osteoblastogenesis ↓Osteoclastogenesis ↑Adipogenesis ↓Lifespan of osteocytes	Increased PPARγ2, Prostaglandin J2, noggin Decreased IL-11, IGFs
Glucocorticoid excess (Manolagas, 2010)	↓Osteoblastogenesis ↓Osteoclastogenesis ↑Adipogenesis ↑Lifespan of osteoclasts ↓Lifespan of osteoblasts ↓Lifespan of osteocytes	Decreased Cbfa1 and TGF-β R Decreased BMP-2 and IGF-I action Decreased Bcl-2/Bax ratio Increased PPARγ2
Renal bone disease: secondary hyperparathyroidism (Montalban et al., 1999, Fahrleitner-Pammer et al., 2009)	↑Osteoblastogenesis ↑Osteoclastogenesis	Increased PTH; decreased calcitriol (?) IL-6 (?)RANK-L/OPG system
Renal bone disease: adynamic bone disease (Ozkaynak et al., 1991, Hoyland and Picton, 1999)	↓Osteoblastogenesis ↓Osteoclastogenesis (?)↓Lifespan of osteoblasts (?)↓Lifespan of osteocytes	(?) IGF-I (?) BMP-1

Table 1.3: Cellular changes and pathophysiological basis in the common metabolic bone disorders (adapted from (Manolagas, 2000)

1.8 Cytokine signalling

Growth factors and hormones are clearly extremely important effectors of the communication network necessary for the well being of bone in health and disease. At the core of this network is a cytokine receptor superfamily, consisting of cell surface receptors and receptor coupled intracellular signalling mechanisms. These determine the timing, nature and strength of the cellular response to an external cytokine stimulus. The basic components of this cellular apparatus are receptor associated Janus tyrosine kinases (JAK) and cytoplasmic transcription factors called 'signal transducers and activators of transcription' (STATs).

1.8.1 STAT Transcription Factors

STATs were first discovered as mediators of interferon-induced gene expression (Darnell et al., 1994). They comprise a family of latent proteins within the cytoplasm that facilitate gene regulation after cell receptors encounter various extracellular peptides. The STAT family consists of seven members that are activated by many cytokines and growth factor (Table 1.4). Their role in cell signalling and development has been elucidated through analysis of transgenic mice lacking individual STAT genes (Akira, 1999, Aaronson and Horvath, 2002).

The STAT proteins are unique among transcription factors, in that they contain an Src homology-2 (SH2)-binding domain (Shuai et al., 1994). Tyrosine phosphorylation is essential for their dimerization and subsequent nuclear translocation. Receptors that catalyze this phosphorylation include those with intrinsic tyrosine kinase activity, such as epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) as well as those that lack such intrinsic activity but have Janus kinases (JAKs) non-covalently associated (Leaman et al., 1996, Ihle et al., 1995). Phosphorylated STAT monomers form dimers that translocate to the nucleus and bind to specific DNA target sequences to regulate gene transcription (Ihle et al., 1997, Ihle et al., 1998, Ihle, 2001b). JAK associated receptors often have a cytokine ligand, including IFN-α, -β and -γ;

interleukins -2, -7, -10 -13, and -15, as well as EPO, growth hormone and other polypeptides (Schindler et al., 2007). STAT dimers and heterodimers, but not monomers, are capable of DNA binding. The known DNA binding heterodimers are STAT1:2 (requires the presence of another protein, p48) and STAT1:3. Homodimeric DNA binding STATs include STAT 1, 3, 4, 5 (STAT5A and 5B interact in a manner equivalent to a heterodimer) and 6 (Schindler and Darnell, 1995). In most cases, STAT activation is transient. Inactivation is carried out by several mechanisms, including dephosphorylation of STAT proteins in the nucleus and degradation through the ubiquitin-proteosome pathways (Kim and Maniatis, 1996).

Factor	Mass (kDa)	Activating Cytokine	Reference
STAT1	91	IFN-α, IFN-γ, EGF, PDGF, FGF, ACRII,	(Darnell et al., 1994)
STAT2	113	IFN-α/β (with STAT1)	(Darnell et al., 1994)
STAT3	92	IL-6, LIF, CNTF, OM, CT-1, EGF, G-CSF, IL-10, leptin	(Darnell et al., 1994) (Zhong et al., 1994)
STAT4	89	IL-12	(Zhong et al., 1994) (Ihle and Kerr, 1995)
STAT5A & 5B	77, 80	IL-2, IL-3, IL-5, IL-7, IL-15, GM-CSF, Prolactin, Epo	(Pallard et al., 1995) (Azam et al., 1995)
STAT6	94	IL-4, IL-13	(Lin et al., 1995) (Quelle et al., 1995)

Table 1.4 The STAT family and their activating cytokines

1.8.2. The suppressors of cytokine signalling

The range of action and potency of cytokines mandate that their actions are tightly regulated to avoid detrimental consequences (Yasukawa et al., 2000). A variety of counter-regulatory measures exist to control the magnitude and duration of cytokine activity. At the extracellular level, these include specific cytokine antagonists, such as IL-1Ra, and soluble receptors, such as sIL-6R. Control can also be exerted within the cell; for example specific phosphatases, such as SHP1 and SHP2, can dephosphorylate JAK/STAT proteins (Matozaki and Kasuga, 1996). In addition, a relatively recently discovered family of cytokine inducible proteins can downregulate cytokine-activated JAK/STAT signalling pathways. These proteins, which represent an important mechanism for self limiting the cellular cytokine response have been designated the suppressors of cytokine signalling (SOCS) (Krebs and Hilton, 2001). The first member of the SOCS family to be discovered, cytokine-induced SH2 domain containing protein (CIS), was described as an immediate early-response gene, the expression of which could be induced by several cytokines (Yoshimura et al., 1995). The second family member was discovered, independently, in three different laboratories and given the names SOCS1, JAK-binding protein (JAB) and STAT induced STAT-inhibitor (SSI) (Endo et al., 1997, Starr et al., 1997, Naka et al., 1999). This confusing nomenclature has been resolved - with SOCS now the most widely used description. At present, the SOCS family contains eight members, SOCS1-SOCS7 and CIS. Each member contains a characteristic central SH2 domain and a carboxy-terminal 40 amino acid 'SOCS box'. Database inspection has revealed a large number of SOCS- related proteins in humans and mice. While these proteins all contain the 40-residue C-terminal motif SOCS box, their physiological significance is unclear (Hilton et al., 1998). SOCS1 and SOCS3 also contain a conserved 12-residue sequence – the kinase inhibitory region (Yasukawa et al., 1999).

Generally, the SOCS genes are not highly expressed in unstimulated tissues, but can be induced rapidly by various cytokines, including those belonging to the interferon, interleukin, and colony-stimulating factor families (table 1.4). In addition, growth hormone, prolactin, leptin, lipopolysaccharide, angiotensin II and growth factors, such as epidermal growth factor (EGF) have been shown to induce the expression of various SOCS (Alexander and Hilton, 2004, Yoshimura, 2009). Individual SOCS proteins are able to inhibit multiple cytokines, although it is not yet apparent how the specificity of SOCS inhibition is regulated. Of interest is the structural homology between SOCS pairs such as SOCS1/SOCS3, CIS/SOCS2 and SOCS6/7 (Dalpke et al., 2008).

Gene	Main Affected Cytokine
CIS	STAT5 signalling (EPO, IL-2, IL-3)
SOCS1	IFN-γ, IFN-α, IL-4, IL-12, LPS
SOCS2	GH, IGF-I
SOCS3	Gp130, IL-2, IL-6, G-CSF, leptin, EPO
SOCS4	Unknown
SOCS5	IL-4, EGF
SOCS6	?insulin
SOCS7	?insulin

Table 1.5. Differential inducibility of the SOCS genes by various cytokines

The most well characterised SOCS family members, CIS, SOCS1, SOCS2 and SOCS3, act in a classical negative feedback loop to inhibit cytokine signal transduction. Both *in vitro* and *in vivo* studies show that cytokine induced SOCS expression is usually dependent on the JAK/STAT signalling pathway (Starr et al.,

1997). Indeed, the promoters of many SOCS genes contain STAT- responsive DNA elements (Naka et al., 1997). However, it is also becoming clear that non-JAK/STAT-dependent regulation of SOCS expression can occur, as has been described in the case of IL-10 stimulated SOCS3 expression in neutrophils (Cassatella et al., 1999). IL-10 upregulation of SOCS3 expression in monocytes is linked to the ability of this cytokine to suppress the actions of many pro-inflammatory cytokines, such as IFN-γ, serving to highlight the potential for cross-regulation between different cytokine signalling pathways via the regulation of SOCS proteins (Ito et al., 1999, Alexander et al., 1999). Furthermore, SOCS1 is highly induced by LPS, implying that SOCS1 also inhibits the toll-like receptor (TLR)-NF-κB pathway (Mansell et al., 2006).

The ability of the SOCS molecules to inhibit cytokine-activated JAK/STAT signalling involves several distinct mechanisms, including receptor interaction, direct JAK inhibition and targeting receptor complex and other signalling proteins for proteasomal degradation. SOCS1 can directly associate with high affinity with all four JAK molecules directly inhibiting their catalytic ability (Nicholson et al., 1999, Yasukawa et al., 1999, Alexander, 2002). The SH2 domain is responsible for the association between SOCS1 and JAK2, and the N-terminal amino acid region possesses kinase inhibitory activity. SOCS3 may function through an interaction with activated cytokine receptors, though it does not appear to interfere with STAT recruitment. For example, it has been shown to associate with the activated GH receptor and inhibit STAT5 signalling without preventing STAT5: receptor binding (Hansen et al., 1999). It has been shown to bind the gp130 receptor at a specific phosphorylated receptor tyrosine identical to that used by the SH2-domain containing tyrosine phosphatase-2 (SHP2), so it may compete for receptor phosphotyrosine docking sites (Schmitz et al., 2000, Nicholson et al., 2000). Finally, the conserved SOCS box domain interacts with elongins B and C to target proteins for degradation through ubiquitination (Zhang et al., 2001, Kamura et al., 1998). So, the SOCS proteins seem to combine specific and

direct inhibitory actions on components of the signalling cascade with a generic mechanism that targets these components for ubiquitin mediated proteasomal degradation. Consistent with this model, the activation of JAKS and STATS can be prolonged in the presence of proteasome inhibitors (Verdier et al., 1998).

It might be inferred from the above discussion that that the SOCS proteins negatively regulate a variety of non-specific, and often unrelated, cytokines. However, *in vivo* data indicate a much more restricted and refined role. The most direct evidence of SOCS function comes from studies utilising gene deletion or overexpression of individual SOCS genes (table 1.6).

Gene	Phenotype in knockout mice	Phenotype when overexpressed in transgenic mice
CIS	Not extensively examined, but reported to have few abnormalities (Marine et al., 1999a). Possible increased haematopoiesis and disturbed lactation.	**Widespread overexpression**: low body weight, lactation failure, fewer splenic γδ T cells, NK cells and NKT cells, preferential T_H2 differentiation, reduced IL-2 signalling (Matsumoto et al., 1999). **T-cell-targeted overexpression**: little effect *in vivo*, altered T-cell receptor responses *in vitro* (Li et al., 2000).
SOCS1	Neonatal fatality caused by fatty degeneration of the liver with haematopoietic infiltration of multiple organs, lymphopenia, apoptosis in lymphoid organs and aberrant T-cell activation. Primarily associated with deregulated responses to IFN-γ (Marine et al., 1999b, Starr et al., 1998, Naka et al., 2001).	**T-cell-targeted overexpression**: suppression signalling by multiple cytokines. T-cell developmental abnormalities with relative increase in $CD4^+$ T cells, fewer γδ t cells and spontaneous T-cell activation (Fujimoto et al., 2000).
SOCS2	Gigantism with evidence of deregulated GH and/or IGF-I signalling (Metcalf et al., 2000).	Gigantism
SOCS3	Mid-gestational embryonic lethality due to placental insufficiency (Roberts et al., 2001), embryonic lethality with erythrocytosis (Marine et al., 1999a).	**Haematopoietic cell-targeted overexpression**: embryonic lethality with increased T_H2 differentiation and anaemia (Marine et al., 1999a).

Table 1.6: The biological functions of the best-characterised SOCS proteins: evidence from SOCS gene manipulation in mice

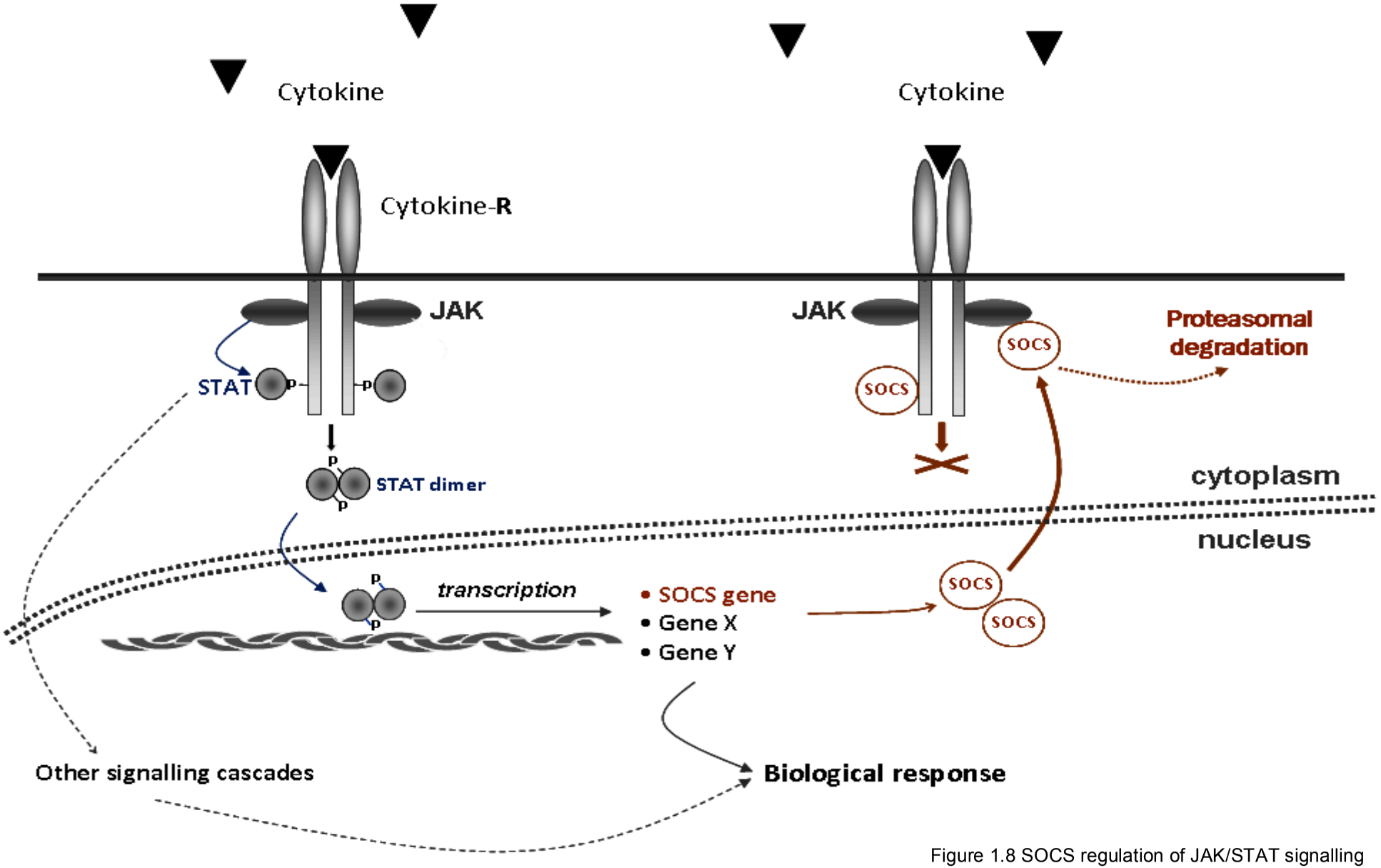


Figure 1.8 SOCS regulation of JAK/STAT signalling

Figure 1.8

In unstimulated cells JAKs and STATs are inactive and, typically, SOCS genes are not expressed. After binding of cytokines, receptor aggregation occurs, bringing receptor associated JAKs together and allowing cross-phosphorylation and activation. These kinases then phosphorylate multiple target proteins, including tyrosine residues in the cytoplasmic domains of the receptor and receptor associated STAT monomers. Phosphorylated STAT monomers form dimers that translocate to the nucleus and bind to specific DNA target sequences to regulate the transcription of biological response genes, including SOCS. The ability of the SOCS molecules to influence signalling involves several disparate mechanisms. In essence, they combine direct inhibitory actions on specific components of the signalling cascade with more generic mechanisms that target components for ubiquitin mediated proteasomal degradation. The disappearance of SOCS proteins once they have acted to shut down signalling allows the cycle of cytokine stimulation to be completed and the cell to return to its cytokine-responsive state. See text for references and (Alexander and Hilton, 2004, Dalpke et al., 2008, Yoshimura, 2009) for detailed reviews.

1.8.3 SOCS in immune, infectious and inflammatory disease

The regulation of cytokine signalling by differential SOCS expression is implicated in the pathogenesis of various immune, infectious and inflammatory diseases. Evidence is emerging for the particular involvement of SOCS1 and SOCS3 in the latter, including rheumatoid arthritis, dermatitis and inflammatory bowel disease. In many circumstances, SOCS expression appears to exert a negative influence on inflammation (Chen et al., 2004, Fujimoto et al., 2004). For example, in a mouse model of colitis, colonic expression of SOCS3 is increased, as it is in intestinal T cells from patients with Crohn's disease (Suzuki et al., 2001, Niemand et al., 2003). SOCS3 inhibition causes STAT3 overactivity and increases disease. However, in asthma, disease severity correlates with the magnitude of SOCS3 expression (Tang and Raines, 2005), perhaps indicating a role in promotion as well as inhibition of inflammatory processes.

1.8.4 The suppressors of cytokine signalling in bone

The presence and function of the SOCS family of cytokine regulators within skeletal tissue has not been studied in detail. In the rat osteosarcoma cell line, UMR106, SOCS1 and CIS appear to play a role in GH signalling (Morales et al., 2002). Pre-treatment with $1,25(OH)_2D_3$ allows a prolonged response to subsequent JAK2/STAT5 mediated GH signalling as well as permitting a response to repetitive GH stimulation. Since pre-treatment with $1,25(OH)_2D_3$ is also associated with the inhibition of subsequent GH induced expression of SOCS3 and CIS, it has been suggested that the release of the JAK2/STAT5 pathway from the negative influence of SOCS proteins explains the effects of $1,25(OH)_2D_3$. The smaller size of SOCS1 knockout mice has prompted further skeletal evaluation (Abe et al., 2006). Undercalcified areas were found in the skull, sternum and certain regions of cortical bone in SOCS1 knockouts and the mineralisation activity of primary cultured calvarial cells from these animals was significantly impaired. Furthermore *in situ* hybridisation demonstrated a significant decrease in osteocalcin, a late marker of osteoblastic differentiation, although early

markers were comparable. Recent genome wide association staudies have identified SOCS2 as one of the 20 loci that influence human adult height (Weedon et al., 2008). An insight into how SOCS may be involved in the counterbalance of positive and negative signals in bone, and on the osteoclast in particular has previously been demonstrated. Expression of SOCS, predominantly SOCS3, can be induced in osteoclast progenitors by exposure to RANK-L and M-CSF, both of which are positive influences on progenitor recruitment. In turn, SOCS3 inhibits STAT-1 phosphorylation and suppresses the signalling of the potentially negative influence of IFN-ß (Hayashi et al., 2002).

A possible role for SOCS in inflammatory skeletal diseases has been inferred from animal models of arthritis (Rottapel, 2001). Overexpression of SOCS3 in joint macrophages and fibroblasts blocks STAT3 activation and attenuates disease severity (Shouda et al., 2001). Recently, a role for SOCS1 as a negative regulator in the experimental methylated BSA/IL-1 arthritis model has been demonstrated in double knockout SOCS1-/- IFN-γ -/- mice (Egan et al., 2003). These animals do not develop the severe spontaneous inflammation and perinatal lethality manifested by single knockout SOCS1 -/- mice and have been developed to explore the *in vivo* role of SOCS1. Induction of this particular form of arthritis, which is known to proceed independently of IFN-γ, in these mice led to disease of similar duration, but markedly increased severity. This has created interest in SOCS1 as a possible therapeutic target in inflammatory disease (Ivashkiv and Tassiulas, 2003).

1.8.4 The suppressors of cytokine signalling in uraemia

The uraemic state is associated with resistance to the action of growth hormone (GH). This manifests clinically as impaired growth and short stature in children with chronic renal disease. The basis of this resistance remains unclear, though several mechanisms have been suggested, including decreased GH receptor (GHR) gene expression (Tonshoff et al., 1994, Tonshoff et al., 1997), increased IGF-I binding to

serum proteins (Tonshoff et al., 1995) and post-receptor defects in IGF-I action (Ding et al., 1996). Partial GH resistance is reproducible in the uraemic rat (Chan et al., 1993). Binding of GH to its membrane receptor induces intracellular signalling via the JAK2/STAT5 (and to a lesser degree STAT3 and STAT1) pathways. The concomitant induction of SOCS expression allows negative feedback at the level of the GHR-JAK2 complex (Ram and Waxman, 1999, Tollet-Egnell et al., 1999). Whereas SOCS1 and SOCS3 appear to be stronger inhibitors than SOCS2 or CIS in transfection models, *in vivo* evidence indicates a clear role for the latter; deletion of SOCS2 causes IGF-I hyper-expression and gigantism in mice (Metcalf et al., 2000), while CIS overexpression causes severe growth retardation (Matsumoto et al., 1999). This makes the JAK-STAT signalling pathway an attractive target for developing a post-receptor paradigm to explain to uraemic GH resistance. This has been further explored in the 5/6th nephrectomy uraemic rat model, where the response to GH treatment, in terms of weight gain and hepatic IGF-I and IGFBP-I mRNA levels, appears abrogated in comparison to sham operated pair fed controls (Schaefer et al., 2001). To determine the basis of this resistance, both ligand/receptor binding and downstream signal transduction through the JAK-STAT pathway were further investigated. Hepatic GHR mRNA levels were significantly decreased in the uraemic animals, though there was no difference in GHR protein level. GH binding to microsomal and plasma membranes was unchanged. Amounts of JAK2, STAT1, STAT3, and STAT5 protein were also unchanged. However, GH induced tyrosine phosphorylation of JAK2, STAT5, and STAT3 was significantly lower in the uraemic animals. This was associated with a two-fold increase in SOCS2 expression in GH treated uraemic animals and a 60% elevation in SOCS3, independent of GH treatment. Thus there appeared to be a post-receptor defect in GH signal transduction characterised by impaired phosphorylation of and nuclear translocation of GH activated STAT proteins. This was mediated, at least in part, by overexpression of the SOCS proteins. The authors speculated that the persistent proinflammatory cytokine release and sub-clinical inflammation associated with the uraemic state might contribute to GH resistance through an increase in SOCS

protein production. The mechanism for the increase in SOCS mRNA levels in the presence of impaired GH-activated JAK2/STAT signalling in uraemia raises important questions. It is possible that SOCS gene expression is induced through cytokine signalling via a different Janus kinase. Alternatively, the increase in SOCS expression in uraemia might itself be mediated by GH, but through a non-STAT pathway. In this regard, there is evidence that nuclear STAT-binding sites are not essential for GH activation of the SOCS3 promoter (Paul et al., 2000). A similar molecular basis has been proposed for the resistance to erythropoietin therapy seen in many ESRD patients, suggesting uraemia may exert an influence beyond GH in terms of JAK/STAT dependent cytokines (Allen et al., 1999).

SOCS have also been implicated as modulators of cell function in experimental models of immune complex glomerulonephritis, where increased glomerular and tubulointerstitial expression of SOCS3 corresponds to proteinuria (Gomez-Guerrero et al., 2004). In addition SOCS2 is demonstrably upregulated in the skeletal muscle of ESRD patients undergoing haemodialysis treatment (Raj et al., 2005). There is also evidence to suggest that SOCS may act as negative regulators of angiotensin II signalling in cultured mesangial and tubular epithelial cells and therefore potential regulators of renal injury (Hernandez-Vargas et al., 2005). Increased monocyte SOCS3 has been demonstrated in CKD patients and appears to correlate with progressive loss of estimated GFR, while lymphocyte SOCS1 expression correlates with other markers of cardiovascular disease such as systolic hypertension and pulse wave velocity (Rastmanesh et al., 2008). There is also an early indication of differential expression according to dialysis modality, with CIS expression significantly increased in lymphocytes of pre-dialysis and peritoneal dialysis, but not haemodialysis patients (Rastmanesh et al., 2009). However, multiple potential confounding factors, such as concurrent inflammation, mean this observation requires further study.

1.9 Apoptosis

1.9.1 Introduction

Apoptosis, or programmed cell death, is the process by which cells commit to their own demise (Kerr et al., 1972). Accordingly, it is sometimes referred to as cellular suicide (Kidd et al., 2000). It is a highly regulated, often endogenously driven, process affecting cells of diverse lineage and it plays a critical role in both development and homeostasis of most tissues. Defects in apoptotic cell death regulation are thought to contribute to many diseases, including disorders of cell accumulation, incorporating cancer, as well as cell loss, such as stroke, left ventricular failure and neurodegenerative diseases (Reed, 2000). The term apoptosis is primarily a morphological one. Characteristic features of an apoptotic cell include chromatin condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage. The cell eventually breaks into small membrane enclosed fragments, termed apoptotic bodies. These are cleared, without producing an inflammatory response, by phagocytosis (Hock et al., 2001). The nuclear changes are particularly distinctive; DNA fragmentation occurs under the action of an endogenous deoxyribonuclease, specific for nuclear, rather than mitochondrial, DNA. Oligonucleosomal fragmentation results, with degraded DNA forming a 200-bp ladder pattern when separated by gel electrophoresis (Wyllie, 1980).

The effector processes underpinning apoptosis are now mostly known and involve activation of both the caspase and Bcl-2 families in response to both physiological and noxious stimuli (Hotchkiss et al., 2009). The term caspase is derived from *c*ysteinyl-directed *asp*artate-specific prote*ase* (Wyllie, 1980) and it is activation of this family that underlies the majority of the apoptotic phenotype. At present, 12-14 mammalian caspases are known, although not all function during apoptosis. These proteases are present as inactive zymogens in virtually all animal cells and are triggered into an active state through proteolytic processing at preserved aspartic acid residues. Cleavage generates large (20kDa) and small (10kDa) subunits that organise into an active heterotetramic (two large and two small subunits) structure (Thornberry and

Lazebnik, 1998). The caspases essentially form a cascade in which initiator caspases are either activated following cytokine-receptor interaction at the cell membrane, or in response to signals within the cell. Thus, caspases 8 is activated when ligands of the TNF family bind to their receptor (often termed the extrinsic pathway), while caspase 9 is activated at the mitochondrial membrane (often termed the intrinsic pathway). Initiator caspases then activate, by cleavage, a set of effector caspases, principally caspase 3, but also caspases 6 and 7, which proceed to cleave proteins at many intracellular locations. This process accounts for much of the distinctive morphology; for example, apoptotic cellular blebbing is caused by cleavage of ROCK-1, a rhokinase isoform (Coleman et al., 2001) and cell shrinkage by cleavage of focal adhesion kinase (FAK) (Wen et al., 1997). Caspases may also be responsible for releasing nucleotides that serve as migration signals for macrophages that engulf apoptotic cells (Elliott et al., 2009).

Bcl-2 family members possess related domains that facilitate interactions within the group (Adams and Cory, 2007). Bcl-2 and its closest relatives, including Bcl-x_L, possess four such domains, designated BH 1,2 and 4. These define a hydrophobic groove, while BH3, a short region within the groove, is able to facilitate binding. These molecules all support cell survival, whilst shared BH domains permit interaction with the pro-death molecules, Bax and Bak. These can punch a wide pore through mitochondrial membranes, allowing leakage of critical molecules (Kuwana et al., 2002). Among those escaping molecules are cytochrome c and dATP, which can activate caspase 9, which itself is held in the vicinity through association with APAF-1 (Van Cruchten and Van Den Broeck, 2002). It is the concentration of Bax or Bak relative to the Bcl-2 - Bcl-x_L or other pro-survival molecules that determines whether this rise in mitochondrial permeability will occur, with subsequent activation of the intrinsic pathway (Rosse et al., 1998). The remaining members of the Bcl-2 family only possess the BH3 domain (Willis et al., 2007). These proteins (including bid, bad, bim and bmf) all promote cell death and may act as sensors of the various forms of cellular injury

(Wyllie, 2010). In addition to the above, a degree of interdependence between the pathways is recognised; for example, caspase-8, classically a feature of the extrinsic pathway, can, like caspase-9, activate the bcl2 protein bid in the outer mitochondrial membrane with release of cytochrome c (Li et al., 1998). Under certain circumstances, cells utilise a different set of proteases to initiate caspase activation. These include the aspartic protease cathepsin D, the serine protease AP24 (apoptosis protease 24), and calpain. Much like the caspases, calpain is a cysteinyl protease, but it does not recognise a defined cleavage site within target substrates (Reed, 2000). Instead it acts on the cellular proteins responsible for the maintenance cytoskeletal integrity and is regulated by Ca^{2+} (Harwood et al., 2005).

Until recently, apoptosis was considered the only form of regulated death during cellular homeostasis, with necrosis regarded as an uncontrolled process. However, accumulating evidence suggests that necrosis can also occur in an ordered fashion (Laster et al., 1988, Vandenabeele et al., 2010). The initiation of programmed necrosis, or 'necroptosis', by death receptors, such as tumour necrosis factor receptor 1, requires the kinase activity of receptor-interacting protein-1 (RIP1, also known as RIPK1) and RIP3 (also known as RIPK3). Its subsequent execution involves the destruction of mitochondrial, lysosomal and plasma membranes (Holler et al., 2000, Declercq et al., 2009, Vanlangenakker et al., 2008). Necroptosis may have a role in the in the pathogenesis of various diseases, including ischaemic injury, neurodegeneration and viral infection (Vandenabeele et al., 2010). Furthermore, an additional death pathway is now recognised for the recycling of cellular contents. During this process, intracellular materials are packaged into vesicles and targeted to the lysosome or vacuole for turnover. This process, termed autophagy, is currently thought to be predominantly cytoprotective (Galluzzi et al., 2007, Kroemer et al., 2009).

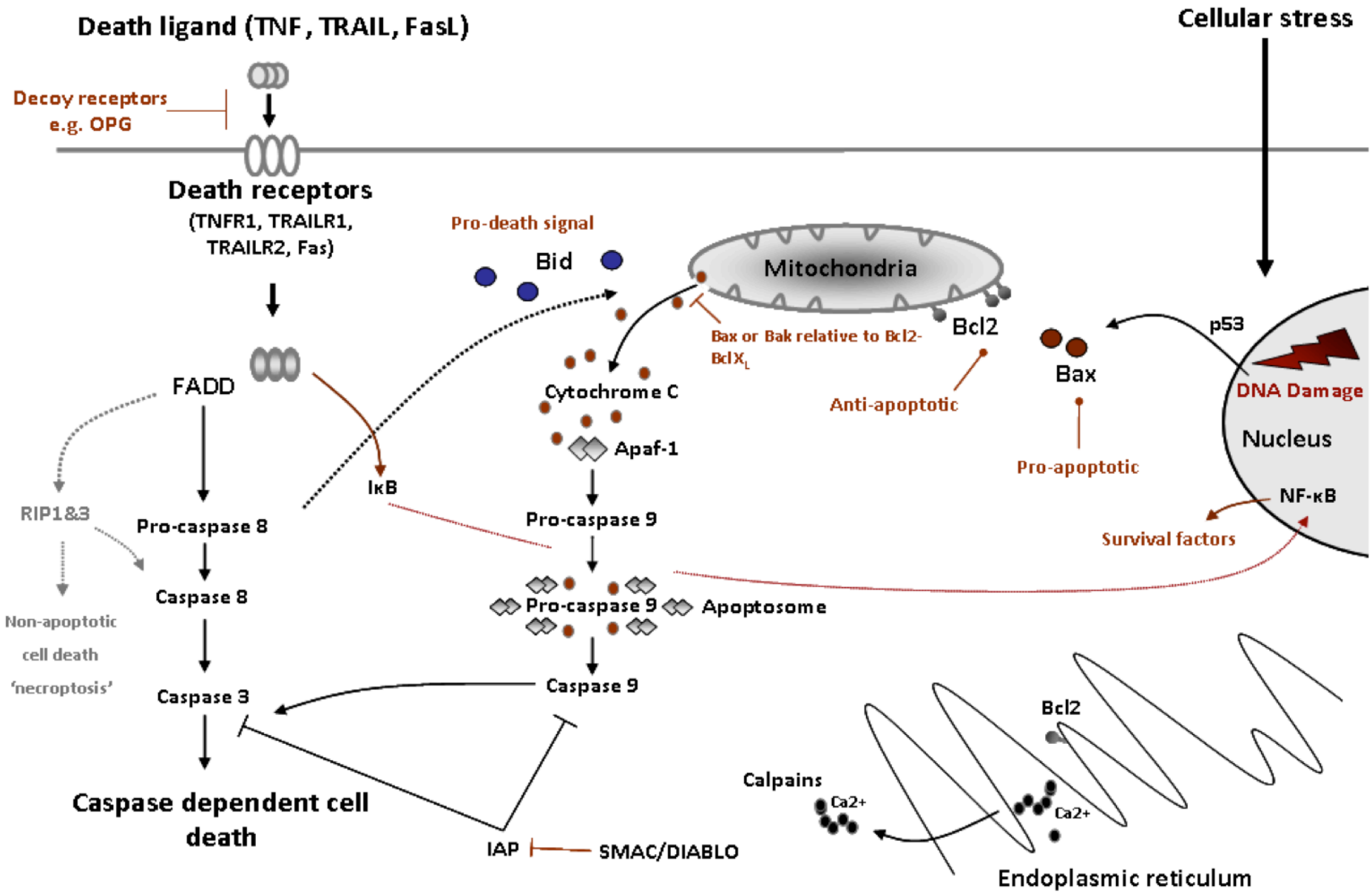


figure 1.9 a schematic overview of apoptotic pathways

Figure 1.9 continued

The extrinsic apoptotic pathway is activated following ligand binding to death receptors (TNFR1, TRAILR1, TRAILR2, Fas). All death receptors contain a death domain (DD), a conserved domain of ~60 amino acids, in their cytoplasmic tail (Feinstein et al., 1995). The DD interacts with adapter proteins, including Fas associated death domain (FADD) (Chinnaiyan et al., 1995). TNFR1 may also promote survival signalling through activation of NF-κB via IκB (Hsu et al., 1995).

The intrinsic pathway involves release of mitochondrial factors including cytochrome c, which forms a molecular complex called the apoptosome with Apaf-1 and pro-caspase 9 and leads to the generation of active caspase 9 (Li et al., 1997). Several factors influence mitochondrial permeability; for example, p53 induces Bax to oligomerise and translocate to mitochondria where it antagonises the anti-apoptotic effects of Bcl2 and $BclX_L$ and disrupts mitochondrial integrity.

The extrinsic and intrinsic pathways converge to a caspase cascade that results in cell shrinkage, DNA fragmentation and the other morphological features of apoptosis.

IAPs (inhibitor of apoptosis proteins) are a group of cytoplasmic proteins that add another layer of regulation through potent caspase inhibition. They are regulated by proteins released from mitochondria, including SMAC/Diablo (Chai et al., 2000).

See text for further detail and references.

1.9.2 The role of apoptosis in skeletal tissue

Skeletal mass can be influenced either through regulating the proliferation and activation of osteoblast/osteoclast precursors or by modifying cellular longevity through apoptosis (Weinstein and Manolagas, 2000). During bone formation and fracture healing, osteoblasts can be seen to undergo an organised developmental progression concluding with apoptotic cell death. This is most marked in areas of rapid bone turnover. The molecular pathways, regulation and overall significance of cell death in mature bone remain poorly understood (Xing and Boyce, 2005).

The rate of bone synthesis is to a large extent determined by the number of functioning osteoblasts. As these cells complete the task of matrix synthesis and leave the remodelling site many undergo progressive phenotypic change, with some becoming lining cells and others osteocytes. The majority of the cells, however, cannot be accounted for in this way and it is probable that these 'missing' cells die by apoptosis (Weinstein et al., 1998). Osteoclasts also die by apoptosis and are removed by phagocytosis once the resorbing cycle is completed (Hughes and Boyce, 1997). This fine balance between cell proliferation and dropout determines the amount of cells available to performed the work of bone formation.

The same cytokines and growth factors that stimulate osteoclast and osteoblast development appear to exert influence over their subsequent apoptosis. TGFß promotes osteoclast apoptosis while inhibiting osteoblast apoptosis. IL-6 and other gp130 family cytokines are anti-apoptotic for osteoblasts and antagonise the pro-apoptotic effects of glucocorticoids. IGF-I, PDGF and both acidic and fibroblast growth factors (FGFs) have all been linked to osteoblast survival *in vitro* (Hill et al., 1997, Jilka et al., 1998, Debiais et al., 2004). IGFs and FGF upregulate calbindin-D28k, which binds to, and inhibits, caspase-3, blocking TNF induced apoptosis in osteoblastic cells (Bellido et al., 2000). One study found that IGF-II induces apoptosis in osteoblasts, which can be overcome by overexpression of Bcl-2 protein (Gronowicz et al., 2004).

TNFα is another of the few factors shown to be pro-apoptotic in cell culture systems (Tsuboi et al., 1999). Furthermore, an *in vivo* role is suggested by its involvement in the regulation of bone loss following ovariectomy (Kimble et al., 1997). The relationship of RANK-L, RANK and OPG to the death receptor family has engendered speculation as to their role in the apoptotic process. RANK-L and OPG have been implicated as regulators of cell survival in other situations. Indeed, RANK-L was initially discovered by two groups attempting to identify and clone genes involved in the regulation of apoptosis in dendritic cells (Suda et al., 1999). RANK-L has been shown to promote the survival of T-cells (Wong et al., 1997), dendritic cells (Wong et al., 1997), mammary epithelial cells (Fata et al., 2000) and osteoclasts (Lacey et al., 2000). In dendritic cells this appears to involve a Bcl-X_L–dependent mechanism. OPG both promotes and inhibits cell survival depending on the model studied. It protects endothelial cells from apoptosis induced by serum withdrawal or activation of NF-κB (Malyankar et al., 2000), while eliciting death signals in others through its two death domain homologous regions (Yamaguchi et al., 1998). In addition, OPG is the only known soluble receptor for TRAIL and has been shown to inhibit TRAIL-mediated apoptosis in Jurkat cells (Emery et al., 1998, Holen and Shipman, 2006). In addition, OPG appears to be a survival factor, *in vitro,* for prostate cancer cells (Holen et al., 2002), breast cancer cells (Holen et al., 2005) multiple myeloma cells (Shipman and Croucher, 2003) and colon cancer cells (De Toni et al., 2008). It remains unclear how relevant such findings are to cells of osteoblastic lineage.

Several widely used drugs exert their skeletal effects through the manipulation of apoptosis in bone cells. In particular, the promotion of osteoclast apoptosis is an important therapeutic goal in disorders characterised by excessive bone resorption, of which osteoporosis is the most important (Weinstein and Manolagas, 2000). Oestrogen promotes osteoclast apoptosis and has undoubted efficacy for the prevention of bone loss in postmenopausal osteoporosis (Hughes et al., 1996, Prestwood et al., 1994). Bisphosphonates also have the promotion of osteoclastic apoptosis at the core of their

mode of action in bone (Luckman et al., 1998) and this stimulated interest in their utility in other situations, where they have been shown to induce tumour cell apoptosis, inhibit tumour growth and encourage immune reactions against tumour cells (Sun et al., 2010). The osteoblast is also an important therapeutic target. Protection from the induction of, or a reduction in, the amount of osteoblastic apoptosis would increase the pool of mature bone forming cells. A decrease in both TNFα and dexamethasone induced apoptosis has been achieved with both bisphosphonates and calcitonin (Plotkin et al., 1999). PTH is particularly interesting, since constant high concentrations induce bone resorption, while intermittent low concentrations promote bone formation and increase bone mineral density. These latter, anabolic, effects may be explained by the inhibition of osteoblast apoptosis (Jilka et al., 1999).

1.9.3 SOCS in the regulation of apoptosis

There is evidence that the suppressors cytokine signalling, through their privileged position in intracellular signalling, may play a significant role in the processing and regulation of apoptotic and anti-apoptotic signals. The abolition of SOCS gene expression in SOCS1 null mice was noted to produce apoptosis in liver and lymphoid tissue (Naka et al., 1998). In addition, SOCS protein appears to play a role in leukaemia inhibitory factor (LIF) dependent survival of embryonic stem cells (Duval et al., 2000). Forced overexpression of SOCS1 or SOCS3 in this system leads to a reduction in cell viability. LIF activates at least two pathways: the JAK/STAT pathway and the ras/mitogen activated protein (MAP) kinase pathway; the JAK/STAT system has been shown to be relevant to cell proliferation and differentiation (Hoey and Schindler, 1998), while the MAP kinase pathway is also important for apoptotic signalling (Kummer et al., 1997). It is likely that there is a degree of interdependence between the pathways, with nuclear translocation of STATS leading to the transcriptional increase of early response genes such as c-Fos, c-Jun, JunB, JunD and c-Myc. In other cell systems SOCS appear to have a protective role. For example SOCS3 ameliorates cytokine induced apoptosis in pancreatic ß-cells (Karlsen et al.,

2001, Bruun et al., 2009) and SOCS1 suppresses TNFα induced cell death, with the latter regulated through p38 MAP kinase rather than JAK-STAT signalling (Morita et al., 2000). SOCS1 suppresses TNFα dependent apoptosis in cardiomyocytes via ERK1/2 pathway activation (Yan et al., 2008) and SOCS3 has been shown to be an important regulator of involution, tissue remodelling and apoptosis that is required after the cessation of lactation (Sutherland et al., 2007). In addition, SOCS3 appears to be a key component of the survival apparatus of prostate cancer cells (Puhr et al., 2009, Horndasch and Culig, 2011b). It is overexpressed in castration-resistant tumours and its knockdown results in increased cell death, mediated through both apoptotic pathways. Overexpression of Bcl-2 protects those cells with reduced SOCS3 from harm. Suppression of SOCS3 increases the susceptibility of renal cell carcinoma to IFN-α, raising the possibility that reducing SOCS expression may be a potential future strategy for augmenting the effects of anti-tumour therapy (Tomita et al., 2011).

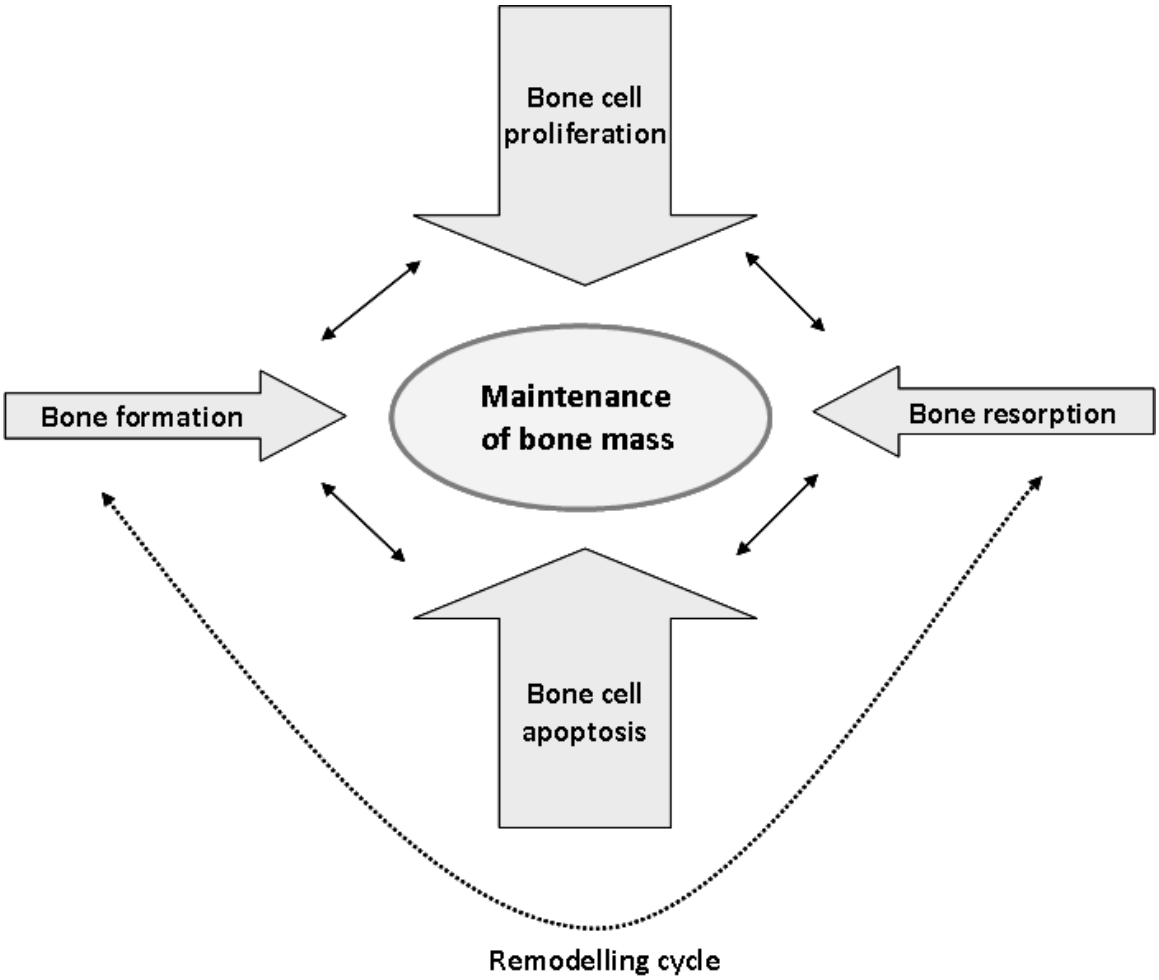


Figure 1.10. A summary of the interrelated processes contributing to skeletal mass and discussed in detail in this chapter.

1.10 Hypotheses

Chapter 2

Uraemic toxins contained in uraemic serum obtained from kidney failure patients will alter osteoblastic IL-6 and OPG release from human osteoblastic cells. These effects may be modifiable by subjecting the serum to heat treatment (to denature peptides/proteins), charcoal stripping (to remove hydrophobic molecules) and haemodialysis (to remove molecules by size).

Chapter 3 and 4

Given the relevance of cytokine signalling to their function in both health and disease, human osteoblastic cells will express the suppressor of cytokine signalling (SOCS) genes SOCS1, SOCS2, SOCS3 and CIS and their pattern of expression following stimulation with relevant osteotropic stimuli will provide an insight into their role. The functional importance of SOCS will be elucidated through the study of two key pathways for osteoblastic cells; namely cytokine secretion, specifically IL-6 and OPG, and cell death pathways.

Chapter 2

Cytokine release from human osteoblastic cells in uraemia

2.1 Background

Since bone remodelling requires coupling of osteoclastic bone resorption and osteoblastic bone formation, disruption of these linked processes may play a role in the aetiology of renal bone disease. A large number of osteotropic factors influence the principal mediators of osteoclastic resorption, namely osteoprotegerin (OPG) and RANK-L (Aubin and Bonnelye, 2000, D'Amelio et al., 2009). Amongst these, interleukin-6 (IL-6) plays a key role in the initiation and maintenance of osteoclastogenesis (Adebanjo et al., 1998, Sims and Walsh, 2010) and it has been implicated in the pathogenesis of a wide variety of pathologies characterised by accelerated remodelling and resorption, including renal bone disease (Langub et al., 1996, Ferreira et al., 2009).

To explore the hypothesis that the uraemic toxins contained in uraemic serum may increase osteoblastic cytokine release, the effect of human uraemic serum on the release of OPG and IL-6 from human osteoblast-like cells was examined. MG-63 cells were cultured for 24h in media containing sera pooled from either healthy male volunteers or haemodialysis (HD) patients alongside matching charcoal stripped sera. The concentrations of OPG and IL-6 were then measured in harvested supernatants. In other experiments, individual serum samples collected from patients prior to, and during, a dialysis session were assayed for IL-6, interleukin-1β (IL-1β) and soluble IL-6 receptor (sIL-6R) using specific enzyme-linked immunosorbent assays (ELISAs). The capacity of charcoal stripping of serum and both sham and therapeutic HD to modify osteoblastic IL-6 release was investigated.

In order to study the effects of a uraemic environment on SOCS expression, MG-63 cells were incubated with pooled serum from either male haemodialysis patients or healthy volunteers. mRNA was isolated from the cells prior to PCR amplification with SOCS specific primers.

2.2 Materials and methods

2.2.1 Osteoblast-like cell lines

MG-63

MG-63 is an osteoblast-like cell line cloned from a human osteosarcoma. It has the phenotype of a immature osteoblast. Constitutively the cells have relatively low levels of alkaline phosphatase, do not express osteocalcin and do not form a mineralised matrix *in vitro,* though these functions can be stimulated by 1,25 dihydroxyvitamin D_3. The cells are able to show a cAMP response to PTH stimulation and are high constitutive producers of growth factors and cytokines.

SaOS-2

The SaOS-2 cell line is also derived from a human osteosarcoma. It has many of the features of a well-differentiated osteoblastic cell, including constitutive expression of high levels of alkaline phosphatase, cAMP response to PTH stimulation, and expression of osteonectin and bone sialoprotein. These cells can form bone when implanted *in vivo* and a mineralised matrix in long-term cultures *in vitro*. However, they do not appear to express osteocalcin at any stage of their development.

2.2.2 Cell culture

MG-63 cells were cultured in Modified Eagles Medium α with 10% (v/v) fetal bovine serum (FBS) and antibiotics (benzylpenicillin 100units/mL, streptomycin 10 µg/mL, amphotericin 2.5 µg/mL (all Invitrogen, Paisley, UK) [hereafter called medium] in sterile T75 culture flasks (Merck, Darmstadt, Germany) and incubated at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 (IR 1500 Automatic CO_2 incubator, Flow laboratories Ltd). SaOS-2 cells were cultured in Roswell Park Memorial Institute (RPMI, Invitrogen) with 10% (v/v) FBS (plus antibiotic). When confluent, the cells were subcultured; i.e. the medium was removed and the cells washed with 10mL of Phosphate Buffered Saline pH 7.4 (PBS). Two mL of PBS solution containing 0.05% Trypsin (Life Technologies, Paisley, UK) was then added to the cells, which were

incubated at 37ºC for approximately 5min until they had detached from the culture flask. Eight mL of culture medium was added and mixed gently using a 10 mL stripette (Merck, Darmstadt, Germany) to ensure a homogeneous suspension and prevent further action of trypsin. The cells were then harvested by centrifugation at 1200 x *g* for 5 minutes (Megafuge 2.0R, Heraeus Equipment Ltd). The supernatant was removed with care so as not to disturb the pelleted cells, which were then resuspended in culture medium and aliquoted into fresh culture flasks.

2.2.3 Cell count

After being harvested by trypsin treatment and centrifugation (see above), an aliquot of pelleted cells was resuspended in 10 mL of culture medium and a 1:1 dilution of the cell suspension made with 0.1% v/v of Trypan blue (Sigma Aldrich Co, Dorset, UK). 100 µL of cell suspension-trypan blue mixture was transferred to a haemocytometer chamber over which a cover slip was placed. Using one chamber of the haemocytometer, the number of dead and viable cells were counted in the middle $1mm^2$ square and at least two of the four corner squares. Each square on the haemocytometer (with the cover slip in place) represents a total volume of $0.1mm^3$ ($10^{-4}\,mm^3$). Therefore,

Live cells per ml = mean number of unstained cells per square x 10^4 x 2

Total cells = cells per ml x volume in which cells suspended x 2

$$\text{Cell viability} = \frac{\text{number of unstained cells}}{\text{Total number of cells}} \times 100$$

2.2.4 Cell freezing

After being harvested, cells were gently washed with 5 mL of PBS, recentrifuged, and the buffer removed. The pelleted cells were then resuspended in 1 mL of Cell Culture

Freezing Medium (GIBCO Life Technologies, Paisley, UK) and transferred to a cryovial (Merck, Darmstadt, Germany), which was placed in a polystyrene container at -20ºC for 2h, followed by a minimum of 2 hours at -70ºC, and finally, transferred to liquid nitrogen for long term storage.

2.2.5 Human blood samples

Normal serum samples were obtained from ten healthy male volunteers with no history of febrile illness in the preceding four weeks. Males alone were chosen, so that important differences in sex steroid concentrations were not introduced. Uraemic serum was obtained from ten male ESRD patients receiving HD treatment (renal diagnoses: - small kidneys - uncertain cause 3; hypertensive nephrosclerosis 2; renovascular disease 1; membranous glomerulonephritis 2; focal and segmental glomerulosclerosis 1; adult polycystic kidney disease 1). All patients had been on HD for at least one year (mean 26 months, range 13-62 months) and had a mean age of 39 years (range 28-67 years). All patients underwent three, 4h dialysis sessions per week using cellulose acetate dialysers and bicarbonate buffering. Venous access in all subjects was by primary arteriovenous fistulae. Blood was sampled from the arterial access port prior to passing through the dialyser at the start of a HD treatment session. Serum was obtained from blood samples taken into plain glass tubes and allowed to stand upright at room temperature for 20min before separation by centrifugation at 2000 rpm and removal of serum with a sterile pipette and immediate storage at -20^{0} C.

2.2.6 Sham dialysis procedure

Blood samples were also obtained from five additional patients during an individual HD session using a modified cellulose dialysis membrane. The characteristics of these patients are shown (table 1). Samples were taken prior to dialysis and at 30, 60, 120, 180, 240 and 300min into their treatment. The first 60min comprised extra-corporeal circulation alone, with no dialysate flow and no programmed ultrafiltration (hereafter referred to as sham dialysis). After 60min, dialysate flow was introduced and continued for the remainder of the dialysis treatment (figure 2.1). During HD treatment, blood is exposed to dialysate, a solution containing physiological concentrations of electrolytes, across a semi-permeable membrane. Pores in this membrane allow small molecules (e.g. urea – MW 60Da) and electrolytes, but not larger ones (e.g. plasma proteins such as albumin – 60,000 Da), or blood cells to pass through. Concentration differences across the membrane allow molecules to diffuse down a gradient. This allows waste products to be removed and desirable molecules or electrolytes to be replaced. Water can be driven through the membrane by hydrostatic force, termed ultrafiltration (Steddon, 2006).

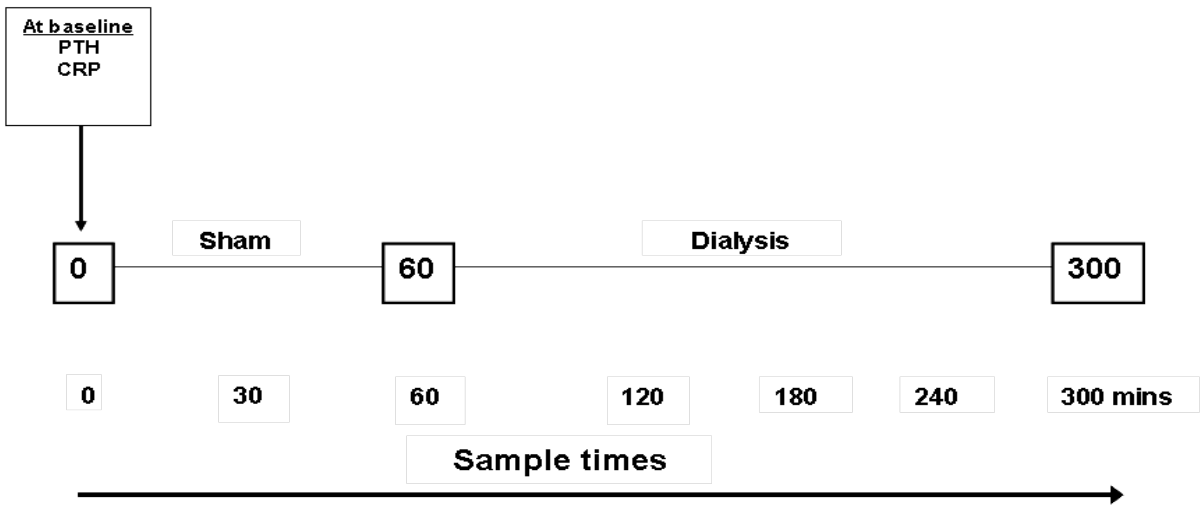


Figure 2.1. Protocol for blood sampling during 'sham' and 'standard' dialysis

Patient	Cause of ESRD	Years on dialysis	PTH (Pmol/L)	CRP (mg/L)
1	Reflux	4	136	24
2	Stone disease	2	3.6	<5
3	Hypertension	12	0.3	<5
4	Hypertension	4	30.9	13
5	Hypertension	1	56.7	27

Table 2.1 Characteristics of the five patients who underwent serum sampling throughout an individual dialysis treatment.

Individual normal and uraemic serum samples were assayed for IL-6, soluble interleukin-6 receptor (sIL-6R) and interleukin-1 beta (IL-1β). IL-1β is known to increase the release of IL-6 from both MG-63 and SaOS-2 cells and was measured to assess any contribution to the differences in measured IL-6. SIL-6R is a 50 kDa ligand protein derived from the surface shedding of the gp80 component of the IL-6 receptor (Kishimoto, 2005). Its ability to bind free IL-6 means it is a potential source of interference with the measured concentration of IL-6. Some aliquots of human sera were 'inactivated' by charcoal stripping; a process that removes hydrophobic structures including steroids and growth factors. Two grams of activated charcoal were added to 50 mL of serum and mixed at room temperature for 4h. Serum was recovered by centrifuging for 30min at 3500 rpm. The supernatant was re-centrifuged for a further 20min at 20,000 rpm at room temperature. Prior to use the serum was filtered through 0.45 μm and then 0.2 μm filters (Minisart, Sartorius AG, Göttingen, Germany). Additional aliquots were heat treated in a water bath (65ºC for 30min) as an alternative method of growth factor and hormone inactivation.

2.2.7 Ethical approval

Ethical approval for these procedures was obtained from the relevant local body (East London and the City Health Authority, reference P/00/241). All patients provided written informed consent.

2.2.8 Experimental design

Cells were seeded into 24 well plates at a density of 200,000 cells/well. They were allowed to adhere for 24h before aspiration of media, washing with sterile phosphate buffered saline (PBS) and incubation with α-MEM buffer containing antibiotics (as before) and 10% charcoal stripped (CS) foetal calf serum (CS-FCS) to provide a non-stimulating (growth arrested) environment for a further 24h. To examine the effects of human sera on IL-6 secretion, cells were then incubated for 24 hours in media supplemented with antibiotics and the human sera of interest at a 10% concentration. Negative controls were provided by refreshing cells with 10% CS-FCS and positive controls (for IL-6) by using 10% CS-FCS to which IL1-β (R&D Systems, Abingdon, Oxon, UK) had been added (at 100 IU/ml). At the end of the 24h incubation period media were collected and stored at -20^{0}C for no longer than one month before assay. Cells were washed with PBS and centrifuged before being subjected to three freeze/thaw cycles between -70^{0}C and room temperature to allow cell lysis for the measurement of cellular protein. All samples were assayed in at least quadruplicate and individual experiments repeated three or more times. In separate experiments the concentration of OPG in pooled 10% normal and 10% uraemic serum was determined and the effect of these sera on OPG secretion from both MG-63 and SaOS-2 examined and compared to serum free controls.

2.2.9 Alkaline Phosphatase detection

Alkaline phosphatase (ALP) activity of the cultured cells was confirmed by staining confluent flasks with 5-bromo-4-chloro-3-indoyl phosphate (BCIP). Positive blue

staining occurs in the presence of ALP and the colour is intensified by oxidation with nitroblue tetrazolium chloride (NBT) (Roche Diagnostics, Burgess Hill, UK).

2.2.10 Cytokine assays

IL-6, OPG, IL1-β and soluble interleukin-6 receptor (sIL-6R) were assayed using the relevant DuoSet ELISA Development kits (R&D Systems, Abingdon, UK). Each contains the components required for the development of a sandwich ELISA to measure a particular cytokine or growth factor in cell culture supernatants. The supplied capture antibody is diluted to working concentration in PBS and added in a volume of 100 µL per well to a 96 well microplate (e.g. mouse anti-human IL-6 at a working concentration of 2µg/mL). The plate is then sealed with an adhesive strip and incubated overnight at room temperature. Each well is then aspirated and washed three times with 400 µL of wash buffer. After the last wash, residual wash buffer is removed by blotting vigorously against paper towels. The plates are 'blocked' by adding 300 µL of reagent diluent containing 1% BSA to each well, with a 1h incubation at room temperature. The wash step is repeated prior to the addition of sample (100 µL per well), or standard (e.g. recombinant human IL-6 in two fold serial dilutions from a high standard of 600 pg/mL), in reagent diluent. The plate is covered and incubated for two hours at room temperature before further washing x 3. Detection antibody (e.g. biotinylated goat anti-human IL-6 in a working concentration of 200ng/mL) is added (100 µL/well). The plate is re-covered and incubated for a further 2h at room temperature, followed by a further wash. Streptavidin conjugated to horseradish-peroxidase (streptavidin-HRP) is added (100 µL/well) and the plate is covered and incubated away from direct light for a further 20 minutes at room temperature. Following a final wash (x 3), substrate solution (100 µL/well) is added prior to a further 20min incubation at room temperature and away from direct light. A stop solution (50 µL/well) is added and the plate gently tapped to ensure mixing. The optical density of each well is determined immediately at 450nm using a microplate reader. Examples of standard curves for the IL-6 and OPG Duosets are presented in appendix 1 (figure..).

2.2.11 Protein assays

Cellular protein in the experiments presented in this chapter (section 2.2.8 and 2.3.6) was assayed using the Bradford dye-binding protein assay (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) in a spectrophotometric microtitre based format. Absorbance was measured at 595 nm and samples quantified with a standard curve prepared from known concentrations of bovine serum albumin.

2.2.12 Isolation of total RNA from MG-63 and SaOS-2 cells

Total RNA isolation was carried out using the RNeasy Mini Kit (Qiagen, Crawley, UK), which is designed to isolate total RNA from small quantities of starting material. The principle of this method involves a specialised high-salt buffer system, which allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica gel membrane. All reagents were supplied with the kit and the protocol was followed as in the manufacturers handbook.

Cells were disrupted in the presence of 350 µL of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. 10 µL of ß-mercaptoethanol (ß-ME) 14.5M (Sigma-Aldrich Co) was added to 1 mL of buffer prior to use. The lysate was then homogenised to shear genomic DNA and reduce the viscosity of the lysate by centrifugation for 2 minutes at maximum speed through a QIAshredder column.

One volume of 70% ethanol (Merck, VWR Inc, Leicestershire, UK) was added to the homogenised lysate and mixed, in order to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample was then applied to an RNeasy spin column, where the total RNA binds to the membrane, and centrifuged for 15s at 9500 x *g*. Contaminants were washed away with 350 µL of buffer RW1 (containing 2.5-10% chaotropic salts), followed by centrifugation for 15 seconds at 9500 x *g*. At this stage, an incubation mix of 10 µL of RNase-free DNase I solution and 70 µL of buffer

RDD (RNase free DNA digest buffer) was added directly onto the spin-column membrane, which was incubated at room temperature for 15 minutes. DNase I was used to provide efficient on-column digestion of DNA during RNA purification.

The DNase was then removed by a second wash with 350 µL of buffer RW1, followed by centrifugation for 15s at 9500 x *g*. Next, the spin column was washed twice with 500 µl of ethanol based buffer RPE to remove all contaminants, and centrifuged for 15s at 9500 x *g* and for 2min at maximum speed to dry the silica gel membrane. Finally the RNA was eluted by applying 50 µL of RNase free water directly onto the silica gel membrane, followed by centrifugation at 9500 x *g* for 1min.

2.2.13 RNA quantitation for cDNA synthesis

The concentration of the isolated RNA was determined using a UV spectrophotometer. RNA was diluted 1:50 with RNase-free water and the absorbance measured at 260 and 280nm. The spectrophotometer was blanked against a cuvette containing only RNase free water. The RNA concentration was calculated from the formula

$$40 \times \text{dilution factor} \times A_{260} = [\text{RNA}]\ \mu g/ml$$

The purity of the RNA was estimated from the ratio of A_{260}/A_{280} with a value of between 1.8 and 2.0 indicating little or no contamination by protein.

2.2.14 First-strand complementary DNA (cDNA) synthesis

This procedure was carried out using the first-strand cDNA synthesis kit (Amersham Biosciences, UK), which has been designed to provide all the reagents necessary to generate full-length first strand cDNA from an RNA template, using a variety of primers. First strand cDNA synthesis is catalysed by Moloney Murine Leukaemia virus (M-MuLV) reverse transcriptase. All reagents were supplied with the kit and the protocol was followed as in the manufacturer's handbook.

The mRNA or total RNA sample, purified as described, was heat denatured at 65ºC for 10min, and immediately placed on ice. The bulk first strand cDNA reaction mix (containing reverse transcriptase, RNase/DNase free BSA and dNTPs) was gently mixed by pipetting to obtain a uniform suspension. 11 µL of the above reaction mix was added to a sterile microfuge tube together with 1 µL of dithiothreitol (DTT) solution (200 mM), 1 µL of a 1 in 25 dilution of Not I-d$(T)_{18}$ bifunctional primer (5 µg/µL) and 20 µL of heat denatured RNA. The solution was mixed by pipetting and then, incubated for 1h at 37ºC, resulting in a double stranded RNA:cDNA heteroduplex, which was stored at -70ºC until amplified by PCR.

2.2.15 Polymerase chain reaction

Intron spanning primers for the house keeping glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene were used in the PCR reaction as an internal control and to normalise for variable quantities of cDNA used in each amplification. These primers were designed to produce a 762 bp product. Primers for the suppressors of cytokine signalling genes SOCS 2, 3 and CIS were selected from the available literature. Intron spanning primers for SOCS 1 were designed using Primer 3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi) and subjected to a Blast search (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). All primers were ordered at MWG (https://ecom.mwgdna.com/register/index.tcl) (Eurofins MWG Operon, Ebersberg, Germany).

Primer name	Length (bp)	Direction	5'-3' sequence
GAPDH	762	Forward Reverse	TCC CAT CAC CAT CTT CCA GTC CAC CAC CCT GTT GCT
SOCS1	470	Forward (Alternative Forward) Reverse	GGC AGC TGC ACG GCT CCT (AGC ATG CGC GAG AGC CGC) CTG TCG CGC ACC AGG AAG
SOCS2	375	Forward Reverse	ACA GAG ATG CTG CAG AGA TG CTC GGT CAG ACA GGA TGG TA
SOCS3	313	Forward Reverse	TGC GCC TCA AGA CCT TCA GC CAG CTT GAG CAC GCA GTC
CIS	314	Forward Reverse	CAG TGC AGG AGG CCA CAT AG GGA GGA TCT GCT GTG CAT AG

Table 2.2: Primer details

A master mix of PCR reaction components was set up on ice, based on a final reaction of 100 µl per sample. The final concentrations were as follows:

Components	Volume	Final Concentration
10 x PCR buffer minus Mg	10 µL	1x
10mM dNTP mixture	2 µL	0.2 mM each
25 mM $MgCl_2$	12 µL	3.0 mM
Primer mix (10µM each)	4 µL	0.5 µM each
Template cDNA	2 µL	-----
Taq DNA polymerase	0.5 µL	2.5 U
Autoclaved distilled water to	100 µL	

In the case of multiple reactions, a master mix was prepared to minimise reagent loss and enable accurate pipetting. The contents of each tube were mixed and overlaid with 50 µl of mineral oil. A negative control (without template DNA) was used in each amplification. Thermal cycling parameters for GAPDH and SOCS primers were optimised for use with a Techne PHC-3 thermal cycler as shown below.

Primers	T (ºC) Duration (secs)				Cycles
GAPDH	94 60s	55 90s	72 90s	72 10min	28
SOCS1	94 60s	55 90s	72 90s	72 10min	28
SOCS2	94 60s	55 90s	72 90s	72 10min	28
SOCS3	94 60s	60 90s	72 90s	72 10min	28
CIS	94 60s	55 90s	72 90s	72 10min	28

Table 2.3: Thermal cycling conditions

2.2.16 Agarose gel electrophoresis

The PCR product was run on a 1.5% Agarose gel (RNase and DNase-free Agarose, Life technologies Ltd) containing 0.2 µg/mL ethidium bromide (Sigma-Aldrich Co) in 1 x TBE (Tris-Borate EDTA) buffer. The gel was poured into a gel former (Horizon 58, Life technologies) and after setting at room temperature, covered in 1 x TBE buffer. Two µL of loading dye (Sigma-Aldrich Co) were added to each sample before loading. Horizontal gel electrophoresis was carried out at 80 V for approximately 40min. A 100bp DNA ladder molecular weight marker (Amersham Biosciences, UK) was used for size determination. The resulting DNA bands were visualised using a UV

transilluminator (Hoefer Scientific Instruments, Holliston, MA, USA) and images were taken using a Polaroid direct screen instant camera DS34 loaded with Polaroid 667 film.

2.2.17 Scanning densitometry

Scanning densitometry was carried out using a commercial system (GDS 7500 and GelBlot Pro Software; Ultraviolet Products Ltd, CA, USA). Saved images were transferred to a remote desktop computer where analysis was performed. Each lane of the Agarose gel was scanned individually and a common background subtracted. Results, corresponding to the peak areas of specific bands, are expressed as a ratio of the specific SOCS to GAPDH.

2.2.19 Statistical analysis

Comparisons between normal and uraemic serum cytokine concentrations were analysed by unpaired t-test while other results of more than two groups were analysed by one-way or repeated measures ANOVA with Tukey's post-hoc test for inter-group comparisons. $P<0.05$ was considered significant (NS = not significant) and all results are expressed as mean ± standard error of the mean (S.E.M.).

2.3 Results

2.3.1 Serum cytokine concentrations

The serum concentrations of IL-6 were lower in normal subjects than in serum taken before dialysis from stable HD patients (0.4 ± 0.1 pg/mL vs. 7.0 ± 1.6 pg/mL respectively P=0.003). However, no significant differences in the concentrations of sIL-6R or IL-1β were seen in sera taken from normal subjects compared with ESRD patients (sIL-6R: 65 ± 26 µg/L vs. 96 ± 24 µg/L, NS; IL-1β: 7.6 ± 1.6 pg/mL vs. 8.7 ± 2.5 pg/mL, NS). In those subjects followed through a dialysis session, no significant changes in the serum levels of IL-6, sIL-6R or IL-1β were found (table 2.4).

	NORMAL SUBJECTS (n=10)	PRE-DIALYSIS (n=8)	40 MIN (n=8)	80 MIN (n=8)
IL-6 (pg/mL)		P=0.003	P=ns	
	0.4 ± 0.1	7.0 ± 1.6	6.3 ± 0.8	7.0 ± 1.0
sIL-6R (µg/L)		P=ns	P=ns	
	65 ± 26	96 ± 24	84 ± 18	79 ± 12.3
IL-1β (pg/mL)		P=ns	P=ns	
	7.6 ± 1.6	8.7 ± 2.5	8.9 ± 2.5	8.9 ± 2.1

Table 2.4 Effect of haemodialysis on serum cytokines in eight subjects followed for 80 minutes during a single haemodialysis session (results are expressed as mean±S.E.M.). The serum concentrations of IL-6 were lower in normal subjects than in serum taken before dialysis from stable HD patients (*P*=0.003). No significant differences in the concentrations of sIL-6R or IL-1β were found and no significant changes in the serum concentrations of IL-6, sIL-6R or IL-1β were seen during 80 min HD treatment.

2.3.2 Serum effects on osteoblast OPG release

The concentration of OPG was significantly higher in 10% uraemic serum than in 10% normal serum (2199.3 ± 31.2 pg/mL vs. 1767.704 ± 46.3 pg/mL, p<0.0001). Figure 2.2.

There were no significant differences in OPG secretion following the incubation of MG-63 cells with 10% normal or uraemic sera (7558.65 ± 193.7 pg/mL and 7764.52 ± 214.5 pg/mL respectively, NS) or serum free controls (7662.43 ± 140.0 pg/mL). Figure 2.2.

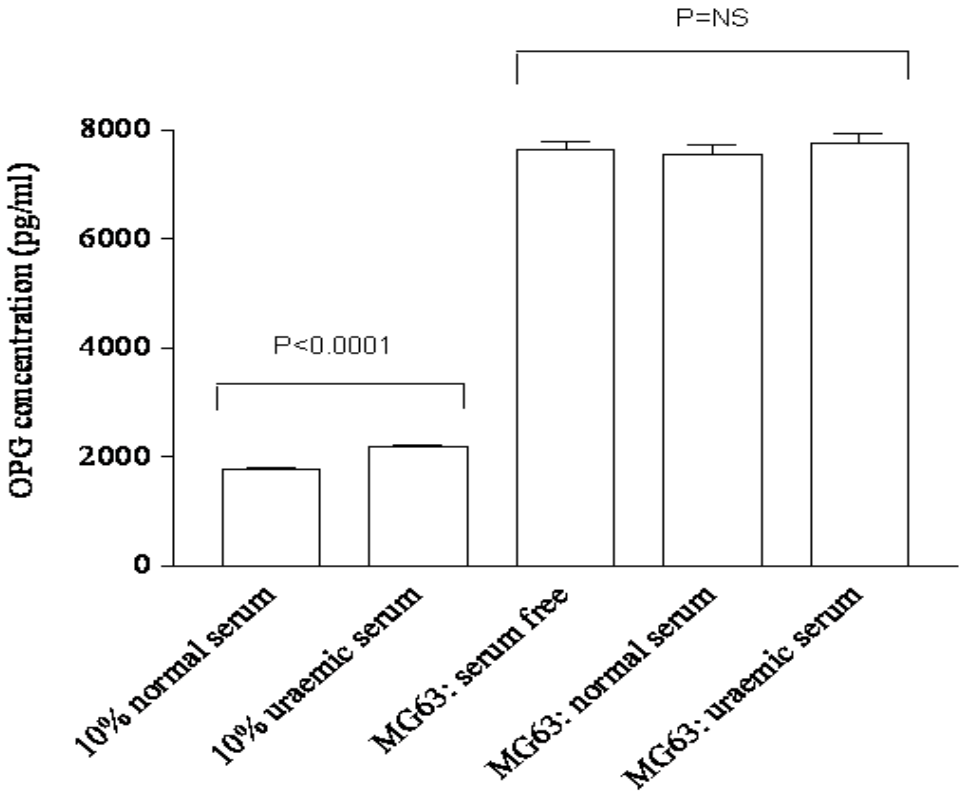


Figure 2.2. The concentration of OPG was significantly higher in pooled 10% uraemic serum than in pooled 10% normal serum (p<0.0001). The release of OPG from MG-63 following 24 hours incubation with these sera was similar and not significantly different from serum free controls

There were also no significant differences in secretion of OPG from SaOS-2 cells following incubation with either sera of interest (2233.88 ± 76.1 pg/mL vs. 2120.46 ± 62.08 pg/mL, NS).

2.3.3 Serum effects on osteoblast IL-6 release

Significant differences in IL-6 secretion were seen in MG-63 cells when incubated with the various sera ($P<0.0001$ by one-way ANOVA, figure 2.3). Serum that had been charcoal-stripped, from either normal or uraemic subjects, resulted in similar amounts of basal IL-6 release (1134 ± 127 pg/mL vs. 853 ± 78 pg/mL, NS). Untreated normal serum augmented IL-6 release by ~6-fold (to 6878 ± 595 pg/mL) over basal (charcoal-stripped) concentrations. This increase was significantly attenuated with untreated uraemic serum, which increased release by ~2-3-fold (to 2579 ± 169 pg/mL) over basal concentrations. The augmentation of IL-6 release by normal sera was significantly greater than that by uraemic sera (6878 ± 595 pg/mL vs. 2579 ± 169 pg/mL, $P<0.001$). Heat treatment of uraemic serum had no effect on the release of IL-6 from MG-63 cells (1952 pg/mL ± 172 heat treated vs. 1872 pg/mL ± 165 non-heat treated) (figure 2.4). IL-1β (100IU/mL) was by far the most powerful stimulus to IL-6 production (31802 ± 3250 pg/mL), whilst serum free media was the weakest (187 ± 33 pg/mL).

Quantitatively similar results were observed with SaOS-2 cells after incubation with human sera (figure 2.5). The augmentation of IL-6 release was 6-fold greater with normal serum compared to uraemic serum (374 ± 6 pg/mL vs. 61 ± 4 pg/mL, respectively).

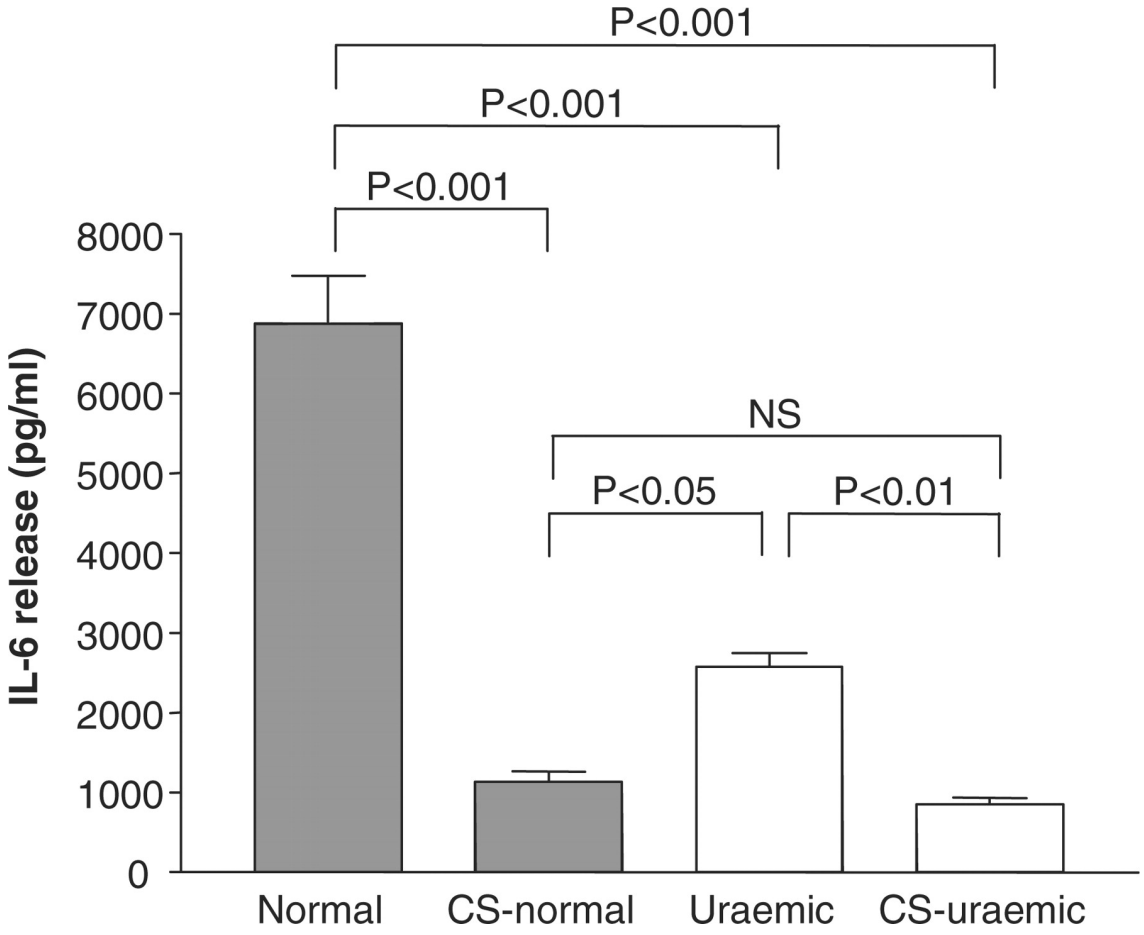


Figure 2.3: IL-6 release from MG-63 cells after 24h incubation with media containing normal and uraemic serum. CS = charcoal stripped. Significant differences between treatments were found ($P<0.0001$ by one-way ANOVA; for each group n=12). The data were reproduced in two further experiments.

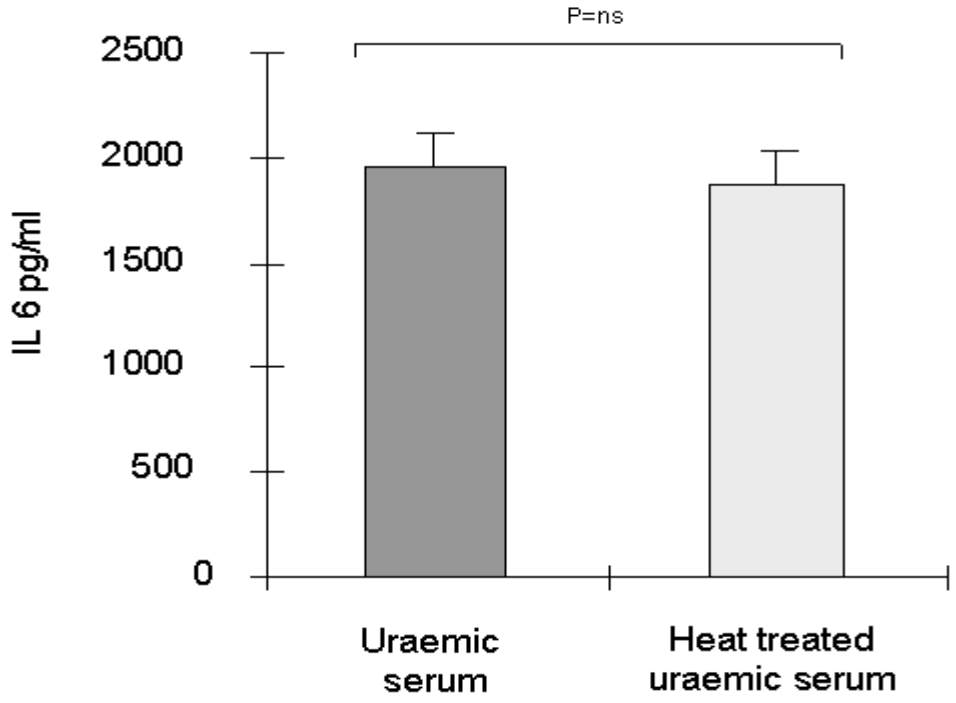


Figure 2.4: The heat treatment (via incubation in a water bath at 65ºC for 30min) of uraemic serum did not induce a significant difference in the release of IL-6 following subsequent 24h incubation with MG-63 cells as compared to non-heat treated uraemic serum (n=6, p=ns).

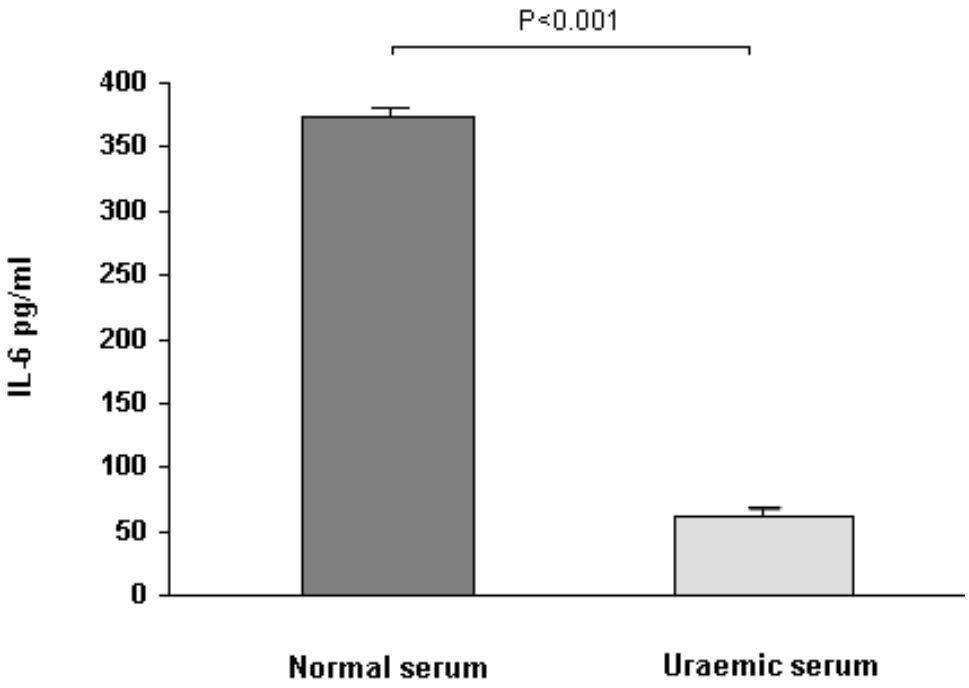


Figure 2.5: The incubation of SaOS-2 cells with media containing uraemic serum release caused a significant reduction in the release of IL-6 release at 24h compared to incubation with normal serum (n=3, p<0.001).

2.3.4 Effect of haemodialysis on osteoblastic IL-6 release in response to uraemic serum

Having found significant differences in IL-6 production, further experiments were designed to explore the capacity of haemodialysis to restore the ability of uraemic serum to promote IL-6 production to the same degree as normal serum (figure 2.6). The initial period of sham dialysis was necessary to explore the possible consequences of both heparinisation and extracorporeal circulation of blood for subsequent effects in cell culture. Therapeutic dialysis aided the investigation into the potential removal of mediators of the effect. Although for some individual patients there was variability in osteoblastic IL-6 secretion at different time points, no significant differences or trends were evident.

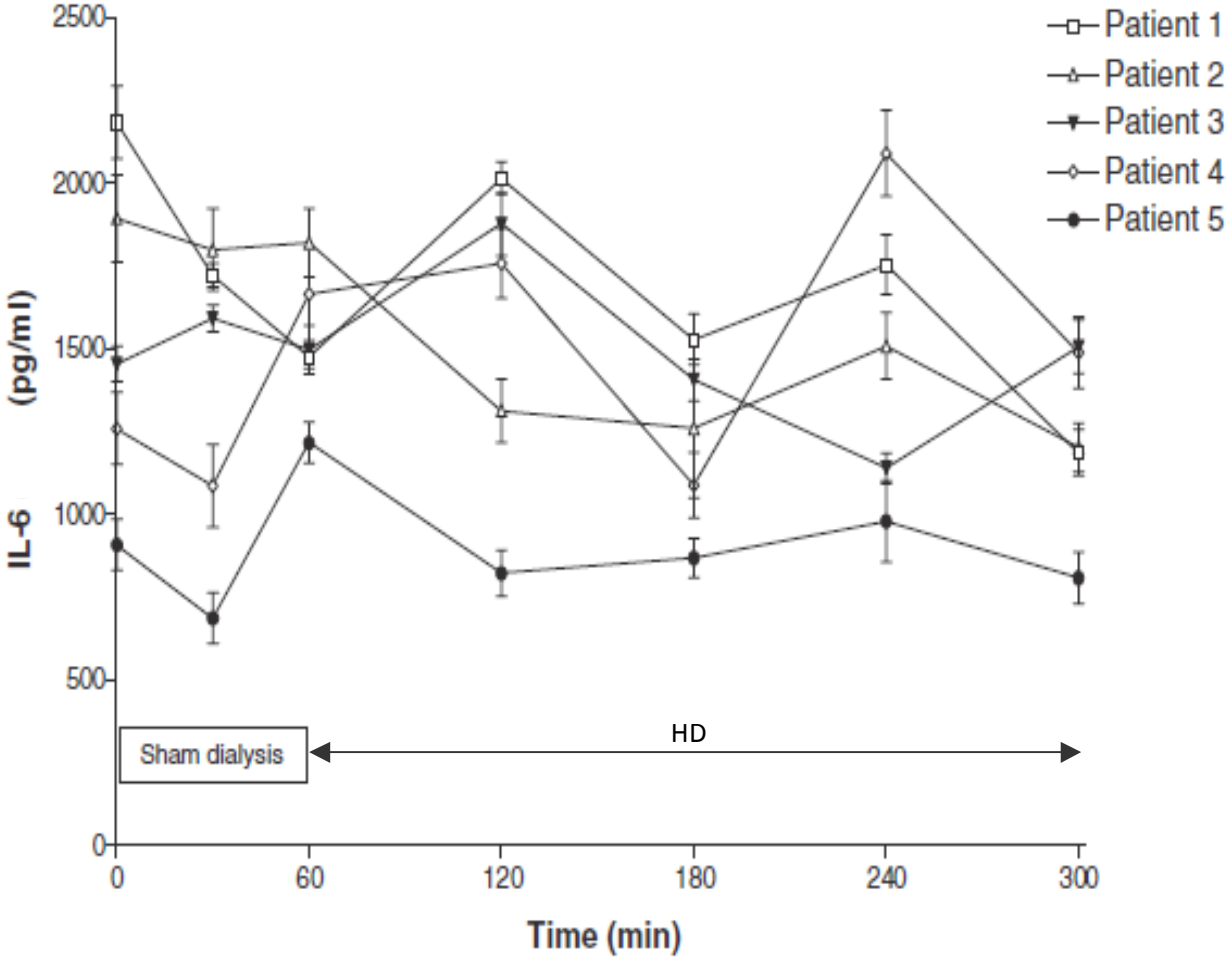


Figure 2.6 IL-6 release from MG-63 cells after 24h incubation with media containing sera harvested from five individual patients during a single haemodialysis session. The first 60 min represent 'sham' dialysis, with no dialysate flow. The abscissas depict the time of blood sampling. No significant differences or trends were evident.

2.3.5 Alkaline phosphatase activity

Positive staining after exposure to NBT and BCIP, confirmed the osteoblastic phenotype of both cell lines (see section 2.2.9).

2.3.6 Cellular protein

There were no significant differences in extracted cellular protein concentrations in any of the groups under study (figure 2.7).

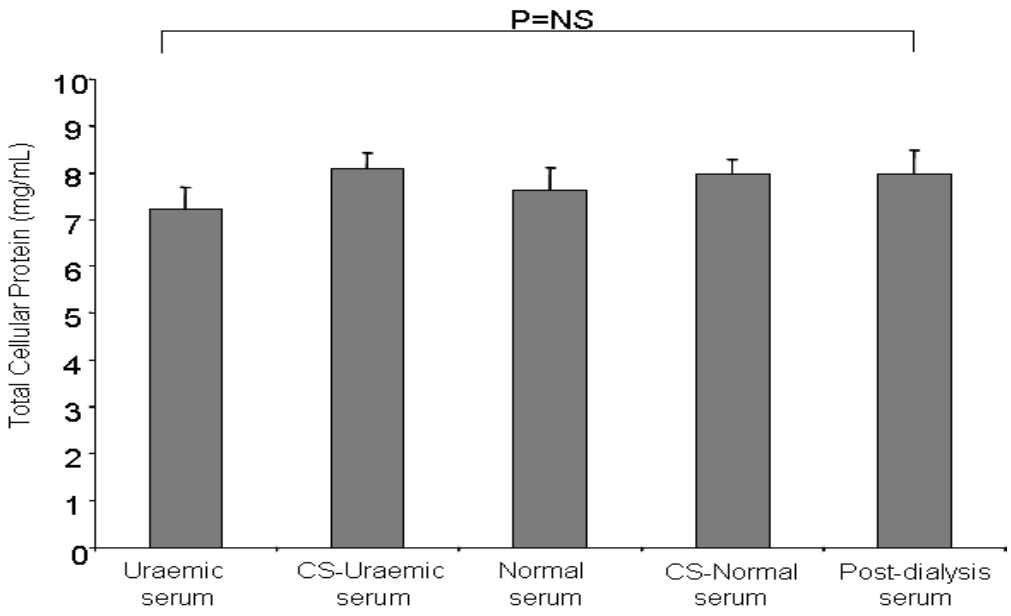


Figure 2.7: There were no significant differences in extracted cellular protein concentration between the different groups under study (n=6, p=ns). CS denotes charcoal stripping of the relevant serum.

2.3.7 Expression of SOCS mRNA in MG-63 cells

After harvesting and counting, MG-63 cells were plated at a density of 0.5 x 10^6 cells per well of a 6-well plate in 3 mL medium and incubated at 37ºC for 24h. mRNA was extracted from the cells with subsequent cDNA synthesis and PCR amplification using SOCS specific primers. Results, indicating constitutive expression of SOCS2, SOCS3 and CIS mRNA, are shown in figure 2.8.

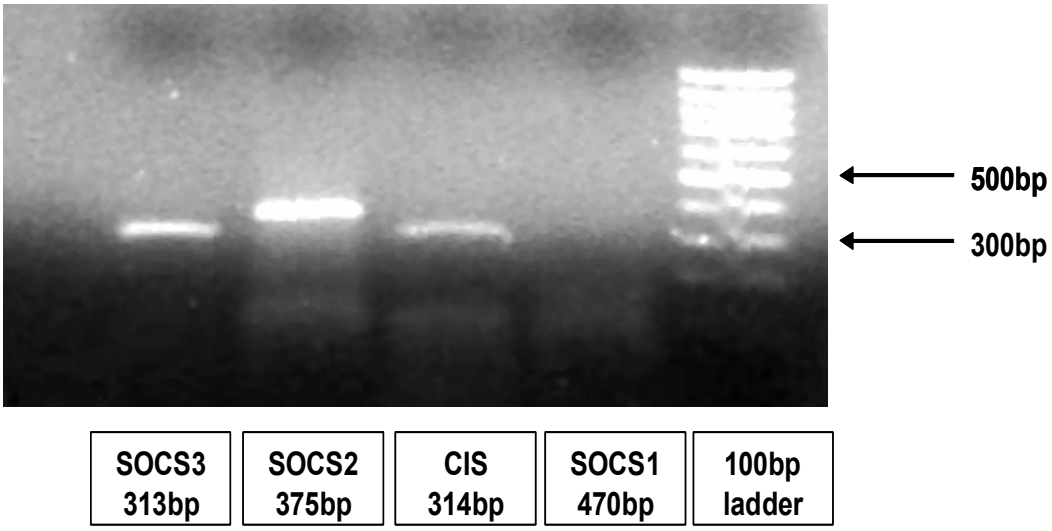


Figure 2.8: Constitutive expression of SOCS mRNA in unstimulated MG-63 cells. SOCS3, SOCS2 and CIS are shown with products corresponding to expected sizes. The failure to detect a product corresponding to the expected size of SOCS1 is discussed in the text. In the case of SOCS3 product identity was further confirmed by restriction enzyme digest (data not shown).

The difficulty in amplifying human SOCS1 is unexplained but widely recognised. It seems to be restricted to human sequence as mouse primers for SOCS1 amplify the murine gene consistently and reproducibly.

2.3.8 Effect of normal and uraemic serum on SOCS expression

In order to explore the effects of a uraemic environment on SOCS expression, serum was collected from male haemodialysis patients prior to a routine dialysis treatment. All patients were medically stable, had no evidence of intercurrent infection and gave express verbal consent. The serum was pooled and filtered before use. Serum was also collected from healthy non-uraemic control subjects.

After being harvested and counted, MG-63 cells were plated at a density of 0.5 x 10^6 cells per well of a 6-well plate in 3 mL medium and incubated at 37ºC for 24h. The cells were then growth arrested in serum-starved media for 12h, before stimulation with either 10% pooled uraemic serum (in media) or 10% normal serum for 4 hours. As before, mRNA was isolated from the cells followed by cDNA synthesis and PCR amplification with SOCS specific primers. The integrity of RNA for each specimen was verified using human GAPDH primers. Results for SOCS3 are shown in figure 2.8 below. Uraemic serum did not increase expression of SOCS3 in comparison to normal serum after 120 minutes, but did increase expression at 240min. No consistent pattern of expression of SOCS2 or CIS was seen in response to either normal or uraemic serum.

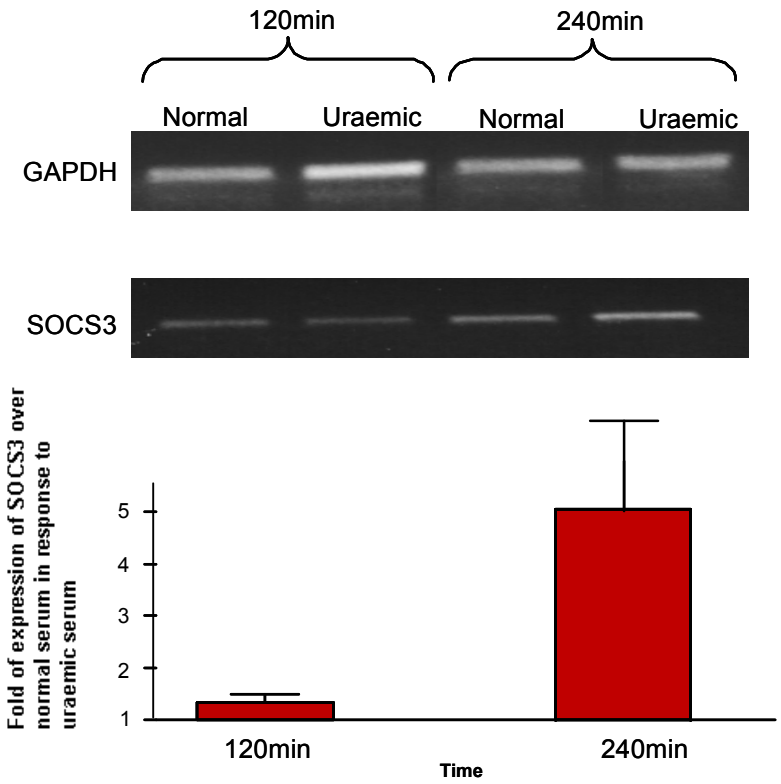


Figure 2.9: SOCS3 expression following stimulation of MG-63 cells with 10% uraemic serum. Results (n=2) are shown as fold of expression compared to cells incubated with 10% normal serum for an identical time.

2.4 Discussion

These studies demonstrate that the release of IL-6 from human osteoblastic cells is substantially greater during exposure to media containing serum from non-uraemic subjects than during incubation with charcoal stripped sera from the same individuals. The release of IL-6 is substantially attenuated in the presence of media containing serum from uraemic patients established on maintenance haemodialysis, even though uraemic serum significantly augmented IL-6 release in comparison to charcoal stripped serum. Charcoal stripped serum from normal and uraemic subjects yielded similar results. These findings suggest that uraemic serum itself might be able to influence bone turnover. In agreement with previous observations (McIntyre et al., 1999, Herbelin et al., 1991), the constitutive release of IL-6 from SaOS-2 cells was significantly (~10-fold) lower than from MG-63 cells; however, the effect of both normal and uraemic sera on the more mature osteoblast phenotype (SaOS-2) paralleled those seen in the less mature (MG-63) cells. In both these cell lines, IL-6 release was not augmented with the negative control (CS-10%FCS) but strongly augmented (>10-fold) with 100 IU IL-1β.

Charcoal stripping is a commonly utilized means of removing steroid hormones, including androgen and oestradiol metabolites, cortisol and thyroid hormones from serum (Darbre et al., 1983). The process is also likely to remove other growth factors and cytokines, although its exact quantitative effect in this regard is ill defined. In contrast, heat inactivation denatures serum proteins and peptides, but has minimal effect on steroid hormones. In the present studies, IL-6 production after exposure to uraemic serum was enhanced with respect to exposure to normal or uraemic sera depleted of cytokines and growth factors by charcoal stripping (figure 2.2). Haemodialysis did not have any consistent effect of on the capacity of uraemic serum to augment IL-6 release (figure 2.3). Sequential samples taken from 5 patients during sham dialysis for 60 minutes, followed by conventional haemodialysis for 240min, evoked a similar and near constant level of augmentation. These results imply that, in contrast to charcoal stripping, the factor(s) in uraemic serum affecting IL-6 release

were not removed by haemodialysis. This may be a clue to nature of the molecules involved, as charcoal stripping is able to remove larger, often more complex, molecules than haemodialysis can across standard modified cellulose membranes. Furthermore, heat treatment of uraemic serum had no effect on the release of IL-6, suggesting that the factors removed by charcoal stripping were not simply proteins or peptides.

The difference in IL-6 concentration in media harvested from MG-63 cells cultured with the various sera is not due to pre-existing circulating IL-6. Even though a disparity in the concentration of IL-6 in normal and uraemic sera was present, similar to those previously reported in patients with chronic kidney disease on or off haemodialysis treatment (Herbelin et al., 1991), these were trivial compared to those in the supernatants at the completion of the experiments. Furthermore, the uraemic and normal serum were diluted ten-fold in media before use. Also consistent with previous reports, there was no effect of haemodialysis itself on circulating IL-6 concentrations (Herbelin et al., 1991). IL-6, in the presence of sIL-6R, is known to induce its own synthesis in osteoblasts (Franchimont et al., 1997b) and if an effect from pre-existing IL-6 in the sera were present, then it might be anticipated that uraemic, rather than normal, serum would increase osteoblast IL-6 secretion.

IL-1β is known to increase the release of IL-6 from the cell lines under study (Siddiqi et al., 1998) and, indeed, addition of IL-1β to the media as a positive control led to a dramatic increase in IL-6 secretion. There were no significant differences in the concentration of IL-1β in any of the sera used in the incubation experiments and, as haemodialysis had no effect on the serum concentrations of IL-1β ((table 2.2), it therefore seems unlikely that this cytokine played a significant confounding role. The ability of dialysis membranes to influence IL-6 release has previously been reported. Both circulating IL-1β and IL-1β release from harvested peripheral blood monocytes have been shown to be elevated by dialysis involving bioincompatible (cuprophane) membranes (Lin et al., 1996), though not by the modified cellulose membranes similar

to those used in this study (Qian et al., 1995). Thus, not only is IL-1β unlikely to have contributed to differences in the release of IL-6, but it is also unlikely to have had any tonic effects. The final concentrations that the MG-63 cells were exposed to were all less than those previously reported as having an effect on IL-6 release from this cell line (McIntyre et al., 1999).

SIL-6R is a 50 kDa ligand binding protein derived from surface shedding of the gp80 IL-6 receptor (Mullberg et al., 1993) and might be expected to interfere with measured IL-6 levels by binding to free IL-6, possibly through interference with immunoassay detection. Artefacts of this kind seem unlikely as we found no significant differences in the concentrations of sIL-6R in any of the sera from patients or control subjects and dialysis did not influence the circulating concentrations of sIL-6R in the uraemic patients.

Receptor activator of NF-κB (RANK) is a membrane bound receptor found on osteoclast surfaces, while its ligand, RANK-L, is inducibly expressed on osteoblast cell membranes. Binding of ligand to receptor promotes osteoclastic differentiation and is a key step in the initiation of bone resorption. OPG, also an osteoblast product, is a secreted decoy receptor of RANK-L and functions as an ingenious regulatory mechanism (Suda et al., 1999, D'Amelio et al., 2009). When OPG levels decrease, more RANK-L is available to bind RANK and induce bone resorption and turnover. On the other hand, a rise in OPG tips the balance in firmly in favour of bone formation. Factors that decrease the OPG/RANK-L ratio, thereby favouring bone resorption with higher rates of bone turnover, include TNFα (Hofbauer et al., 1999c), PTH (Yasuda et al., 1998b), calcitriol (Yasuda et al., 1998b), gp130 activating cytokines (e.g. IL-6, IL-11) (Yasuda et al., 1998b) and IL-1 (Hofbauer et al., 1998, Hofbauer et al., 1999c). Those that increase the OPG/RANK-L ratio, thereby decreasing bone resorption and slowing bone turnover include oestrogens (Hofbauer et al., 1999b), and androgens (Hofbauer et al., 2002).

The current results, while consistent with previous observations of elevated circulating OPG levels in uraemic patients (Gonnelli et al., 2005, Sigrist et al., 2009), differ from those obtained for IL-6 and do not support our hypothesis that uraemic serum might differ from normal serum in its ability to promote or inhibit osteoblastic OPG secretion. However, it might have been expected that the amount of OPG measured in the cell supernatants would increase by as least as much as that present in the 10% dilutions of serum in comparison to serum free controls. This was indeed the case for SaOS-2, but not for MG-63, perhaps indicating that both normal and uraemic serum partially abolish the synthesis of OPG in this particular cell line.

There are precedents for components of the uraemic milieu affecting cellular function. In 1976 Wills and Jenkins reported that pre-dialysis serum from uraemic patients was able to inhibit the resorptive effect of parathyroid extract on bone, a phenomenon that was not reproducible by the addition of known uraemic metabolites. Furthermore, this effect could be removed by dialysis, following which there was a net increase in resorption (Wills and Jenkins, 1976). Uraemic toxins have been shown to affect the normal functioning of calcitriol by inhibiting the binding of calcitriol receptors to vitamin D response elements in a uraemic rat model (Patel et al., 1995). It has previously been reported that calcitriol does not have an effect on IL-6 transcription or release from MG-63 cells at concentrations less than 10^{-9} M, far higher than those encountered in the cell culture media (McIntyre et al., 1999). Thus, calcitriol is unlikely to be responsible for the effects on IL-6 release in this cell system. Many small proteins in uraemic serum may have profound effects on cellular functions (Haag-Weber et al., 1994) and it might be expected that some of these, for example PTH, may exert influence in the current model. The previous observation that PTH has no effect on IL-6 production in MG-63 cells at concentrations below 10^{-9} M (McIntyre et al., 1999), coupled with the demonstration that concentrations as high as 10^{-7} M did not increase IL-6 release in primary cell osteoblast cultures (Littlewood et al., 1991) suggests that this is unlikely to be the case. The PTH concentrations in the culture media fell below the lower of these

concentrations. Furthermore, heat treatment would have been expected to destroy PTH and no such effect was observed. Srivastava *et al* reported that uraemic serum contains a humoral substance capable of modulating colony forming unit counts in mice, an effect that could be modified by dialysis (Srivastava et al., 1990). In the current study, an effect of the various sera on cellular proliferation is unlikely to be of relevance as there were no differences in the levels of total cellular protein extracted from the various experiments. This is compatible with, although not proof of, a neutral effect on proliferation. Furthermore, Picton *et al* reported that a variety of uraemic toxins exerted no effect on the proliferation of SaOS-2 cells, despite incubation times up to three times longer than those used in this set of experiments (Picton ML, 1999).

The constituitive expression of mRNA for SOCS2, SOCS3 and CIS indicates that this important group of regulators of cytokine signalling may be operative in MG-63 osteoblatic cells. This further exploration of this observation is a major focus of the studies presented in chapters 2 and 3 of this thesis. The increase in SOCS3 expression in the presence of uraemic serum is consistent with previous data demonstrating impaired GH-activated JAK2/STAT signalling and EPO signalling in uraemia (Schaefer et al., 2001) (Allen et al., 1999). The elevation of SOCS3 in osteoblastic cells raises important questions, as the over expression of SOCS3 could potentially cause some of the aberrations of cytokine signalling seen in uraemic bone.

In conclusion, IL-6 release is attenuated by uraemic serum in comparison to normal serum in human osteoblast-like cells. This was not the case for OPG, where secretion remained the same in both settings. The removal of small molecules, including cytokines and growth factors, through charcoal stripping, removes the ability of serum to augment IL-6 production, though it remains unaffected by either sham or therapeutic haemodialysis. This suggests there may be a common factor(s) in uraemic serum, apparently not influenced by the primary renal diagnoses, causing reduced stimulation

of IL-6 release from osteoblasts and therefore potentially involved in the aberrant local signalling and cellular kinetics in uraemic bone.

Chapter 3

Expression and function of the suppressors of cytokine signalling (SOCS) in human osteoblastic cells

3.1 Background

Uraemia is a highly complex and multi-faceted state and one that is, at best, only partly represented in *in vitro* work by uraemic serum. In particular, uraemic serum is susceptible to multiple confounding factors; including concurrent inflammation, differing concentrations of circulating hormones as well as the possible effects of co-existing co-morbid conditions and drug treatments. These limitations guided subsequent experiments toward exploration of the cellular regulation of osteoblastic cytokine signalling, rather than toward attempts to identify those factors in the uraemic milieu that may interfere with it.

The potency of cytokines require that their effects are closely regulated to avoid harmful consequences. Amongst the measures that control cytokine activity are a family of inducible proteins that downregulate the cytokine-activated JAK/STAT signalling pathway - the suppressors of cytokine signalling or SOCS (Yasukawa et al., 2000). In unstimulated cells JAKs and STATs are inactive and SOCS genes are typically not expressed. After cytokine binding, receptor aggregation occurs, bringing receptor associated JAKs together for cross-phosphorylation and activation. These kinases can then phosphorylate multiple target proteins, including tyrosine residues in the cytoplasmic domains of the receptor and receptor associated STAT monomers (Ihle, 2001a). Phosphorylated STAT monomers form dimers that translocate to the nucleus where they bind specific DNA target sequences to regulate the transcription of biological response genes, including SOCS (Krebs and Hilton, 2001).

The ability of the SOCS proteins to influence signalling involves several disparate mechanisms. In essence, they combine direct inhibitory actions on specific components of the signalling cascade with more generic mechanisms that target components for ubiquitin mediated proteasomal degradation (Piessevaux et al., 2008). The clearance of SOCS proteins once they have acted to shut down signalling allows

the cycle of cytokine stimulation to be completed and the cell to return to its cytokine-responsive state (Hilton, 1999).

While both *in vitro* and *in vivo* studies show that cytokine induced SOCS expression is usually dependent on the JAK/STAT signalling pathway, it is increasingly apparent that non-JAK/STAT-dependent regulation of SOCS expression may also occur (Alexander et al., 1999, Yoshimura, 2009).

The function of SOCS within bone tissue is still only partially understood (Ahmed and Farquharson, 2010). The aim of the studies described in this chapter was to built on the observation of constituitive expression of SOCS2, SOCS3 and CIS in human osteoblastic cells presented and to attempt to determine their functional significance.

3.2 Materials and methods

3.2.1 Osteoblast-like cell lines and cell culture techniques

Cell lines and cell culture protocols were followed as described in section 2.2.1.

3.2.2 RT-PCR

The techniques for isolation of total RNA, RNA quantitation, first-strand complementary DNA (cDNA) synthesis, polymerase chain reaction, agarose gel electrophoresis and scanning densitometry are detailed in sections 2.2.12- 2.2.17.

3.2.3 Western immunoblotting

Sub-confluent MG-63 cells were incubated in medium with and without additional growth factors of interest for either 4 or 24h. At the end of the experiment, the incubation was stopped by the addition of an excess of ice cold PBS. Cells were pelleted by scraping with a cell lifter followed by centrifugation for 5min at 1200 *g*. Cell pellets were then lysed by the addition of ice-cold lysis buffer (pH 7.4):

Lysis buffer

20 mM Tris (pH 7.4)

150 mM NaCl

1% NP-40

0.5% w/v Na deoxycholate

0.1% SDS (Promega Corporation, USA)

Protease inhibitors were added immediately prior to cell lysis at the following final concentrations:

1 mM PMSF (10 mg/mL stock solution in isopropanol)

1 mM orthovanadate (100 mM stock solution)

30 µL of a 5-10 U/mL solution of aprotinin from bovine lung

Typically, 100 µL of lysis buffer was added per 2 x 10^6 cells. This was left on ice for 15min with regular vortex mixing and then centrifuged at 15,000 *g* for 10min at 4ºC.

The protein content of the supernatant was measured using the bicinchoninic acid (BCA) protein quantitation assay (Pierce, Thermo Scientific) in a 96-well plate format. Working reagent was made according to the kit insert. To construct a standard curve, a set of protein standards was made by diluting 2.0 mg/mL BSA stock solution in dH_20. Eight dilutions were made ranging in concentrations between 25 to 2000 µg/mL. 25 µL of each BSA standard was pipetted into the appropriate wells. 200 µL of the working reagent was added to each well, taking care that the samples were mixed well. The microplate was placed on a plate shaker for 30s and then put in an oven at 37°C for 30 minutes. Absorbance was then measured at 550nm on a Dynex MRX II (Dynex, Virginia, USA) plate reader.

3.2.4 Immunoprecipitation of SOCS proteins

SOCS proteins were immunoprecipitated from cell lysates. Lysates (500 µg cell protein) were incubated with 2 µg of polyclonal antibody to SOCS1 through 3 or CIS (Santa Cruz Biotech, CA, USA) for 2h at 4ºC with end over end agitation. The bound SOCS proteins were then immunoprecipitated by incubation with 20 µl protein A-agarose (Santa Cruz Biotech) for 4h at 4ºC with end over end agitation. Immunoprecipitates were collected by centrifugation at 2,500 *g* for 5 min at 4ºC and

pellets washed three times with 500 µL of lysis buffer containing protease inhibitors and each time collected by centrifugation at 2,500 *g* for 5min. After the final wash pellets were resuspended in 40 µL of sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 5min, and a proportion subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described below. The remaining immunocomplex in sample buffer was stored at -70ºC for future analysis.

3.2.5 SDS-PAGE

Fifty µg of protein from each sample (whole cell lysate) or a proportion of immunoprecipitated protein was electrophoresed on a 10% polyacrylamide gel in the presence of SDS. The composition of the solutions for SDS-PAGE are shown below:

Stacking gel

Protogel (30% w/v acrylamide; 0.8% w/v bis-acrylamide)	0.33	mL
Resolving buffer (1.44M Tris-HCl; 0.384% SDS; pH 8.8)	0.63	mL
10% Ammonium persulfate (APS)	12.5	µL
dH_20	1.54	mL
TEMED	2.5	µL

Resolving gel

Protogel (National Diagnostics, UK)	1.67	mL
Resolving buffer (0.5M Tris-HCl; 0.4% SDS; pH 6.8)	1.30	mL
10% APS	50	µL
dH_20	1.98	mL
TEMED	5	µL

Samples were mixed with an equal volume of sample buffer heated to 95ºC for 5 minutes and placed on ice. 10-20 µL (corresponding to 10 or 20 µg of protein) per well was loaded onto the gel. Electrophoresis was carried out at 25 mA per gel for approximately 45 minutes at room temperature in a buffer containing 0.025 M Tris (pH 8.3), 0.192 M glycine, 0.1% SDS.

3.2.6 Immunoblotting and detection of SOCS protein

Acrylamide gels were carefully removed from the electrophoresis apparatus and laid on top of a piece of polyvinyldifluoride (PVDF) membrane (Immobilon P; Millipore, MA, USA) which had been cut to size and pre-wetted in 100% methanol and dH20. Air bubbles were smoothed out with a sterile glass rod and the gel and membrane sandwiched between two sponges soaked in transfer buffer (10mM CAPS [3-cyclohexylamino-1 propane sulfonic acid] pH 11.0, 10% methanol in dH_20). The sandwich was then placed in the transfer apparatus with the membrane facing the anode and electroblotting carried out at 400 mA for 40 mins at room temperature. Following electroblotting the membrane was separated from the gel and any residual acrylamide washed off by soaking in tris-buffered saline-tween 20 (TBS-T) for 2 minutes. Non-specific sites on the membrane were blocked with 3% non-fat dry milk in TBS-T overnight at 4ºC. After washing in TBS-T for 10min at room temperature, the membrane was probed with polyclonal SOCS1-3 or CIS antibody (1:500 v/v in TBS-T + 1% BSA w/v; Santa Cruz Biotech) for 2h at room temperature with constant agitation. The membrane was then washed 5 times for 10 minutes each with TBS-T and incubated with a polyclonal anti-rabbit IgG antibody conjugated to horse radish peroxidase (1:2000; Santa Cruz Biotech) for 1h at room temperature with constant agitation. The membrane was washed as before but with the addition of a final wash with TBS. SOCS protein bands were visualised by chemiluminescence (ECL Chemiluminescence Detection System, Amersham, UK). Equal volumes (~2 mL) of ECL solutions (A and B) were mixed and gently pipetted onto the membrane and left

for one minute without agitation. The solutions were then drained off and the membrane placed onto a lint-free tissue to remove excess solution. The membrane was then immediately covered with Dow Saran wrap (Fisher Scientific UK, Loughborough UK) and exposed to light–sensitive film (Kodak Biomax Light) for various exposure times. The film was developed using an X-ray developer.

3.2.7 Real time PCR with Taqman ® technology

Fluorescence-based kinetic RT-PCR procedures significantly improve the reproducible quantification of mRNAs and overcome many of the limitations of RT-PCR, which is only a semi-quantitative technique. The Taqman assay (Perkin-Elmer/Applied Biosystems, CA, USA) utilises the 5'-nuclease activity of DNA polymerase to hydrolyse a hybridisation probe bound to a target amplicon. Three oligonucleotides are required. Two template-specific primers define the endpoints of the amplicon (and provide the first level of specificity). The third oligonucleotide probe hybridises to the amplicon during the annealing/extension phase of the PCR (and provides additional specificity). This probe contains a fluorescent reporter dye at its 5' end, the emission spectrum of which is quenched by a second fluorescent dye at its 3' end. If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound. If the correct amplicon is amplified, the probe can hybridise after the denaturation step. It then remains hybridised while the polymerase extends the primers until reaching the probe. It is then displaced and hydrolysed, separating the reporter and quencher dyes and releasing quenching of reporter fluorescence emission. As the polymerase will cleave the probe only while it remains hybridised to its complementary strand, the temperature conditions of the polymerisation phase of the PCR must be adjusted to ensure probe binding. Most probes have a Tm of around 70ºC; therefore, the Taqman system uses a combined annealing and polymerisation step at 60ºC to ensure the probe remains bound to its target during the primer extension step. It also ensures maximum 5'-3' exonuclease activity of the polymerase.

3.2.8 Extraction of RNA for real time PCR

The RNeasy 96 procedure (Qiagen) was used to provide the high throughput isolation of high quality RNA. The procedure has been validated for use with sensitive quantitative techniques such as Taqman. RNeasy combines the selective binding properties of a silica gel based membrane with vacuum technology. Cells are lysed with guanidine isothiocyanate (GITC) to inactivate RNases. Ethanol is added to provide appropriate binding conditions and the sample applied to the wells of a RNeasy 96 plate sitting in the QIAvac vacuum manifold. Total RNA binds while contaminants are washed away. High quality RNA is then eluted in a small volume of water, ready for use in complementary DNA synthesis.

3.2.9 RNA quantitation for real time PCR

The most commonly used technique for measuring nucleic acid concentration (as described in section 3.2.3) is the determination of absorbance at A_{260}. This method has several disadvantages, including the relative contribution of proteins and free nucleotides to the signal, the interference caused by contaminants and the relative insensitivity. The use of sensitive, fluorescent nucleic acid stains lessens many of these problems. RiboGreen ® RNA quantitation reagent (Molecular Probes, Invitrogen, Paisley, UK) is an ultrasensitive fluorescent nucleic acid stain for quantitating RNA in solution; based on fluorescein excitation and emission wavelengths, it enables the quantitation of as little as 1ng/mL RNA in a fluorescent microplate reader.

TE assay buffer (10 mM Tris-HCL, 1mM EDTA, pH 7.5) is used for diluting the RiboGreen reagent and RNA samples. A 20×TE stock solution is supplied and can be diluted in diethyl pyrocarbonate (DEPC) water. On the day of the use, an aqueous working solution of the RiboGreen reagent is prepared via the 1 in 2000 dilution of an aliquot of concentrated DMSO stock solution into TE. The working solution is protected from light by covering with foil. An RNA standard curve is prepared from supplied top standards as shown in figure 3.1. RNA samples are diluted 1 in 1000. 100 µl of diluted

sample (or standard) are added in duplicate to a 96-well plate. 100 µL of diluted RiboGreen is then added to all wells. The plate is wrapped in foil and left at room temperature for 5min. Fluorescence is read in a fluorescence plate reader (BMG Fluorostar, BMG Labtechnologies Ltd, Aylesbury, UK) at standard fluorescein wavelengths (excitation ~ 480 nm, emission ~ 520 nm).

TE volume	Volume (µL) of 100ng/mL RNA stock	Volume (µL) of 2000 fold diluted RiboGreen reagent	Final concentration
300	0	100	0
294	6	100	1ng/mL
270	30	100	5ng/mL
150	150	100	25ng/mL
0	300	100	50ng/mL

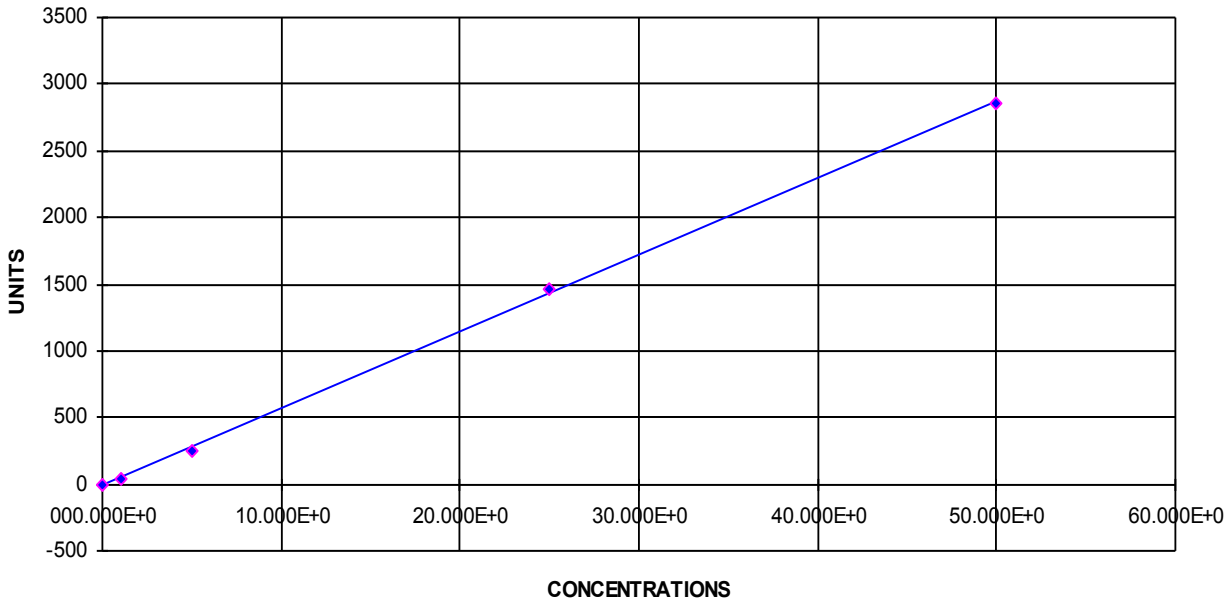


Figure 3.1: standard curve generated by the ribogreen assay. Concentration of RNA (x axis) is plotted against fluorescence (y axis)

3.2.10 First-strand complementary DNA (cDNA) synthesis for real time PCR

Reverse transcription was performed using the Omniscript reverse transcription kit (Qiagen) as recommended by the manufacturers, in which 0.5 µg of RNA and oligo-dT primer were used. Controls with no reverse transcriptase were included.

3.2.11 Primer and probe design

A summary of the primer and probe design is shown in the tables. Primer design for the Taqman is performed to specification through the use of a specialist primer design program, Primer Express (Applied Biosystems, Life Technologies Corp, USA).

Taqman primer guidelines
Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50-150 base pairs recommended)
Keep the G/C content in the 20-80% range
Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
The Tm should be 58-60ºC
The 5 nucleotides at the 3' end should have no more than 2 G and/or C bases

Table 3.1: Primer guidelines

Taqman probe guidelines
Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50-150 base pairs recommended)
Keep the G/C content in the 20-80% range
Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
The Tm should be 68-70º
No G on the 5' end
Select the strand that gives the probe more C than G bases

Table 3.2: Probe guidelines

An important default parameter in Primer Express software is the selection of amplicons in the 50-150 base pair range. Small amplicons are favoured because they amplify more efficiently and are more likely to be denatured during the 92-95ºC step of the PCR, allowing the probes and primers to compete more efficiently for binding to their complementary targets.

Primer	Length (bp)	Direction	5'-3' sequence	Taqman Probe
GAPDH	226	Forward Reverse	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
SOCS1	134	Forward Reverse	CGCCCTTAGCGTGAAGATG GGCGCCGCCACGTA	TTCAGGCCGGCCGCTTTCA
SOCS2	108	Forward Reverse	GCTCGCATTCAGACTACCTACTAACA CATATGATAGAGTCCAATCTGAATTTTCC	CTGGACCAACTAATCTTCGAATCGA ATACCAAGA
SOCS3	134	Forward Reverse	CCAGCCTGCGCCTCAA CGGCACTGAGCAGCAGGT	CAGTGCGCAAGCTGCAGGAGAGC
CIS	94	Forward Reverse	GTCCAGCCGAGTCCCCAC AGTCCGCTCCACAGCCAG	TCCTCTGCGTTCAGGGACCTCGTC

Table 3.3: Taqman primer and probe details

By independently varying forward and reverse primer concentrations, the concentrations that provide optimal assay performance can be identified. Primers are always in large molar excess during the exponential phase of PCR amplification, and by adjusting their initial concentration their effective T_ms can be adjusted to reduce the amount of non-specific primer binding. An initial concentration range of 50 nM-900 nM is tested for each primer. A probe optimisation experiment, in which the probe concentration is varied from 50-250nM, determines the probe concentrations that will provide acceptable results, without using the probe at unnecessary excess (so minimising running costs). Table 3.6 shows the optimised primer and probe concentrations.

	SOCS1 300/300	SOCS2 300/300	SOCS3 300/900	CIS 900/900	GAPDH 200/200
Master Mix	5	5	5	5	5
Forward	0.6	0.6	0.6	1.8	0.4
Reverse	0.6	0.6	1.8	1.8	0.4
Probe	0.2	0.2	0.2	0.2	0.2
Water	1.6	1.6	0.4	0	2

Table 3.4: Taqman master mix with optimised primer and probe concentrations

3.2.12 Construction of standard curves for real time PCR

SOCS are inducible genes not expressed in the steady state. To obtain cDNA to construct SOCS standard curves, DNA from the leukaemic cell line TF-1 after GM-CSF stimulation (previously shown to induce SOCS - unpublished data from our laboratory) was extracted and amplified using our standard RT-PCR primers. However the resulting level of expression was not sufficiently high to generate standard curves. As an alternative approach, plasmids containing the cDNA for the entire coding region of each human SOCS gene were obtained. These were a generous gift of Professor A.

Yoshimura, Fukuoka, Japan. SOCS 1 was contained in the pBluescript II SK + plasmid and SOCS 2,3 and CIS in the pcDNA3.1 plasmid.

Transformation

10 ng of plasmid DNA was added to a 200 µL suspension of JM109 E Coli competent cells (Promega). The mixtures were heat shocked at 42ºC for 45s and then chilled on ice for 10min. One mL of SOC medium was added followed by an agitated 1h incubation at 37ºC. 200 µL of this suspension was streaked onto LB (Sigma Aldrich Co, Dorset, UK) containing agar plates (Agar noble; Difco) containing ampicillin (Merck), X-GAL (Promega) and IPTG (Promega) and left overnight at 37ºC. White colonies were selected and grown overnight in 5 mL of LB broth containing ampicillin at 37ºC.

DNA purification

Small scale purification of plasmid DNA was carried out using the Wizard Plus DNA purification system (Promega). All reagents were supplied with the kit and the protocol was followed as in the manufactures instructions. The bacterial culture was centrifuged for 2min at 10 000 x *g*. The pelleted cells were resuspended in 200 µL of cell resuspension solution (50 mM Tris, 10 mM EDTA, 100 µg/ml RNase A) and transferred to a microcentrifuge tube. The bacteria were lysed with 200 µl of alkaline cell lysis solution (0.2M NaOH and 1%SDS) that was subsequently neutralised with 200 µL neutralisation solution (1.32M potassium acetate). The resulting lysate was centrifuged at 10 000 x *g* for 5 minutes. The supernatant containing plasmid DNA was transferred to the barrel of a syringe containing Minipreps DNA purification resin and the resulting mixture injected into a Minicolumn. The syringe was reused to push 2 mL of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA and 55% ethanol) into the minicolumn. The minicolumn was then transferred to a 1.5 mL centrifuge tube and at 10 000 x g for 2 minutes to dry the resin. The plasmid DNA was finally eluted in 50 µL of water.

To ensure that the plasmid preparation contained the correct size fragments, restriction endonuclease digestion of the plasmid DNA with EcoRI was performed. The purified plasmid DNA was quantitated and serially diluted to construct standard curves for each of the SOCS genes.

Standard curves for GAPDH

cDNA from unstimulated MG-63 cells was amplified using primers specific for human GAPDH yielding a 312 bp product (table 3.5). The primers were designed such that the 5' and 3' ends of the PCR product flanked the sites of the Taqman GAPDH primers. This PCR product was run on an agarose gel before excision for gel purification using the Wizard SV Gel and PCR clean-up System (Promega). The gel slice is transferred to a microcentrifuge tube and Membrane Binding Solution added at a ratio of 10µl of solution per 10 mg of agarose gel slice. The resulting mixture is vortexed and incubated at 65°C for 10min (until the gel slice dissolves). The dissolved gel mixture is then transferred to an SV Minicolumn sitting in a collection tube and incubated for 1 minute at room temperature followed by centrifugation at 10 000 x *g* for 1min. The flow-through is discarded. The column is then washed by the addition of 700µl of Membrane Wash Solution (diluted with 95% ethanol) and the minicolumn assembly centrifuged for 1min at 10 000 x *g*. The flow-through is again discarded and the wash step repeated. The column is then recentrifuged into an empty collecting tube to ensure drying. The DNA is eluted from the column into 50 µl of nuclease-free water. Once purified the GAPDH DNA is quantitated and serially diluted for use as a template for the standard curve.

3.2.13 The Taqman assay

The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) is a high-throughput real-time PCR system that allows the detection and quantitation of nucleic acid sequences. It operates in a 384-well plate format and is able to detect fluorescence between 500nm and 660nm.

A generic master mix is used for all assays. Assays are run using the same universal thermal cycling parameters. This eliminates the need for optimisation of the thermal cycling parameters and allows multiple assays to be run on the same plate. These thermal cycling parameters are shown in table 3.7.

Times and Temperatures			
Initial steps		**Each of 40 cycles**	
		Melt	**Anneal/extend**
Hold	**Hold**	**Cycle**	
2 min 50ºC	10 min 95ºC	15 sec 95ºC	1 min 60ºC

Table 3.5: Taqman assay thermal cycling conditions

3.2.14 Relative quantitation of gene expression

Accurate and reproducible quantification of mRNA using fluorescence-based PCR is based on the concept of the threshold cycle. During the Taqman assay, fluorescence values, representing the amount of product amplified to a particular point in the amplification reaction, are recorded as the cycle progresses. The more template present at the beginning of the reaction, the fewer number of cycles are needed to reach a point in which the fluorescent signal becomes statistically significant above background. This point is defined as the C_t. It always occurs during the exponential

phase of amplification so quantification is not affected by limitation of reaction components during the plateau phase.

Quantification of mRNA transcription can be either relative or absolute. Relative quantification determines the changes in steady state transcription of a gene and is often adequate. A relative standard consists of a sample, the calibrator, which is used to create a dilution series with arbitrary units. During the PCR assay, the target C_t is compared directly with the calibrator C_t and is recorded as containing either more or less mRNA. For normalisation to an endogenous control (GAPDH in this instance), standard curves are prepared for both the target and the endogenous reference. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. Then, the target amount is divided by the endogenous reference amount to obtain a normalised target value.

Plate setup

Figure 3.2 shows the 384-well plate setup for the relative quantitation of the SOCS mRNA. The target and endogenous reference (GAPDH) are amplified in separate tubes. Dilutions of cDNA prepared from SOCS DNA (in either the pcDNA3.1 or pBluescript II SK plasmids) was used to construct SOCS standard curves. Dilutions of purified GAPDH cDNA from MG-63 cells was used to construct the standards for GAPDH. Standards are placed in the wells on the left of the plate (coloured in the template).

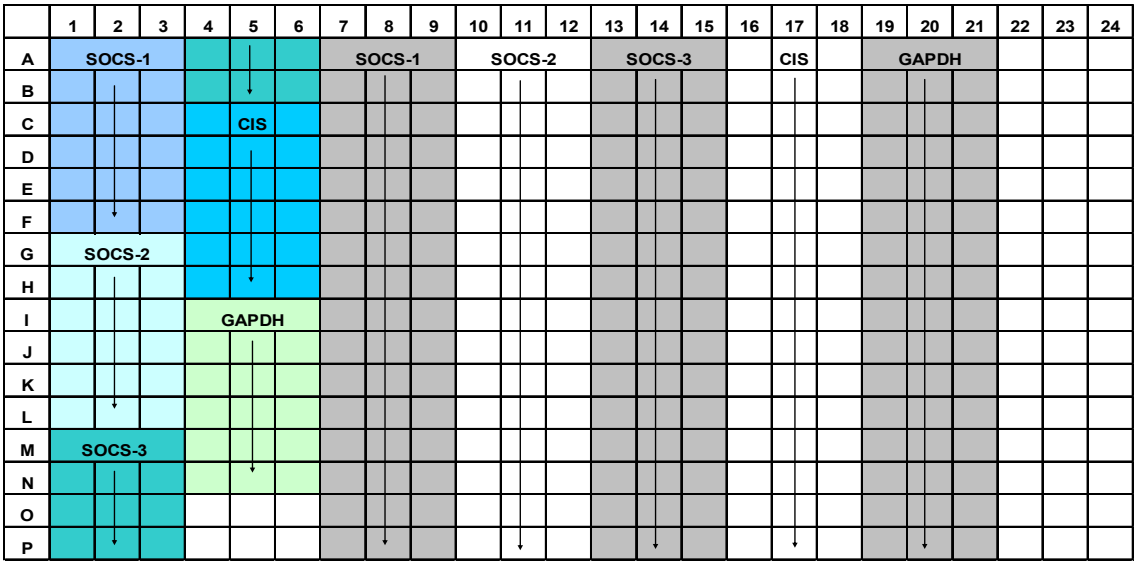


Figure 3.2: Plate setup for the Taqman assay

The unknown samples to the right of the plate are cDNA prepared from total RNA isolated from either MG-63 or SaOS-2 cells.

Setting thresholds

After performing the PCR, a threshold is set for fluorescence detection by examining the semi-log view of the amplification plots (fluorescence versus cycle number). This is above the noise, but still in the linear region of the semi-log plot (horizontal red line in the figure 3.3 below).

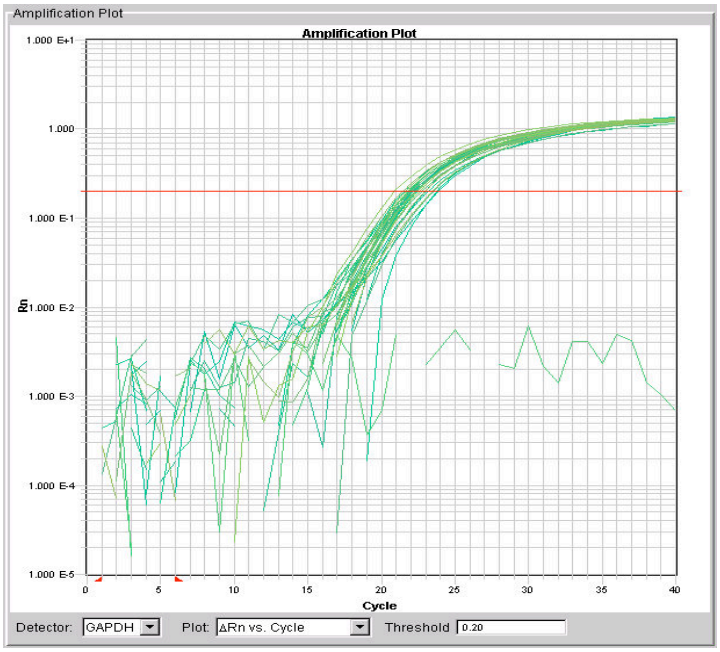


Figure 3.3: An amplification plot (fluorescence vs. cycle number) for GAPDH of several cDNA samples. The horizontal red line shows the threshold for fluorescence detection

Constructing a relative standard curve

To analyse the experiment, results are exported to an Excel (Microsoft) spreadsheet and plotted as an XY scatter graph on the worksheet with the log input amount (DNA standard) as the X values and the C_t as the Y values. A linear regression trendline is added with intercept, slope and R^2 values displayed.

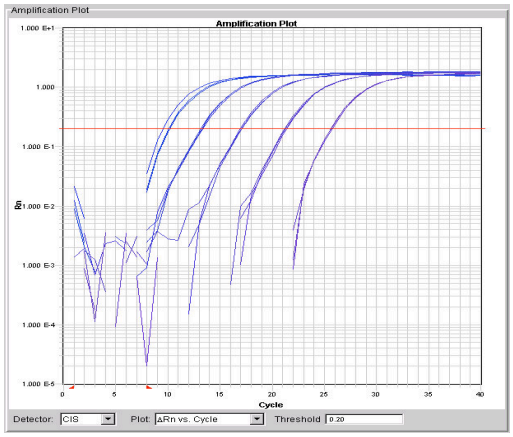


Figure 3.4: an amplification plot of serial (1 in 5) dilutions of CIS DNA

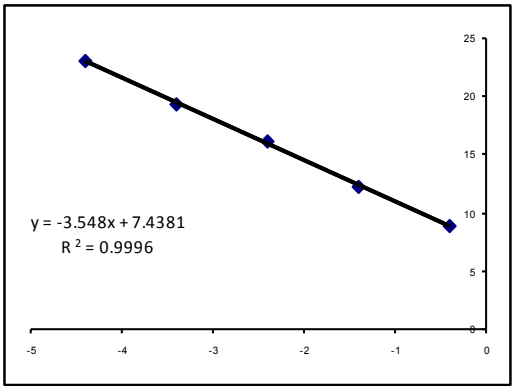


Figure 3.5: An XY scatter plot of log DNA input amount (x) against C_t (Y)

For the line shown in the figure, the log input is calculated by entering the following formula in one cell of the work sheet:

= ([cell containing C_t value] – b)/m

where b = y-intercept of standard curve line

and m = slope of standard curve line

The input amount for samples is calculated by entering the following formula in an adjacent cell (where ^ represents 'to the power of'):

= 10^ [cell containing input amount]

These steps are repeated to construct a standard curve for the endogenous reference (using the C_t values determined with the labelled GAPDH probe). The input amount is calculated for each sample cDNA from both SOCS and GAPDH standard curves. The value for SOCS input amount is then divided by the GAPDH input amount that yields the relative input amount.

3.2.15 Regulation of SOCS expression by cytokines: interrogation by real time PCR

Poor reproducibility and the failure to detect SOCS1 mRNA proved to be significant limitations of standard semi-quantitative RT-PCR methods for the investigation of SOCS expression. In view of this, a fluorescence-based kinetic PCR technique using Taqman® technology (Applied Biosystems) was used in all subsequent expression experiments. The specificity of the Taqman assay is such that the confirmation of product identity by sequencing is considered unnecessary (Bustin, 2002, Nolan et al., 2006).

3.2.16 Experimental design

After being harvested and counted, MG-63 cells were plated at a density of 0.5 x 10^6 cells per well of a 6-well plate in 3 mL medium and incubated at 37ºC for 24h. The cells were then placed in serum free media for 12h, before incubation with the growth factor or cytokine of interest (IGF-I, IL-1ß, LPS or GM-CSF), for time points between 0 and 8h. An additional untreated sample was collected at each time point. mRNA was isolated from the cells and used in subsequent cDNA synthesis and (real time) PCR amplification with SOCS specific primers. All stimulation experiments were performed on three separate occasions. An identical experimental paradigm was used for SaOS-2 cells.

Each cDNA specimen was divided so that subsequent PCR amplifications could be performed in triplicate, with subsequent calculation of the mean. Each reaction is corrected for GAPDH, expressed as a fold of its own unstimulated control and normalised to time zero (i.e. relative expression at time zero = 1). Data are shown as the mean ± SEM for each treatment and analysed using one-way ANOVA (analysis of variance), and applying a post hoc Bonferroni test. $p<0.05$ was considered statistically significant

Growth Factor	Concentration
IGF-I	10 ng/mL
IL-1ß	100 ng/mL
Lipopolysaccharide (LPS)	100 ng/mL
GM-CSF	2.5 ng/mL

Table 3.6: concentrations of growth factors and cytokines used in real time PCR SOCS expression studies

3.2.17 Functionality: transfection of MG-63 cells with SOCS 1 and SOCS 3 DNA

In these experiments the effects on cytokine production of SOCS1 and SOCS3 over expression in MG-63 cells was examined. MG-63 cells were seeded into 6 well tissue culture plates and allowed to reach 50% confluence. For each well to be transfected, 3 µL GeneJuice Transfection Reagent (Novagen, Merck, Darmstadt, Germany) was added drop wise to 100 µL serum free medium in a sterile tube, before vortexing. Following incubation at room temperature for 5min, either 1 µg SOCS pcDNA3 plasmid (containing the full length SOCS gene) or empty vector was added for each well to be transfected and combined by gentle pipetting. This mixture was incubated at room temperature for 10 minutes before careful drop wise addition to the 6 well plates where cells remained in complete growth medium. The drops were distributed over the entire surface of the dish and the plates gently rocked to ensure even delivery to the adherent cell monolayer.

After 8h the transfection mixture was removed and replaced with complete medium. The cells were then incubated for a further 24h at 37˚C, before washing and either (i) simple media refreshment, (ii) stimulation with IL-1β, or (iii) stimulation with IGF-I for a further 24h. Successful transfection was confirmed by PCR.

3.2.18 Cytokine assays

IL1-β and OPG were assayed in cell culture supernatants using the relevant DuoSet ELISA Development kit (R&D Systems, Abingdon, UK), as described in section 2.2.10.

3.3.19 Functionality: JAK/STAT signalling pathway

In this experiment, 3 separate clones of SOCS3 transfected cells - designated 3.1, 3.2 and 3.3 - were stimulated with IL-6 and the sIL-6R. The sIL-6R is the soluble form of the α subunit of the IL-6 receptor. Unlike other soluble cytokine receptors, it is an agonist - binding to IL-6 and stimulating JAK/STAT signalling via the gp130 subunit. It is added in this experiment as MG-63 cells respond poorly to IL-6 alone. Whole cell lysates were assessed for the activation of STAT3 to examine whether signalling via gp130/STAT3 remained intact.

3.3.20 Assay for activated STAT3

STAT3 activation was assayed using a TransAM Kit (Active Motif). This comprises a 96-well plate coated with an immobilised oligonucleotide containing the consensus binding site of the activate form of STAT3. A primary STAT3 antibody recognises an epitope that is accessible only when it is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a colorimetric readout that can be quantified by spectrophotometry. Briefly, 10 µg of sample extract is diluted in lysis buffer, with 20 µL added to each well. The plate is sealed and incubated for 1 hour at room temperature with continuous gentle mixing. Following a wash x 1, STAT3 antibody (100 µL of a 1:500 dilution in binding buffer) is added and a further 1 hour incubation undertaken. After a further wash, 100 µL of HRP-conjugated antibody (1:1000 dilution in binding buffer). After a further hour, developing solution is added. Stop solution is added when the sample wells turn blue (2-10min). Absorbance is read on a spectrophotometer at 450 nm with a reference wavelength of 650 nm.

3.3 Results

The preliminary data generated by initial studies using conventional RT-PCR are presented in appendix 2.

3.3.1 Results of Taqman SOCS expression studies (figures 3.6-3.11)

IL-1ß

There was a trend to increased expression of all four SOCS genes in MG-63 cells following stimulation with 100 IU/mL IL-1ß, this became significant at 4h for SOCS1 and SOCS3 and at 8h for SOCS2. The response to IL-1ß in SaOS-2 cells, with the exception of an absent SOCS2 response, was similar, with a trend to increased SOCS1, SOCS3 and CIS expression.

GM-CSF

SOCS3 expression was increased at 1h and had doubled by four hours. This effect was not significant however. Effects of GM-CSF on SOCS1 expression were very small and those on SOCS2 and CIS negligible.

LPS

The effect of LPS on SOCS expression in MG-63 cells was most evident for SOCS3 and CIS, though neither reached statistical significance. The effect of LPS on SOCS expression generally became apparent at later time points than for the other stimuli studied.

IGF-I

SOCS1 was induced by IGF-I in MG-63 cells (significant after 2h) as well as CIS (significant after 1h). A trend to increased SOCS2 and SOCS3 expression was seen in SaOS-2 cells.

The pattern of expression of the SOCS genes after stimulation with the above factors in MG-63 and SaOS-2 cells is shown in figures 3.6 – 3.11.

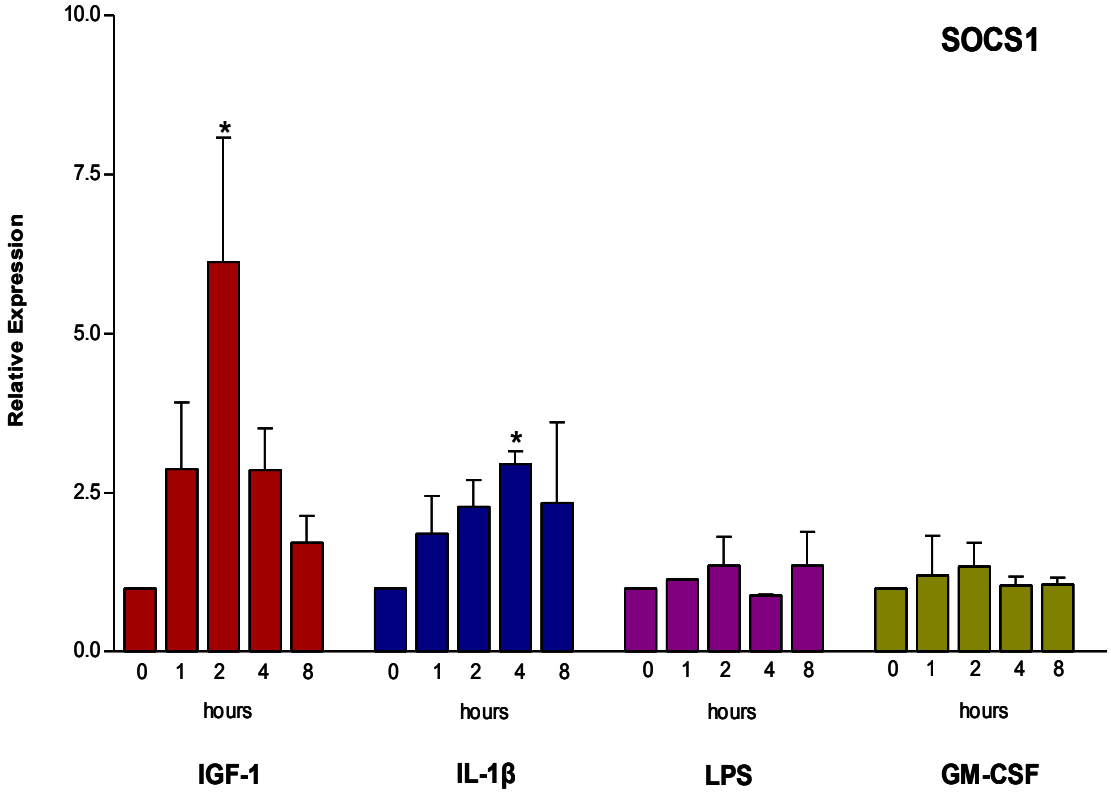


Figure 3.6: SOCS1 expression at 0, 1, 2, 4 and 8 h following stimulation of MG-63 cells with selected growth factors and cytokines, as determined by real time PCR. A statistically significant difference ($p<0.05$) in expression in comparison to time 0 is denoted by *. Expression of SOCS1 increases post IGF-I exposure, reaching statistical significance at 2h and following IL-1ß, reaching significance at 4h. For each treatment n=3. All PCRs were performed in triplicate.

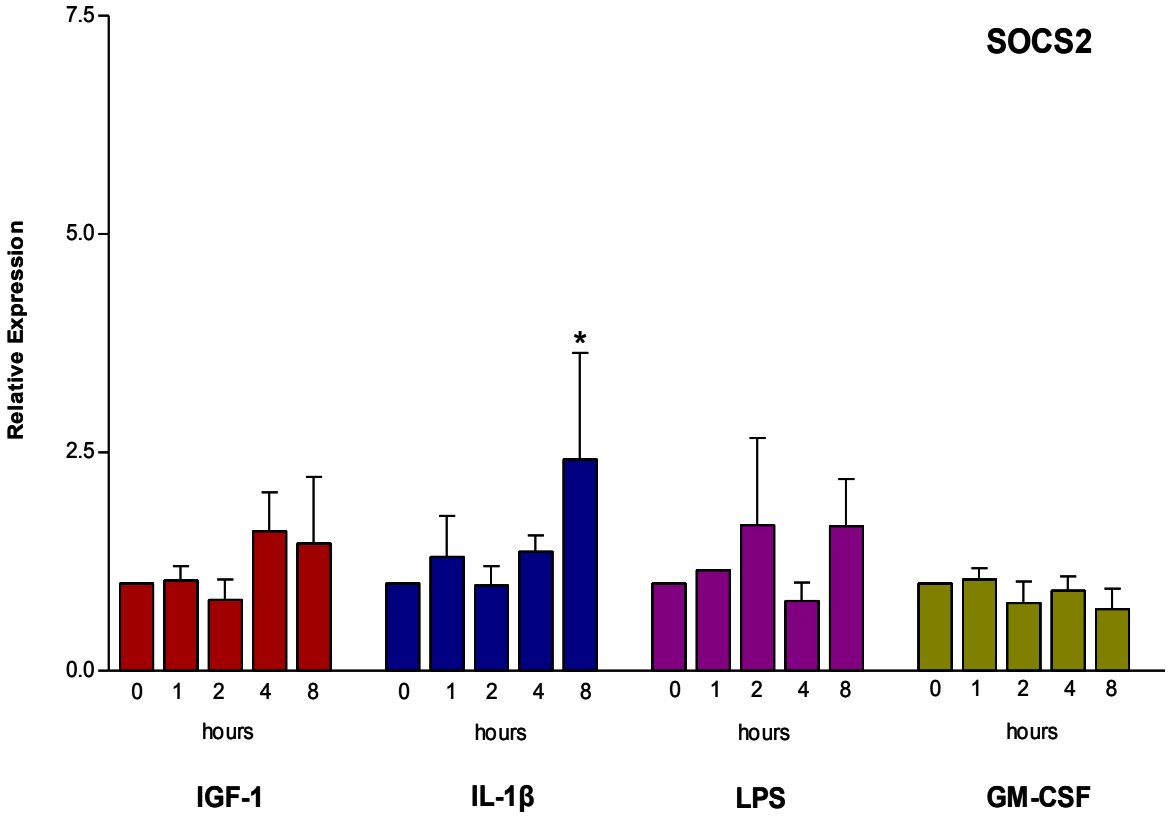


Figure 3.7: SOCS2 expression at 0, 1, 2, 4 and 8 h following stimulation of MG-63 cells with selected growth factors and cytokines, as determined by real time PCR. A statistically significant difference ($p<0.05$) in expression in comparison to time 0 is denoted by *. Expression of SOCS2 post IL-1ß exposure reached statistical significance at 8h. For each treatment n=3. All PCRs were performed in triplicate.

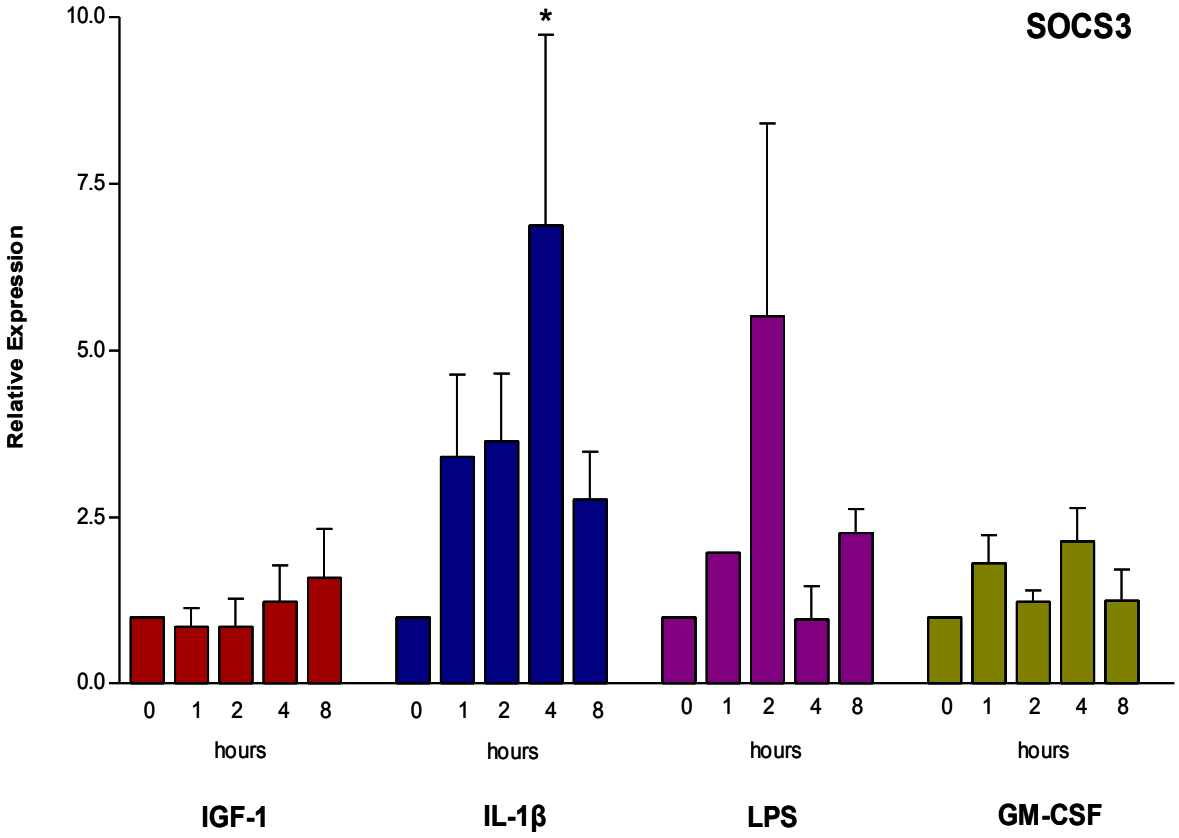


Figure 3.8: SOCS3 expression at 0, 1, 2, 4 and 8 h following stimulation of MG-63 cells with selected growth factors and cytokines, as determined by real time PCR. A statistically significant difference ($p<0.05$) in expression in comparison to time 0 is denoted by *. Expression of SOCS3 post IL-1ß exposure reached statistical significance at 4h. For each treatment n=3. All PCRs were performed in triplicate.

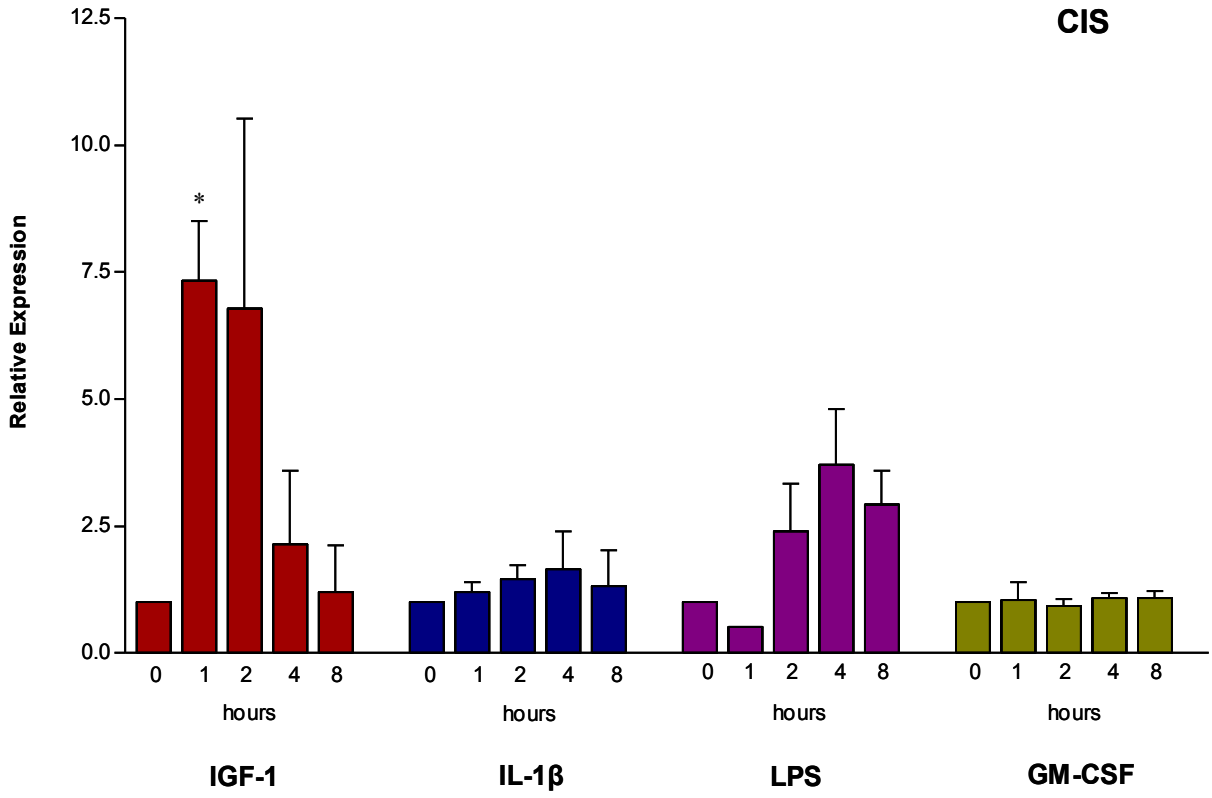


Figure 3.9: CIS expression at 0, 1, 2, 4 and 8 h following stimulation of MG-63 cells with selected growth factors and cytokines, as determined by real time PCR. A statistically significant difference ($p<0.05$) in expression in comparison to time 0 is denoted by *. Expression of CIS post IGF-I exposure reached statistical significance at 1h. None of the exposures led to a statistically significant difference in CIS expression compared to time zero. For each treatment n=3. All PCRs were performed in triplicate.

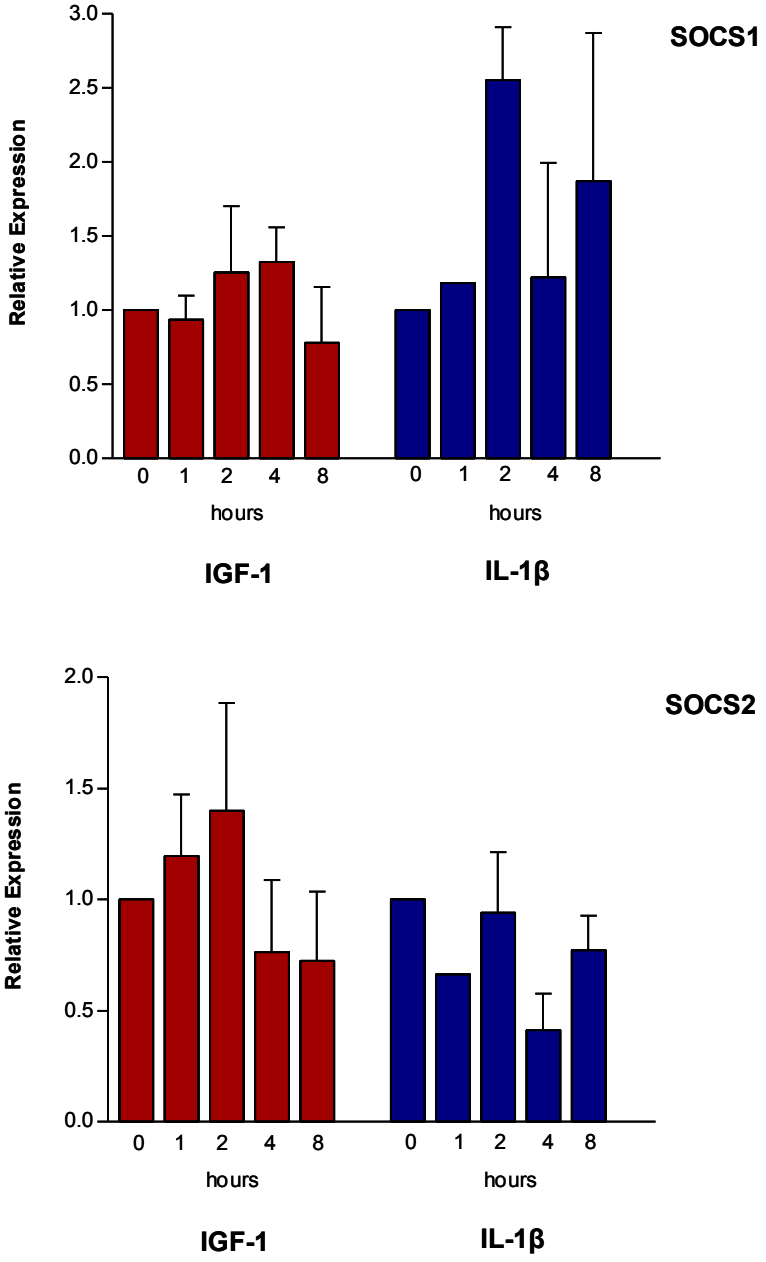


Figure 3.10: SOCS1 and SOCS2 expression at 0, 1, 2, 4 and 8 h following stimulation of SaOS2 cells with IGF-I and IL-1ß, as determined by real time PCR. For each treatment n=3. All PCRs were performed in triplicate.

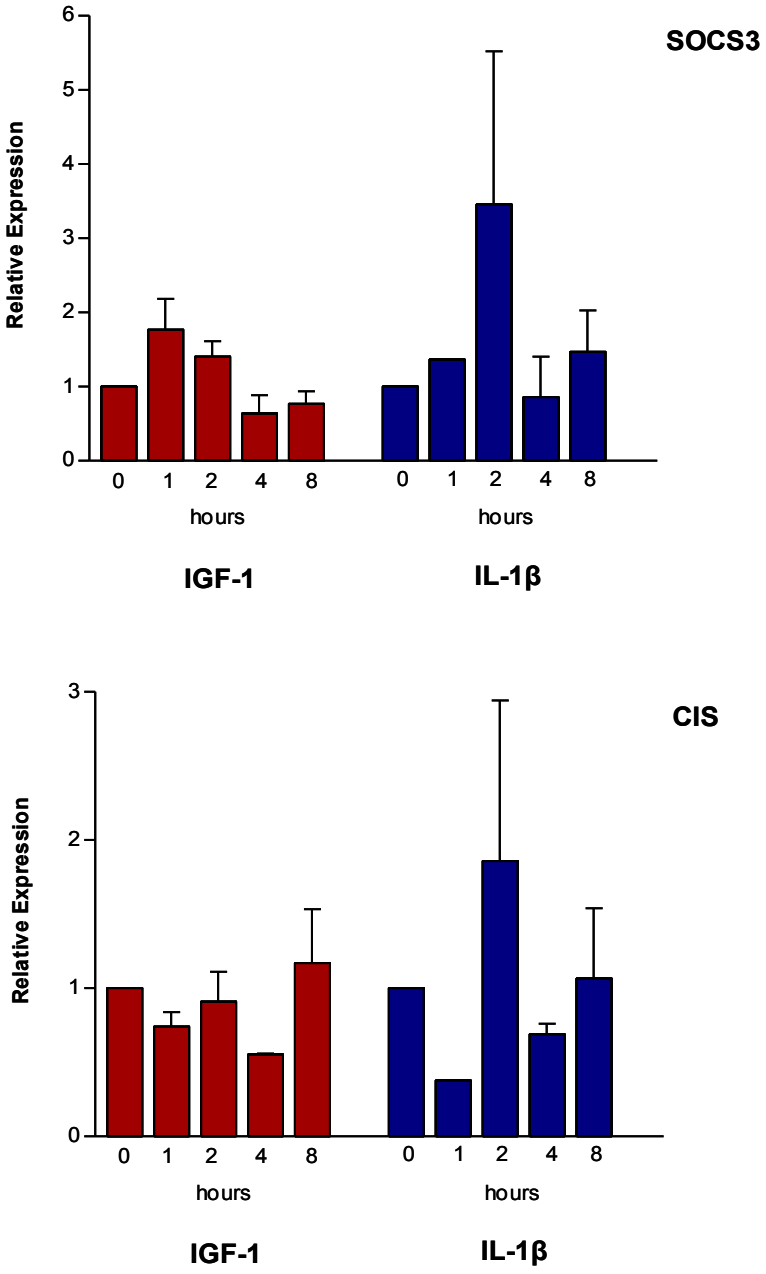


Figure 3.11: SOCS3 and CIS expression at 0, 1, 2, 4 and 8 h following stimulation of SaOS2 cells with IGF-I and IL-1ß, as determined by real time PCR. For each treatment n=3. All PCRs were performed in triplicate.

3.3.2 Detection of SOCS Protein in MG-63 cells

The detection of SOCS protein in whole cell lysates has proved difficult due to the paucity of good quality commercially available antibodies. This experience has been shared with other laboratories with an interest in this area. Immunoprecipitation has been attempted (see 3.2.9) and not appreciably enhanced SOCS protein detection. Due to these difficulties it cannot be viewed as an adequate technique for quantitative purposes. New antibodies are now available but relatively untested for Western blotting applications. Figure 3.12 shows representative Western blots of whole cell lysates from MG-63 cells, in this instance incubated with 10 ng/mL IGF-I for 4 or 24 hours. Small bands were seen at expected molecular weight for SOCS2, SOCS3 and CIS although these tended to be quite faint and often masked by non-specific bands, whilst no band was visible at the expected position of SOCS1.

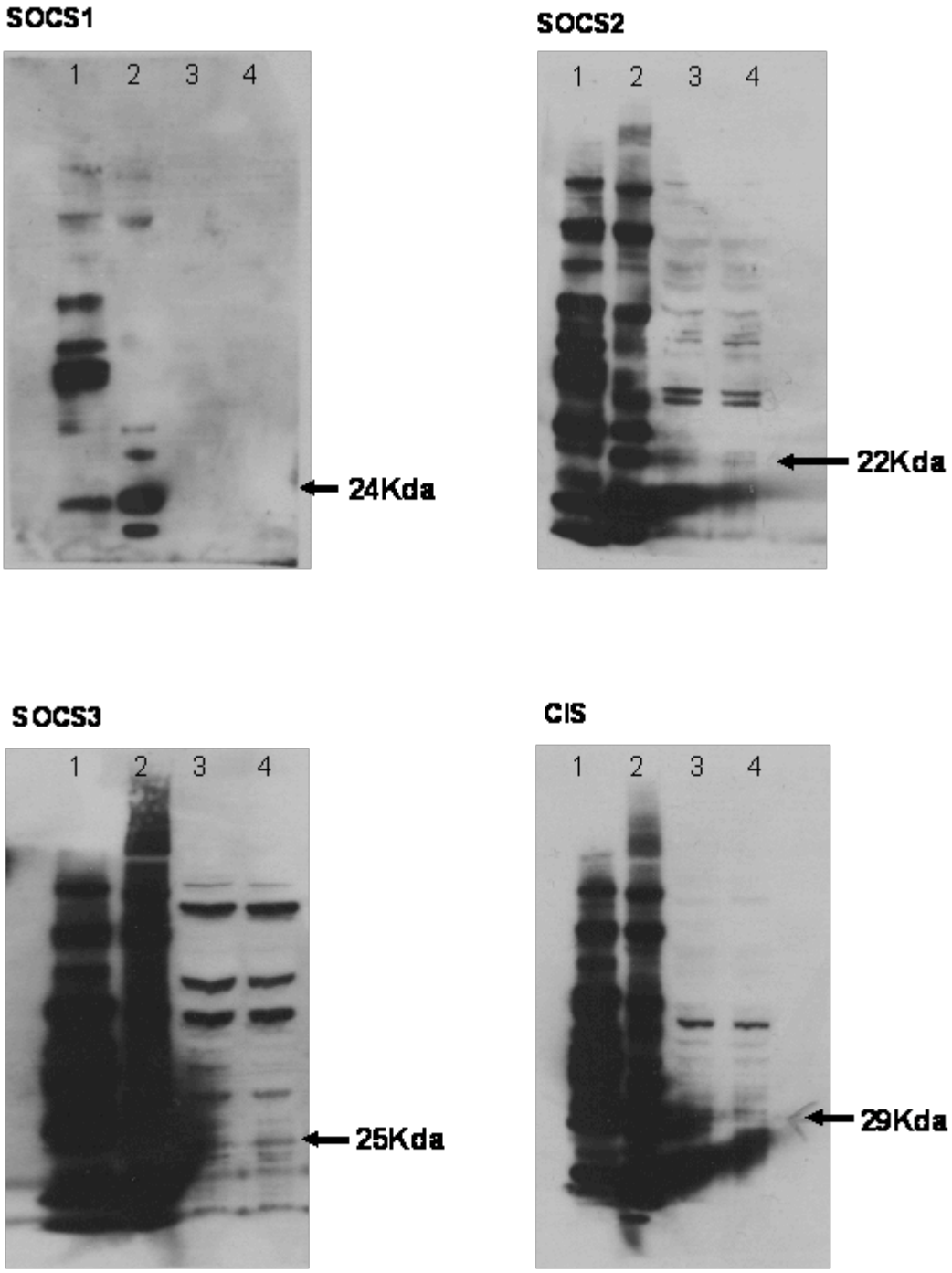


Figure 3.12: The detection of SOCS protein in MG-63 cells. Four western blots prepared from whole cell lysates are shown. Lane 1: 5 µL of Perfect Protein Marker (Novagen). Lane 2: 5 µL of Rainbow Marker (Amersham). Lane 3: 50µg whole cell lysate from MG-63 cells following 6 hours incubation with 10ng/mL IGF-I. Lane 4: 50µg whole cell lysate from MG-63 cells following 24 hours incubation with 10ng/mL IGF-I. Membranes were probed with the appropriate rabbit anti-SOCS antibody at 1:500 dilution. The arrows show the size and position of the expected SOCS protein bands. The bands were very faint and the long exposure time required led to overexposure of markers.

3.3.3 Functionality: IL-6 production following stimulation of SOCS1 DNA transfected MG-63 cells with IL-1β

Basal IL-6 production was dramatically increased in MG-63-SOCS1 cells (see section 3.2.22) compared with MG-63-Empty Vector (EV) cells (n=6, p<0.001). MG-63 SOCS1 cells also demonstrated enhanced production of IL-6 following IL-1β (100IU/mL) stimulation in comparison to MG-63-EV (n=6, p<0.001). These results are summarised in figure 3.13.

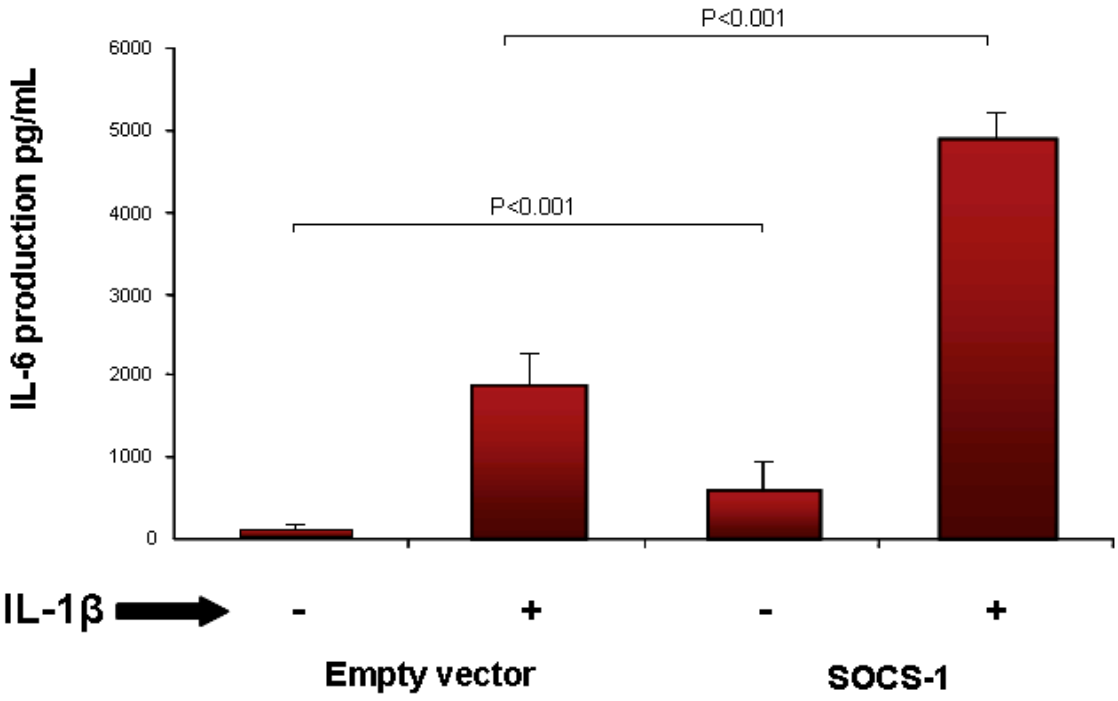


Figure 3.13: Basal IL-6 production was significantly increased in MG-63-SOCS1 cells compared with MG-63-EV cells (n=6, p<0.001). MG-63 SOCS1 cells also demonstrated enhanced production of IL-6 following IL-1β stimulation (100IU/mL) in comparison to MG-63-EV (n=6, p<0.001).

3.3.4 Functionality: IL-6 production following stimulation of SOCS3 DNA transfected MG-63 cells with IL-1β

There were comparable data following transfection with SOCS3 (see section 3.2.22). Basal IL-6 production was dramatically increased in MG-63-SOCS3 cells compared with MG-63-Empty Vector (EV) cells (n=6, p<0.001). MG-63-SOCS3 cells also demonstrated enhanced production of IL-6 following IL-1β stimulation in comparison to MG-63-EV (n=6, p<0.001). These results are summarised in figure 3.14.

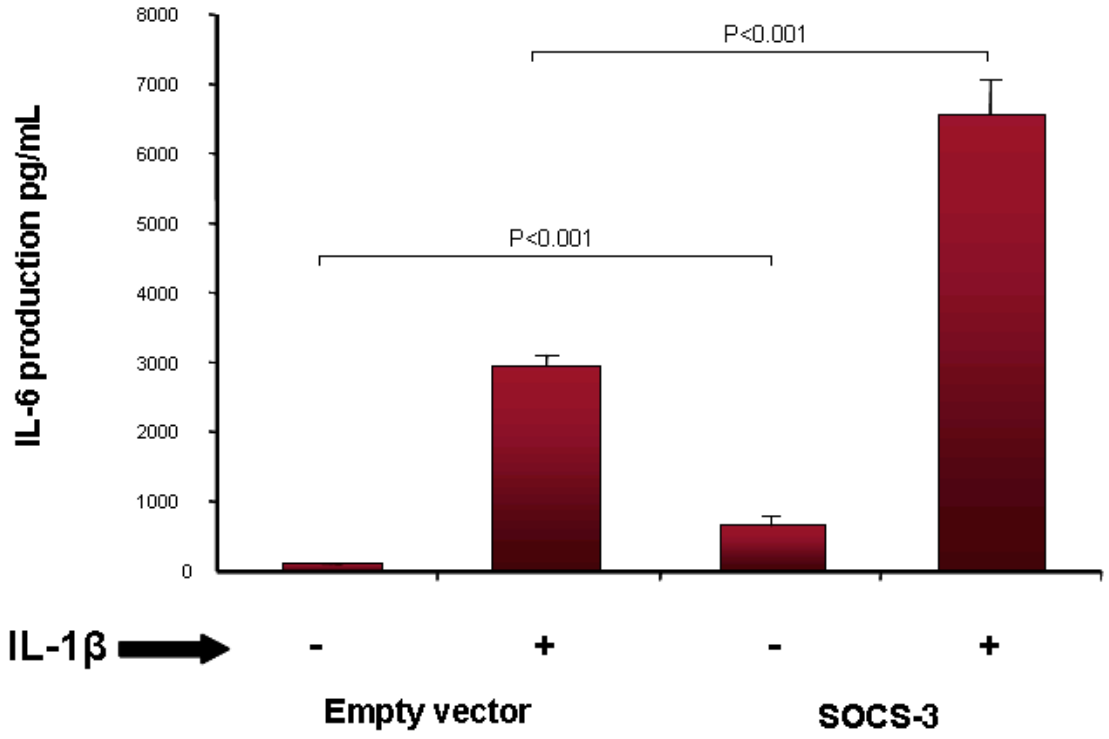


Figure 3.14: Basal IL-6 production was significantly increased in MG-63-SOCS3 cells compared with MG-63-EV cells (n=6, p<0.001). MG-63-SOCS3 cells also demonstrated enhanced production of IL-6 following IL-1β stimulation in comparison to MG-63-EV (n=6, p<0.001).

3.3.5 Functionality: IL-6 production following stimulation of SOCS 1 DNA transfected MG-63 cells with IGF-I

Basal IL-6 production was increased in MG-63-SOCS1 cells compared with MG-63-Empty Vector (EV) cells (n=6, p<0.001). MG-63 SOCS1 cells did not demonstrate enhanced production of IL-6 following IGF-I stimulation in comparison to MG-63-Empty Vector. These results are summarised in figure 3.15.

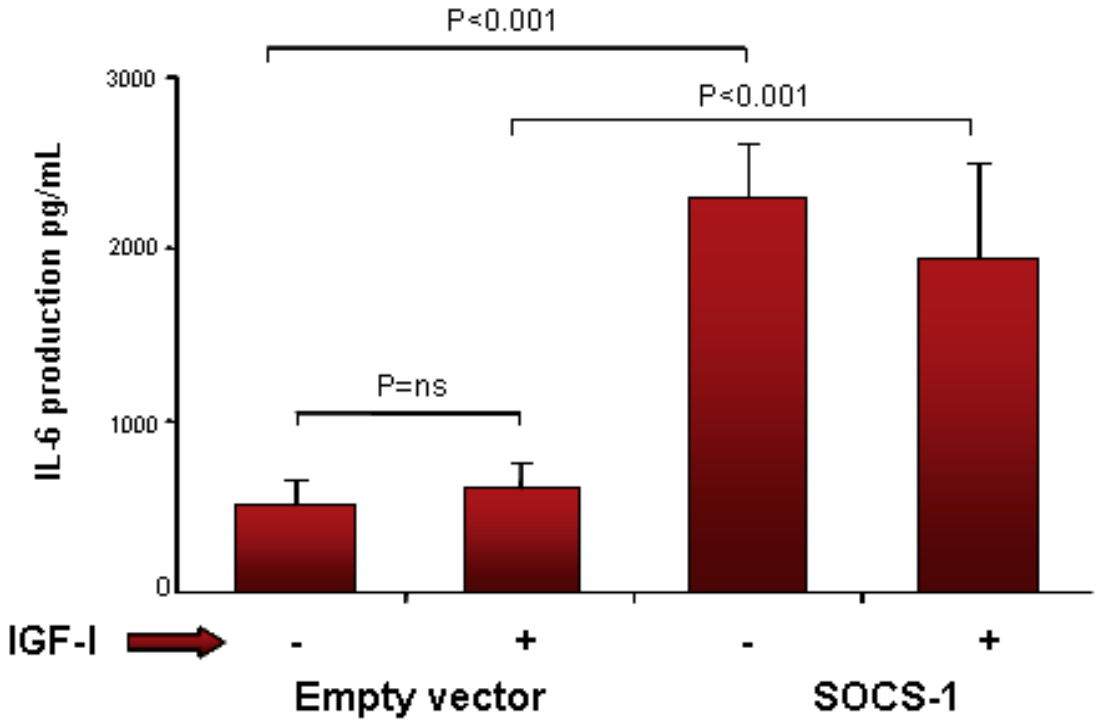


Figure 3.15: Basal IL-6 production was increased in MG-63-SOCS1 cells compared with MG-63-EV cells (n=6, p<0.001). MG-63 SOCS1 cells did not demonstrate enhanced production of IL-6 following IGF-I stimulation in comparison to MG-63-EV (n=6, p=ns).

3.3.6 Functionality: IL-6 production following stimulation of SOCS 3 DNA MG-63 cells transfected with IGF-I

Basal IL-6 production was increased in MG-63-SOCS3 cells compared with MG-63-Empty Vector (EV) cells (n=6, p<0.001). MG-63 SOCS3 cells did not demonstrate enhanced production of IL-6 following IGF-I stimulation in comparison to MG-63-Empty Vector. These results are summarised in figure 3.16.

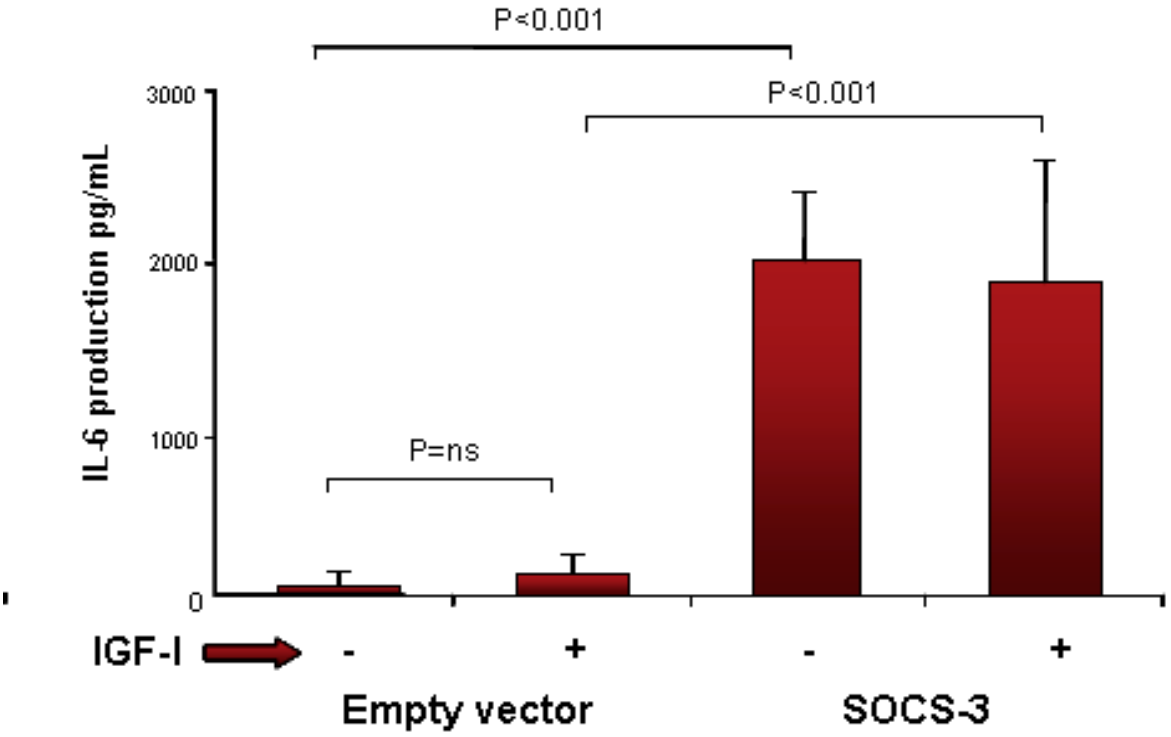


Figure 3.16: Basal IL-6 production was increased in MG-63-SOCS3 cells compared with MG-63-EV cells (n=6, p<0.001). MG-63 SOCS3 cells did not demonstrate enhanced production of IL-6 following IGF-I stimulation in comparison to MG-63-EV (n=6, p=ns).

3.3.7 Functionality: OPG production following stimulation of SOCS 1 DNA transfected MG-63 cells with IL-1β

Basal OPG production was not increased in MG-63-SOCS3 cells compared with MG-63-Empty Vector (EV) cells. No significant differences in OPG secretion were seen in the SOCS1 transfected cells following IL-1β stimulation in comparison to cells transfected with EV. Figure 3.17.

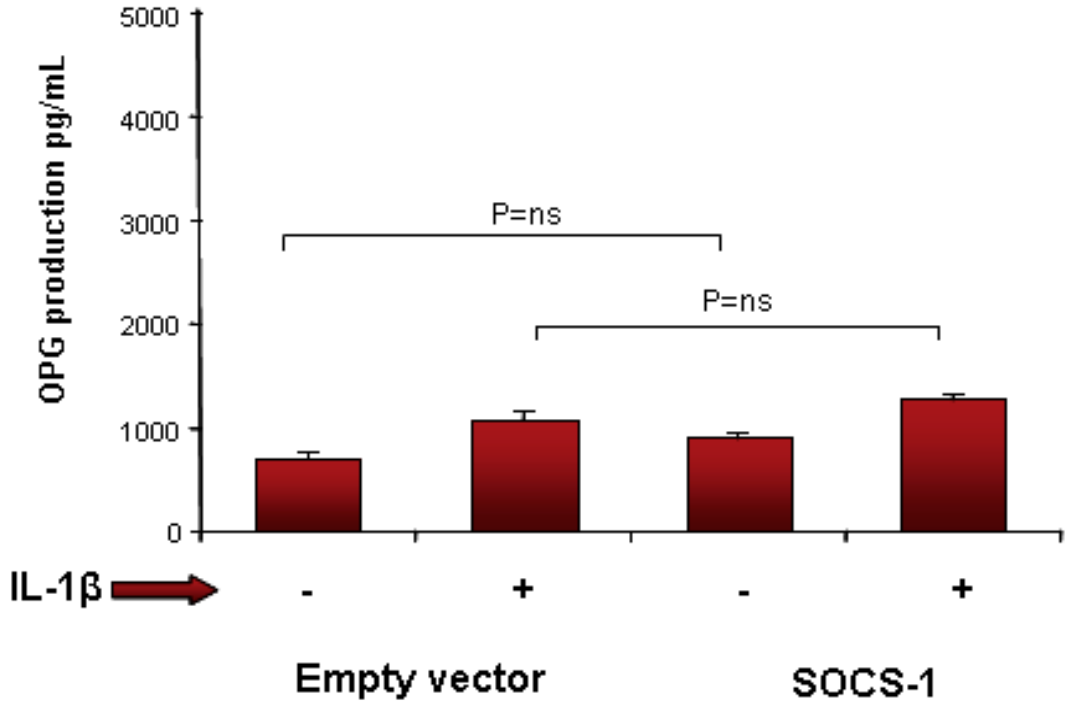


Figure 3.17: Basal OPG production was not increased in MG-63-SOCS3 cells compared with MG-63-EV cells (n=6, p=ns). No significant differences in OPG secretion were seen in the SOCS1 transfected cells following IL-1β stimulation in comparison to cells transfected with EV (n=6, p=ns).

3.3.8 Functionality: OPG production following stimulation of SOCS3 DNA transfected MG-63 cells with IL-1β

Basal OPG production was not increased in MG-63-SOCS3 cells compared with MG-63-Empty Vector (EV) cells. However, significant differences in OPG secretion were seen in the SOCS3 transfected cells following IL-1β stimulation in comparison to cells transfected with empty vector (n=6, p<0.001). Figure 3.18.

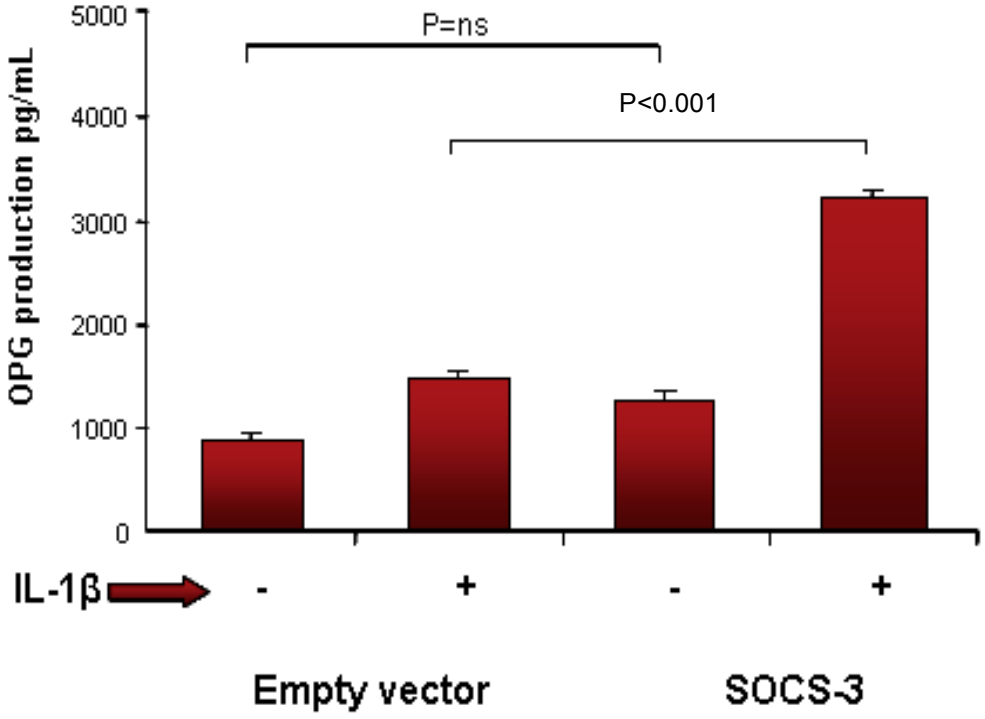


Figure 3.18: Basal OPG production was not increased in MG-63-SOCS3 cells compared with MG-63-EV cells. However, significant differences in OPG secretion were seen in the SOCS3 transfected cells following IL-1β stimulation in comparison to cells transfected with empty vector (n=6, p<0.001).

3.3.9 Functionality: JAK/STAT signalling pathway

In this experiment, 3 separate clones of SOCS3 transfected cells - here designated 3.1, 3.2 and 3.3 - were stimulated with IL-6 and the sIL-6R. The sIL-6R is the soluble form of the α subunit of the IL-6 receptor. Unlike other soluble cytokine receptors, it is an agonist - binding to IL-6 and stimulating JAK/STAT signalling via the gp130 subunit. It is added in this experiment as MG-63 cells respond poorly to IL-6 alone. Whole cell lysates were assessed for the activation of STAT3 (section 3.2.24). In the SOCS3 expressing cells signalling via gp130/STAT3 appears intact, suggesting the increase in IL-6 production seen in SOCS3 transfected cells is not mediated via STAT3 and that transfected SOCS3 is not inhibiting the JAK/STAT pathway.

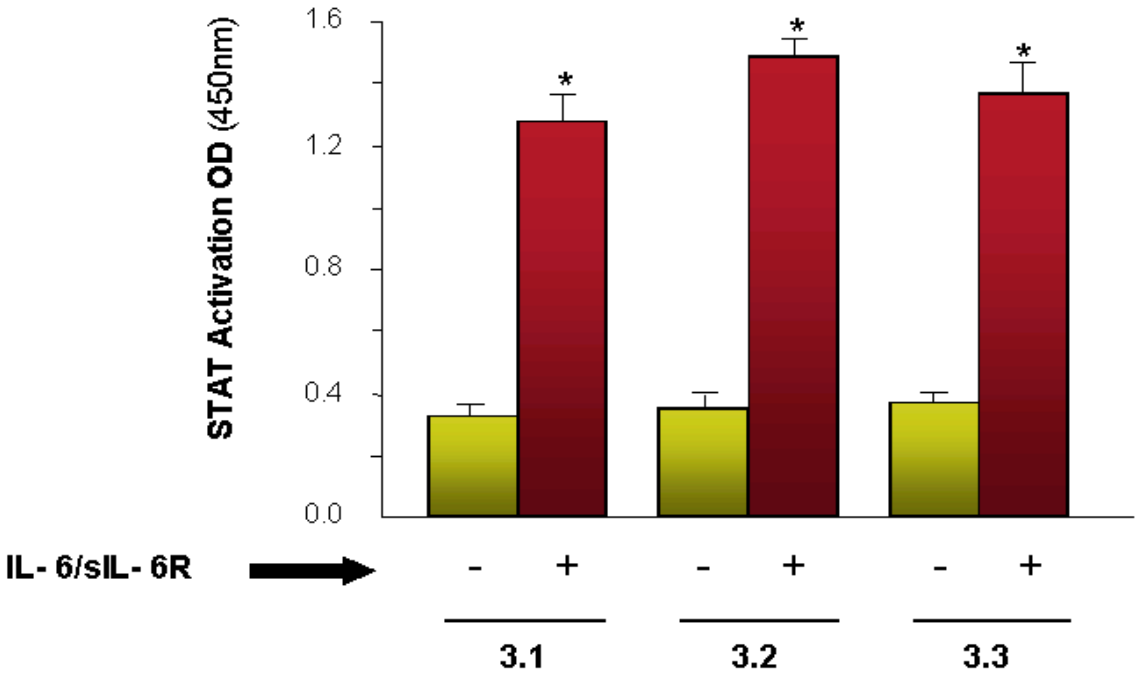


Figure 3.19: STAT3 activation in MG63 cells over expressing SOCS3 DNA following stimulation with IL-6/sIL-6R suggests signalling via gp130/STAT3 is intact and that transfected SOCS3 may not be inhibiting the JAK/STAT pathway. (n=6, * = significant $p<0.01$).

3.4 Discussion

These studies demonstrate that SOCS mRNA and protein are present in the human osteoblastic cell lines MG-63 and SaOS-2. Furthermore, the increased expression seen following stimulation with cytokines and growth factors is potentially compatible with their documented negative feedback function, acting to regulate uncontrolled and potentially harmful over-stimulation. The temporal pattern of SOCS expression in osteoblasts is consistent with studies performed in other cell types (Alexander and Hilton, 2004, Yoshimura, 2009). In general, upregulation occurred within 1 hour, peaked between 2 and 4 hours and diminished, without necessarily returning to baseline, by 8 hours. The constituitive expression of the four SOCS genes was higher, with a more pronounced response to stimulation, in MG-63 than in SaOS-2 cells. This is likely to reflect the different levels of basal cytokine activity exhibited by the cells, with, for example, the constituitive production of IL-6 higher by a factor of several thousand in MG-63 (3.4 ± 0.2 pg/10^6 cells [mean± SE] in SaOS-2 versus 2,898 ± 401 pg/10^6 cells in MG-63) (Dovio et al., 2001). It is possible that a parallel increase in the baseline activity of the SOCS proteins is required in order to keep such autocrine signals in check.

Of the growth factors and cytokines studied, GM-CSF had the least striking influence on SOCS mRNA expression. MG-63 cells express the GM-CSF receptor and display a JAK/STAT mediated proliferative response following ligand binding (Thacker et al., 1994). Since the JAK/STAT apparatus is the classical target of SOCS inhibition, and GM-CSF has been shown to influence SOCS gene expression in other systems (Faderl et al., 2003), a more pronounced effect might have been expected. SOCS3 expression did start to increase at 1h and reach a two-fold increase by four hours, but effects on SOCS1 were small and those on SOCS2 and CIS barely detectable. This could be evidence that SOCS are not involved in the regulation of GM-CSF signalling in osteoblastic cells, though this would probably be an over-simplification of the relationship between relative SOCS expression and the amount of consequent

inhibition - the effect of SOCS has been shown to be highly sensitive in many *in vitro* and in vivo systems, a small increase in expression leading to powerful inhibition of JAK/STAT signalling.

LPS is an integral cell wall component of gram-negative bacteria and is one of the mediators of endotoxic shock. It is recognised by TLR4, a member of the Toll-like receptor (TLR) group (Beutler and Poltorak, 2001). Ligand binding induces the sequential activation of several intracellular signalling molecules, including myeloid differentiating factor (MyD) 88 and TNFR-associated factor (TRAF) 6, with the subsequent recruitment of several downstream pathways, including c-jun NH_2-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK) and NF-κB (Muzio and Mantovani, 2001). Despite the non-JAK/STAT nature of these signal transduction routes, the SOCS family, and SOCS1 in particular, have been shown to be an important regulatory mechanism, perhaps functioning to protect tissues against an overwhelming LPS response (Nakagawa et al., 2002). The effect of LPS on SOCS expression in MG-63 cells was most apparent for SOCS3 and CIS. Interestingly, the effect of LPS was minimal at one hour post-stimulation and became appreciable, though not significant, at 2h. This delay might reflect a secondary SOCS response to LPS induced autocrine cytokine release, rather than to LPS itself. This could also explain why the most obvious increase was seen with SOCS3 rather than SOCS1 as might have been expected. LPS powerfully augments IL-6 secretion in MG-63 cells and it is the regulation of IL-6/gp130 signalling that is increasingly recognised as one of the most important functions of SOCS3 (Croker et al., 2003). Furthermore, it is very interesting to note that since the time the data under discussion were obtained, SOCS3 has gradually emerged as a powerful regulator of LPS in many cell systems, particularly within the CNS (Qin et al., 2007, Brown et al., 2009, Yan et al., 2010).

IL-1ß was one of the first non-JAK/STAT cytokines shown to upregulate SOCS genes, and in particular SOCS3. IL-1ß is a potent stimulator of bone resorption via RANK-L

and IL-6 production in osteoblastic cells. The present data show a trend to increased expression of all four SOCS genes in MG-63 cells, becoming significant at 4 hours for SOCS1 and SOCS3 and at 8 hours for SOCS2. The delay in upregulation of SOCS2 by IL-1ß may again represent a secondary autocrine event. Further evidence for this comes from the absent SOCS2 response to IL-1ß in SaOS-2 cells. It is possible that the lower level of evoked cytokine secretion in these cells reduces the likelihood of a late autocrine response. Apart from SOCS2, the response to IL-1ß in SaOS-2 cells was similar to that in MG-63, with a trend to increased SOCS1, SOCS3 and CIS expression.

The effect of IGF-I on SOCS gene expression is of particular interest. While IGF-I has been implicated in several aspects of cell function, including proliferation, differentiation, transformation and survival, reports of the effect of IGF-I on SOCS gene expression are scarce. The IGF-I receptor (IGF-IR) is a tetrameric type II receptor protein tyrosine kinase composed of two ligand-binding α-subunits and two transmembrane ß-subunits. Binding of ligand to receptor causes conformational change, ß-subunit cross-phosphorylation and the creation of binding sites for downstream signalling molecules (Chen et al., 1998). The major IGF-IR substrates are insulin receptor substrates (IRS) -1 and -2 and Shc, themselves able to recruit the Ras/MAPK and the phosphatidylinositide 3-kinase (PI3-kinase) pathways (O'Connor et al., 1997). MAPK signalling plays an important role in promoting cell growth and regulates a variety of genes, while PI3-kinase is involved in cell survival. Intriguingly, evidence suggests that the IGF-IR can induce activation of STAT3 as well as the phosphorylation of JAK1 and JAK2 (Zong et al., 2000). Although the mechanism of STAT3 activation is not established, several possibilities exist. Firstly, the activated receptor could associate with JAKs and phosphorylate STAT3. Secondly, JAKS may be activated to directly phosphorylate STAT3. Thirdly, JAKS could provide STAT3 recruitment sites in the receptor complex, as is probably the case for JAK2 in response to growth hormone receptor activation. There is also evidence that the SOCS proteins,

particularly SOCS1 and SOCS3, are able to inhibit this IGF-I induced STAT3 activation (Dey et al., 2000) (Zong et al., 2000). The data presented here demonstrates a significant induction of SOCS1 and CIS by IGF-I in MG-63 cells and a trend to increased SOCS2 and SOCS3 in SaOS-2. This discrepancy between cellular phenotypes is consistent with an almost universal observation concerning the SOCS genes; in general the pattern of expression is dependent on cellular, not cytokine, identity. In addition, there is an inconsistency between the response to IGF-I *in vitro* and in vivo. In transfection models SOCS1 and SOCS3 are stronger inhibitors than SOCS2 and CIS, while in transgenic models it is the deletion of SOC2 that causes IGF-I hyper-expression and gigantism and CIS over expression that causes growth retardation (Schaefer et al., 2001, Metcalf et al., 2000).

As well as demonstrating the presence of SOCS mRNA in human osteoblastic cells, with expression increasing in response to a variety of osteotropic stimuli, a functional role for these genes in these cells is strongly suggested by the enhanced basal and IL-1β stimulated IL-6 release following SOCS1 and SOCS3 over-expression, as well as the enhanced IL-1β stimulated OPG release following SOCS3 overexpression. Although SOCS expression, particularly SOCS3, is usually (and intuitively) associated with reduced basal IL-6 activity, the increases seen here are not without precedent. In rat skeletal muscle, exercise-induced increases in SOCS3 expression significantly elevate IL-6 production (Spangenburg et al., 2006). Interestingly, if SOCS3 is cotransfected into cultured myotubes with an NF-κB luciferase construct, NF-κB transcriptional activity is increased nearly 30-fold as is IL-6 promoter activity. However, if it is cotransfected with a mutated construct, no increase in IL-6 activity is seen. It is known that the IL-6 promoter contains a consensus NF-κB-binding site (Xiao et al., 2004) and this could be required for SOCS3 induced increases in IL-6 promoter activity. This is compatible with previous data showing that overexpression of SOCS3 in cultured macrophages results in heightened transcriptional activation of NF-κB (Park et al., 2003). The current finding of intact gp130/STAT3 signalling in cells successfully

transfected with SOCS3, but producing significant amounts of IL-6, would support the involvement of other pathways in osteoblastic cells. The coordination of the interplay between these genes remains uncertain, as they are often involved in relatively contradictory processes. The balance between cytokines with opposing functions is clearly important in many situations, not least in bone remodelling, with this balance likely to be the outcome of intricate cross talk between different intracellular transduction signals. The observation of an enhanced IL-6 response to IL-1β stimulation contrasts with previous studies suggesting SOCS3 deletion, rather than upregulation, is associated with an overzealous IL-6 response in this situation. Perhaps the most important example of this is the severe IL-1 mediated arthritis experienced by mice with SOCS3 partially knocked out (Wong et al., 2006). However, it is now recognised that IL-1 is able to directly induce SOCS3 expression in macrophages, rather than achieving this indirectly through stimulation of IL-6 production (Wong et al., 2006), again suggesting the involvement of NF-κB signalling (Hayashi et al., 2002). Furthermore, in addition to classic gp130/STAT3 signalling, bone growth and remodelling is partially regulated by interactions between gp130 and the Ras/MAPK pathway (Sims et al., 2004). For example, mice carrying a point mutation that favours JAK/STAT signalling through the selective blockade of the Ras/MAPK pathway, display both increased bone turnover and decreased trabecular volume (Sims et al., 2004, Sims, 2009). SOCS3 has previously been shown to positively regulate the Ras/MAPK pathway (Cacalano et al., 2001) providing a additional route for it to potentially influence IL-6 stimulated bone turnover beyond a JAK/STAT paradigm.

IL-1β is involved in the initiation of bone resorption (Kimble et al., 1994) and it's signal to the osteoclast is generally processed via osteoblastic RANKL expression (Hofbauer et al., 1999c). This has made it difficult to understand its' additional positive effect on OPG production (Pantouli et al., 2005). However, the latter may be dependent on phosphorylation of p38 and ERK, rather than NF-κB (Lambert et al., 2007). It is possible that forced expression of SOCS3 may regulate NF-κB signalling, thereby

favouring alternative pathways. This could support OPG, rather than RANK-L, production, and potentially act as a counter-regulatory measure during resorption. Alternatively, OPG could act as a survival factor via TRAIL or other pathway.

Chapter 4

The role of cytokines in human osteoblast survival: potential regulation by the suppressors of cytokine signalling (SOCS)

4.1 Background

In addition to altering the balance between proliferation and activation of osteoblast and osteoclast precursors, the mass of skeletal tissue can be strongly influenced through the regulation of cellular longevity (Weinstein and Manolagas, 2000). During bone formation osteoblasts undergo an ordered developmental progression concluding with apoptotic cell death. The cellular and molecular pathways, control and overall significance of this process are yet to be fully understood, but the same cytokines and growth factors that stimulate osteoblast development appear fundamental to the regulation of subsequent apoptosis (Xing and Boyce, 2005). Evidence suggests that IGF-I exerts an important influence on senescence in cell types as diverse as cardiac myoblasts, neuronal cells, epithelial cells and osteoblasts (Hill et al., 1997, Vincent and Feldman, 2002). In the latter, IGF-I may inhibit caspase-3, one of several effector proteases, through upregulation of calbindin-D28k (Bellido et al., 2000). Such an anti-apoptotic effect would help IGF-I fulfil it's function as a key anabolic factor for the normal growth and maintenance of the skeleton (Zhao et al., 2000). SOCS proteins are increasingly recognised for their role in the processing and regulation of apoptotic and anti-apoptotic signals. This might serve a protective function; for example, SOCS3 ameliorates both IL-1β and TNFα induced apoptosis in pancreatic ß-cells (Karlsen et al., 2001, Bruun et al., 2009); while SOCS1 suppresses the p38 MAP kinase regulated TNFα induced death of fibroblasts (Morita et al., 2000). In other systems, such as embryonic stem cells, SOCS expression may have a opposite effect, leading to a reduction in cell viability (Duval et al., 2000). The effect of IGF-I on SOCS gene expression in osteoblastic cells discussed in section 3.3.7 is of interest, as reports of the utility of IGF-I for inducing SOCS expression are relatively scarce. The downstream role SOCS proteins may play in the regulation of IGF-I functionality is currently unknown, but may potentially include an influence on apoptotic signalling and therefore cell death.

The bone resorbing activity of IL-1β is well described, particularly in common pathological states such as osteoporosis and rheumatoid arthritis (Strand and Kavanaugh, 2004). In an inflammatory context, the relationship between IL-1β and TNFα may play a particularly key role (Kobayashi et al., 2000) and this cytokine combination is demonstrably pro-apoptotic for osteoblastic cells (Tsuboi et al., 1999). IL-1β also stimulates apoptosis in other cell types, including pancreatic β cells (Vanderford, 2010), where it is, at least partially, under the control of SOCS3 (Karlsen et al., 2004). In osteoblasts, IL-1β also induces the release of OPG, a powerful anti-resorptive factor, which possesses the potential to act as a survival factor through acting as a decoy receptor for TRAIL (Pantouli et al., 2005). The data presented in chapter 3 demonstrated the facility for IL-1β to induce SOCS1 and 3 expression in osteoblastic cells (chapter 3.3.7) and for SOCS1 and SOCS3 overexpression to enhance both basal and IL-1β stimulated IL-6 release (chapter 3.3.8-9). The functional implications of these are unknown, but may have relevance to cell survival. The increased IL-1β stimulated OPG release in the setting of SOCS3 overexpression (chapter 3.3.14) is potentially interesting, as it could provide a potential counter regulatory mechanism to block TRAIL mediated cell death.

The studies in this chapter aim to explore the response of osteoblastic cells to the potential pro- and anti-apoptotic stimuli of IL-1β and IGF-I through the study of protease activity and DNA fragmentation. The potential utility of SOCS in the regulation of apoptotic pathways is studied through the targeted overexpression and knockdown of these genes.

4.2 Materials and methods

4.2.1 Osteoblast-like cell lines and cell culture techniques

Cell line and cell culture protocols were followed as described in section 2.2.1 – 2.2.4.

4.2.2 In situ assays for caspase 3, 8 and 9

MG-63 cells were seeded into 6 well tissue culture plates with 2 mL of medium. After 24h the media was removed and refreshed with a further 2 mL. A blank plate (without cells) was also set up. To all plates the cell permeable inhibitor of the relevant caspase (table 4.1) was added to 3 wells whilst the remaining 3 wells were treated with the same volume of DMSO. IGF-I was added to all wells. After a 15 min pre-incubation at 37^0C in a humidified environment of air/CO_2 (19:1), the cell permeable substrate of the relevant caspase was added to all wells Table 2.8) [all inhibitors and substrates from Calbiochem, Merck, Darmstadt, Germany]. After 4, 24 or 48 h incubation period fluorescence was detected at 380 nm excitation and 460 nm emission on a fluorescence plate reader (BMG Fluorostar, BMG Labtechnologies Ltd, Aylesbury, UK). Caspase activity was determined for each treatment as the difference between the mean fluorescence in the samples without the inhibitor, and those with the inhibitor. The caspase activity of each media solution without cells (blank plate) was then subtracted from the activity in the tissue culture plate to ensure that only cellular caspase activity was measured.

4.2.3 In situ assay for calpain activity

MG-63 cells were seeded into 6 well tissue culture plates with 2 mL of medium. After 24 h the wells consisted of a monolayer of cells. The media was then removed and replaced with 2 mL of fresh media. A blank plate (without cells) was also set up. To all plates 50 μmol/L of the calpain specific inhibitor calpeptin (in DMSO) was added to 3 wells whilst the remaining 3 wells were treated with the same volume of DMSO. At this point IGF-I (or other stimulus) was added to all wells, except those in the non-treatment groups. After a 15 min pre-incubation at 37^0C in a humidified environment of air/CO_2

(19:1), 10 μL of the cell permeable calpain substrate N-succinyl-Leu-Leu-Val-Tyr-AMC (1 mmol/L in DMSO) was added to all wells. After an incubation (4, 24 or 48 h) period fluorescence was detected at 380 nm excitation and 460 nm emission on a fluorescence plate reader (BMG Fluorostar, BMG Labtechnologies Ltd, Aylesbury, UK). Calpain activity was determined for each treatment as the difference between the mean fluorescence in the samples without the calpain inhibitor, and those with the inhibitor. The calpain activity of each media solution without cells (blank plate) was then subtracted from the calpain activity in the tissue culture plate to ensure that only cellular calpain activity was measured.

	Substrate	**Inhibitor**
Caspase-3	DEVD-AMC (final concentration 25 μM)	DEVD-CHO (final concentration 2.5μmol/L)
Caspase-8	Z-IETD-AFC (final concentration 25 μM)	IETD-CHO (final concentration 2.5 μmol/L)
Caspase-9	Ac-LEHD-AFC (final concentration 25 μM)	LEHD-CHO (final concentration 2.5 μmol/L)

Table 4.1: Concentrations of substrates and inhibitors for *in situ* assays of caspase-3, 8 and 9.

4.2.4 Caspase-3 end-point Assay

Cell lysates were prepared from cell pellets with 150 μl of mammalian extraction buffer (GE Healthcare Life Sciences, Little Chalfont, UK). Lysates were kept on ice with frequent mixing for 15 min before centrifugation (16000 *g*, 4^0C) to remove cell debris. Samples were then diluted 3:1 in running buffer, before being boiled for 5 min. Caspase-3 activity was measured using the fluorometric substrate Ac-DEVD-AMC as described previously. In summary, for each sample four replicates of 10 μL were

assayed. 90 µL of caspase assay buffer was added to each sample well. Two replicates contained 50 µM of caspase-inhibitor (Ac-DEVD-CHO) in 2 µL and the remaining two containing the same volume of vehicle (DMSO). Each replicate contained 50 µM of the caspase substrate (Ac-DEVD-AMC). Fluorescence was measured on a microplate reader (Fluostar Galaxy; BMG Laboratory Technologies, Aylesbury, UK) with excitation at 380 nm and emission at 460 nm. Fluorescent readings from wells containing inhibitor were subtracted from the fluorescence in wells containing substrate and corrected for the amount of protein. Results were calculated as nmol AMC/min/mg total protein. Protein was measured by performing the Pierce® bicinchoninic acid protein assay (BCA) (ThermoScientific) using 25 µL of lysate (diluted 1:1 in distilled H_2O) according to the instructions of the kit insert for the 96-well format.

4.2.5 Quantification of apoptotic cell death by DNA fragmentation assay

Cell death was detected by a DNA fragmentation enzyme linked immunosorbent assay (ELISA), which quantifies the mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics, Lewes, UK). Cytosolic lysates extracted using 63.2mmol/L imidazole-HCL buffer, pH 7.3, containing 10µmol/L digitonin, were diluted 20-fold in kit lysate buffer. 20 µL of the diluted cell lysate is placed into a streptavidin coated microtitre plate. 80 µL of a mixture of anti-histone-biotin and an anti-DNA monoclonal antibody conjugated with peroxidase (anti-DNA-POD) are added and the plate incubated for 2h at room temperature. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin coated microtitre plate via its biotinylation. Additionally the anti-DNA-POD antibody reacts with the DNA component of the nucleosomes. The plate is then washed 3 times with incubation buffer before 100 µL of ABTS solution is added to each well and the plate incubated at room temperature for 10 min on a plate shaker (250 rpm). The plate is read at 405 nm. DNA fragmentation is expressed as an enrichment factor

(absorbance value for 24h experimental sample/absorbance value of 0h sample) from a time 0h baseline sample per mg of total lysate protein.

4.2.6 Quantification of necrotic cell death by LDH assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant following damage to the plasma membrane. In this assay, LDH activity is calculated using an enzymatic test on cell free supernatants. The assay is based on a two-step principle: in the first step NAD^+ is reduced to $NADH/H^+$ by the LDH-catalysed conversion of lactate to pyruvate. In the second step, a catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), so reducing it to formazan, which can be detected using colorimetric measurements. LDH activity was measured in the incubation media prior to (background control) and at the end of the experiment (24h) using this assay (Cytotoxicity Detection Kit; Roche Diagnostics). Cytotoxicity (%) was calculated by the subtraction of the background control (media without cells) from the 24 h sample and expressed as a percentage of a positive control (2% Triton X-100 = 100% lysed cells), minus background control, corrected for total lysate protein. A background control is used for each separate treatment.

4.2.7 Western blotting for fodrin breakdown products

Fodrin is a universally expressed membrane associated cytoskeletal protein consisting of alpha and beta subunits. The protein is important for maintaining normal membrane structure and supporting cell surface protein function. Alpha fodrin is an important substrate of proteases and is cleaved during cell death. The full length 240kDa protein can be cleaved at several sites within its sequence by activated caspases generating N terminal 150kDa, C terminal 120kDa and 35kDa major products. It is also cleaved by

calpain yielding a 145KDa product. Cleavage of alpha fodrin leads to membrane dysfunction and cell shrinkage.

Cell extracts were prepared as described in section 3.2.8. Ten µg of lysate protein was electrophoresed on a 10% polyacrylamide gel in the presence of SDS (section 3.2.10) before being probed with mouse anti α-fodrin diluted 1:1000 (Affiniti Research Products Ltd, UK). The secondary antibody (1:2000) was donkey anti-mouse labelled IgG conjugated to horseradish peroxidise (Santa Cruz Biotech, Autogen Bioclear, Calne, UK). Protein bands were visualised using chemiluminescence (ECL Chemiliminescence Detection System, Amersham, UK).

4.2.8 Transfection of MG-63 cells with SOCS 1 and SOCS 3 DNA for gene overexpression experiments

This was performed as per the methods outlined in section 3.2.22

4.2.9 SOCS siRNA gene knockdown

MG-63 cells were seeded into six-well plates in DMEM and left to adhere overnight to 50% confluence. Media was then replaced with 1.6 mL of fresh media (with FCS but without antibiotics). SOCS 1, SOCS 3, CIS and non-targeting control siRNA (20 uM) were diluted 1:100 with Opti-MEM I reduced serum medium (Invitrogen Life Technologies) and gently mixed. Lipofectamine (Invitrogen Life Technologies) was then diluted 1:100 with Opti-MEM I, mixed gently and incubated for 5min at room temperature. This was then combined 1:1 with the diluted siRNA (total volume 100 µL), mixed gently and left to incubate at room temperature for 20min to allow the siRNA: Lipofectamine complexes to form.

Each cell culture well had 10 nM of siRNA added, before gentle mixing and incubation at 37˚C for 24h. IL-1β (100 IU/mL) or control was added prior to a further 24h incubation. LDH activity as a marker of necrotic cell death was measured in the

incubation media at the start and the end of the experiment using a colorimetric assay (Cytotoxicity Detection Kit, Roche Diagnostics, Burgess Hill, UK), as described in section 4.2.5. Cytotoxicity (%) was calculated by the subtraction of the background control and expressed as a percentage of a positive control (2% Triton X-100). Caspase-3 activity was determined by end point assay, as described in section 4.2.4. STAT3 activity was determined using the TransAM kit (Active Motif) as described in section 3.2.24.

4.3 Results

4.3.1 Effect of IGF-I on apoptosis in MG-63 cells: caspase-3 activity at 24 h

After being harvested and counted, MG-63 cells were seeded at a density of 0.3 x 10^6 cells per well of a 6-well plate in 2 mL of medium and incubated at 37ºC for 24h. The cells were then refreshed in media containing a desired concentration of IGF-I and incubated for 24 or 48 h. *In situ* assays for caspase-3 were performed and cell lysates examined for DNA fragmentation. Unless otherwise indicated, all results are corrected for cellular protein. For each concentration of IGF-I n=6 and data are shown as mean ± SEM and analysed using Student's t-test.

At 24h, 10 ng/mL IGF-I caused a significant reduction in caspase-3 activity when compared to control ($p<0.002$). Caspase 3 activity was also reduced by 100 ng/mL IGF-I, but to a lesser extent. DNA fragmentation was reduced by both 10 ng/mL and 100 ng/mL IGF-I, the latter reaching statistical significance ($p<0.005$) (figure 4.1).

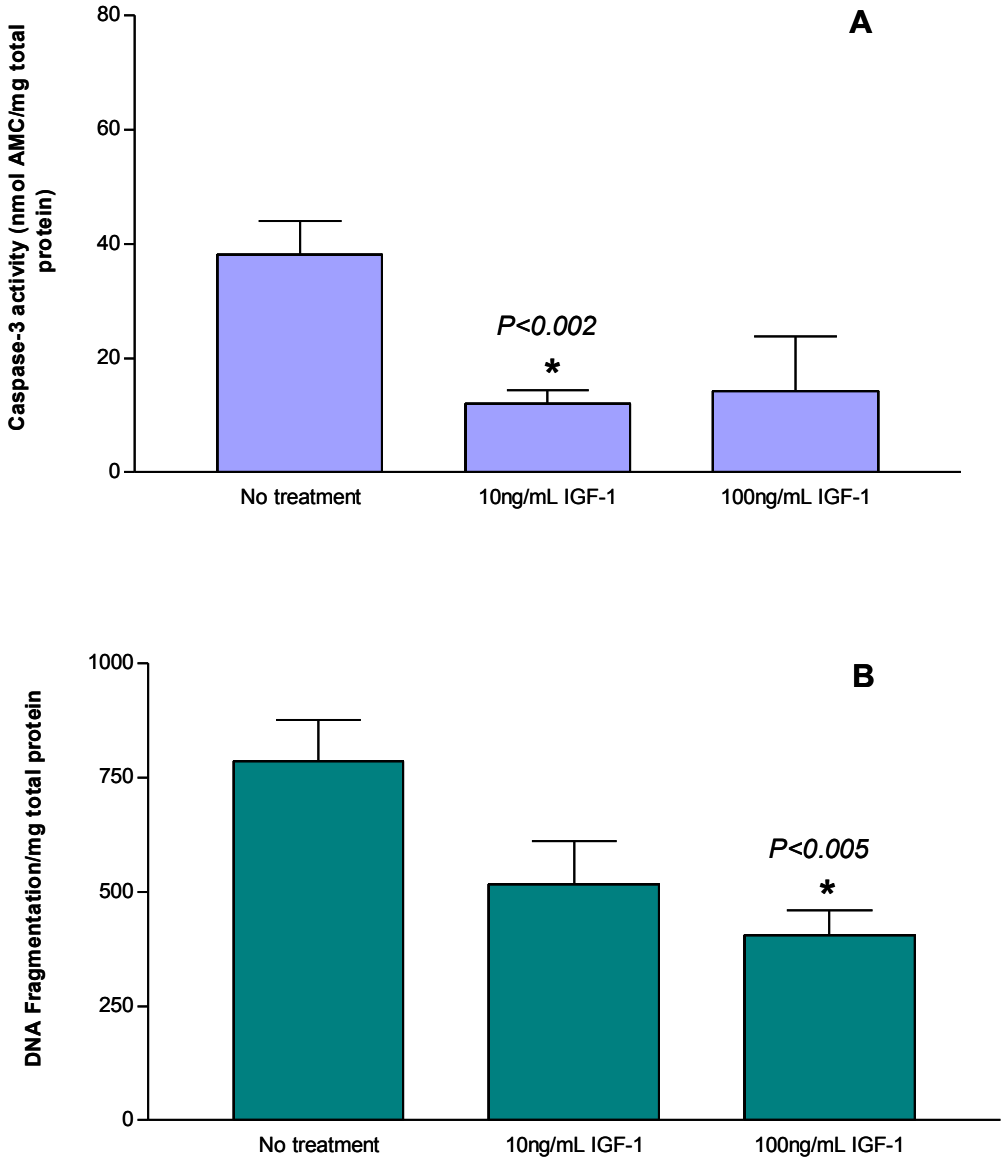


Figure 4.1: Caspase-3 activity (corrected for cellular protein) in MG-63 cells (**A**) and DNA fragmentation (**B**) following 24h incubation with 0, 10 or 100 ng/mL IGF-I. A statistically significant reduction in comparison to control is denoted by *. n=6 for each concentration of IGF-I.

4.3.2 Effect of IGF-I on apoptosis in MG-63 cells: caspase-3 activity at 48h

At 48h, caspase-3 activity is greater in controls than at 24h. There is a reduction in caspase-3 activity with 10 ng/mL IGF-I, though this does not reach statistical significance. Activity was also reduced by 100 ng/mL IGF-I, but to a lesser extent. DNA fragmentation was reduced by both 10 ng/mL and 100 ng/mL IGF-I (figure 4.2).

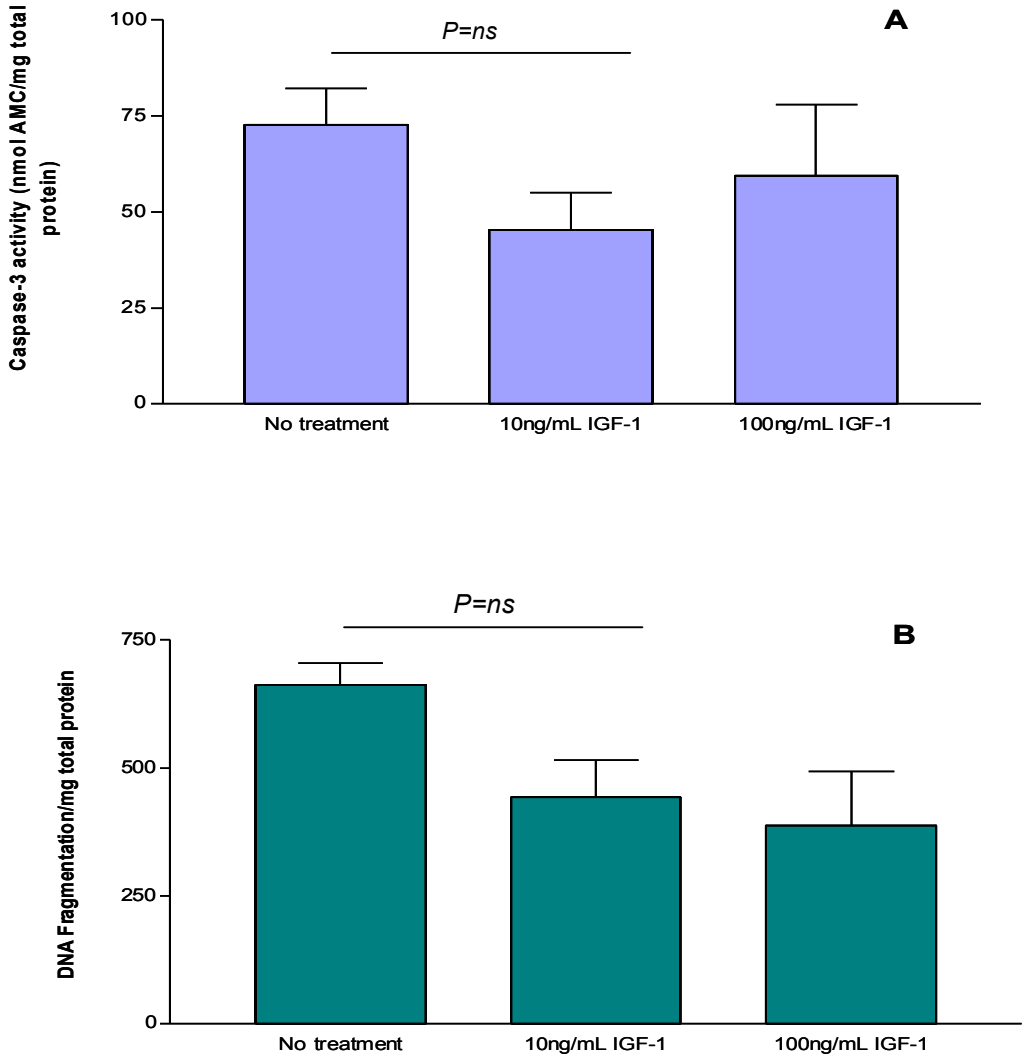


Figure 4.2: Caspase-3 activity (corrected for cellular protein) in MG-63 cells (**A**) and DNA fragmentation (**B**) following 48h incubation with 0, 10 or 100 ng/mL IGF-I. n=6 for each concentration of IGF-I. No statistically significant differences were demonstrated.

4.3.3 Caspase-3 activity: effect of PI3-kinase inhibition

A reduction in caspase-3 activity was seen following 24h incubation with 10ng/mL IGF-I ($p<0.05$). This decrease was abolished in the presence of 100nM wortmannin, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor (figure 4.3).

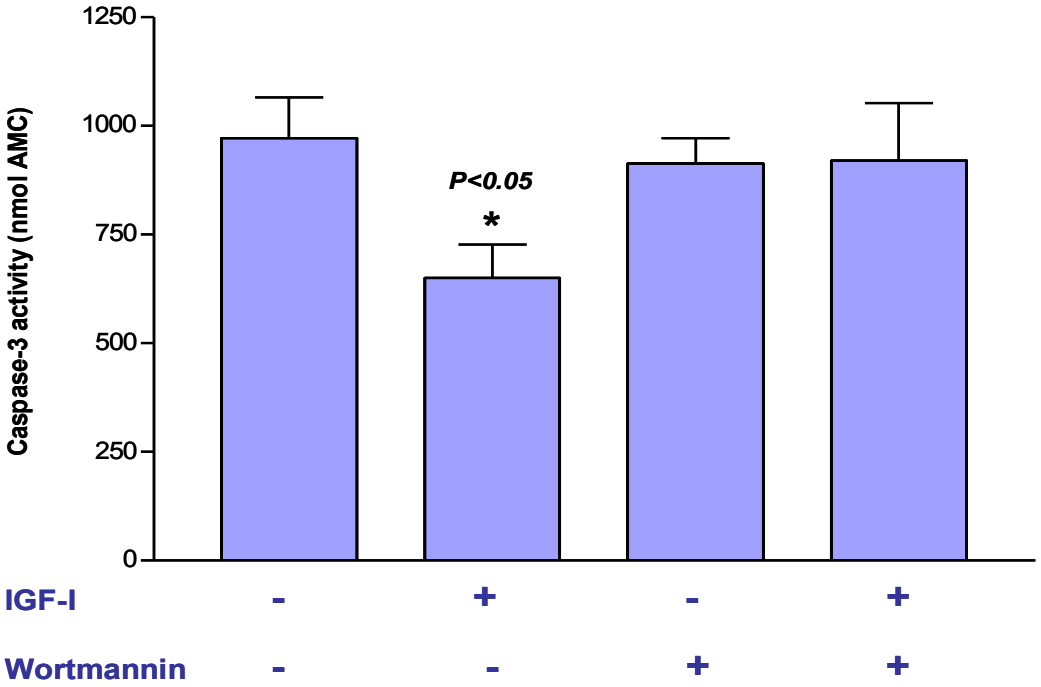


Figure 4.3: Caspase-3 activity is significantly reduced following 24h incubation with 10 ng/mL IGF-I (n=6, $p<0.05$). The reduction is abolished in the presence of 100nM of the PI3-kinase inhibitor, Wortmannin.

4.3.4 IGF-I dose response: caspase-3 activity at 24 h

Caspase-3 activity showed an incremental reduction at escalating concentrations of IGF-I (0, 2.5, 5, and 10 ng/mL IGF-I). At 10 ng/mL this reduction reaches statistical significance ($p<0.001$) (figure 4.4).

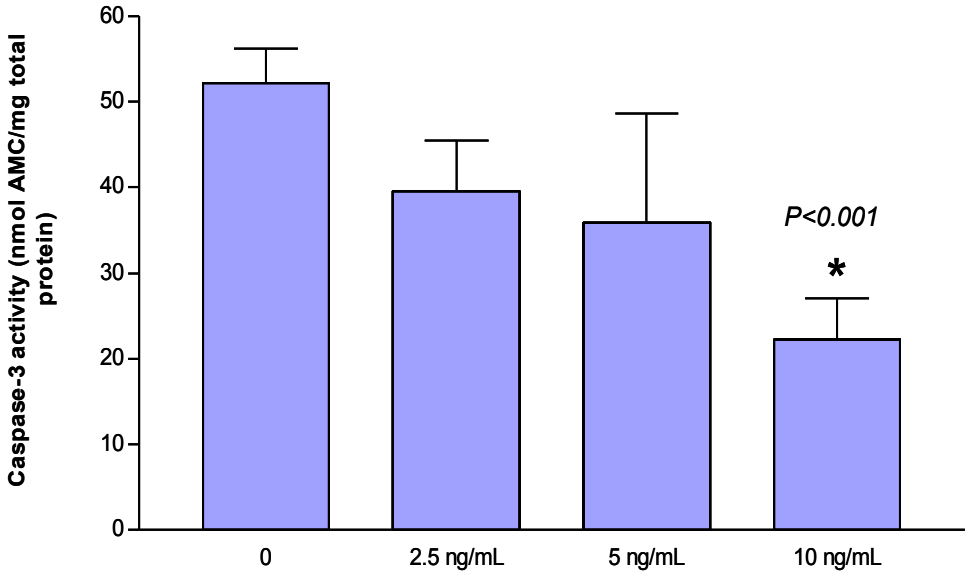
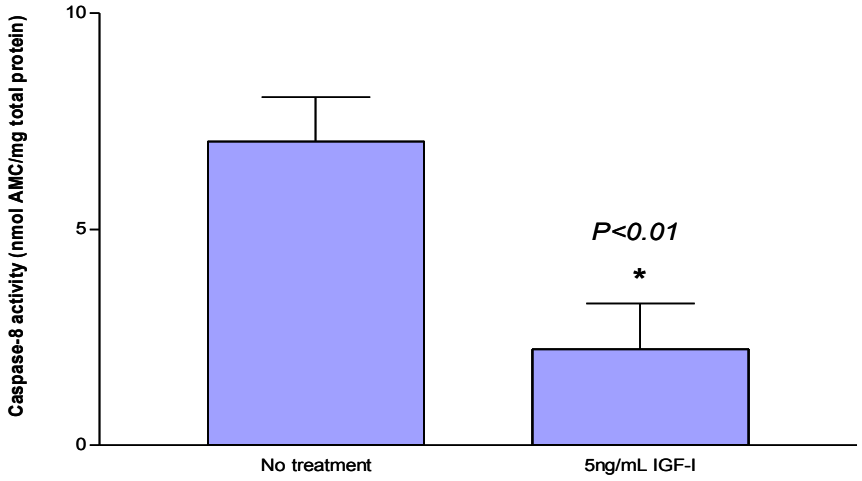


Figure 4.4: Caspase-3 activity (corrected for cellular protein) in MG-63 cells following 24h incubation with 0, 2.5, 5 or 10 ng/mL IGF-I. n=6 for each concentration of IGF-I. A significant difference was shown for 10ng/mL IGF-I ($p<0.001$).

4.3.5 Effect of IGF-I on apoptosis in MG-63 cells: caspase-8 and -9 activity

The activity of both caspase-8 ($p<0.01$) (figure 4.5 A) and -9 ($p<0.005$ respectively) (figure 4.5 B) was significantly reduced after 24h incubation with 5 ng/mL IGF-I. In view of the high baseline apoptosis seen at 48h in preceding experiments, these studies were terminated at 24h.

A



B

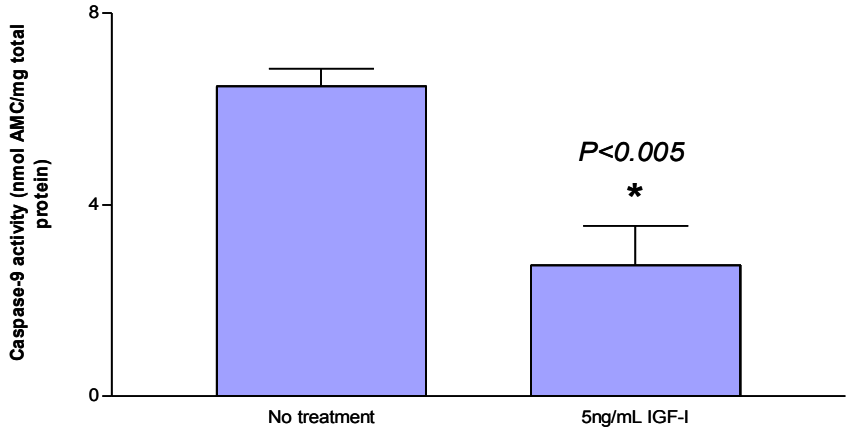


Figure 4.5: A: caspase-8 and B: caspase-9 activity (corrected for cellular protein) in MG-63 cells following 24h incubation with 0 or 5 ng/mL IGF-I. n=6 for each concentration of IGF-I. A significant difference was demonstrated in both cases ($p<0.01$ and $p<0.005$ respectively).

4.3.6 Effect of IGF-I on apoptosis in MG-63 cells: DNA fragmentation

There was consistent and dose-dependent reduction in DNA fragmentation following exposure to 5 or 10 ng/mL IGF-I.

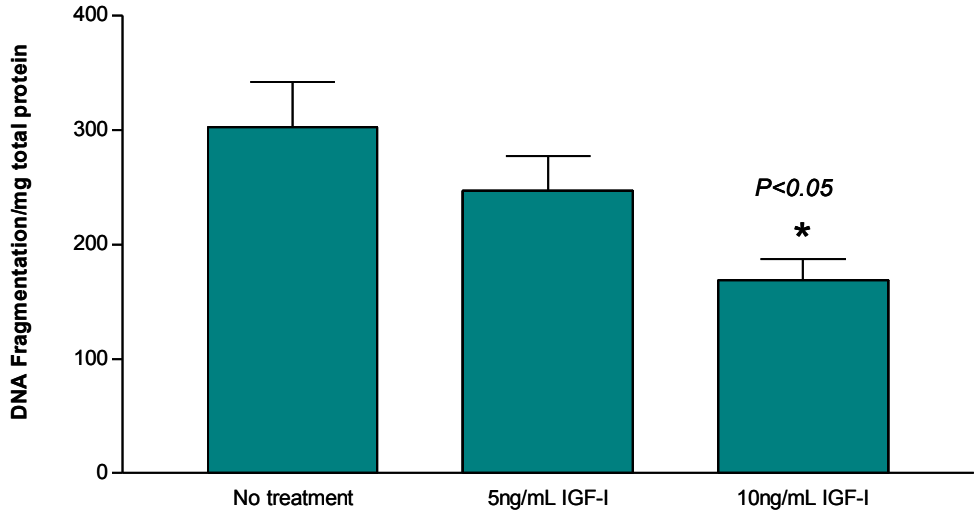


Figure 4.6: DNA fragmentation in MG-63 cells following 24h incubation with 0, 5 or 10 ng/mL IGF-I. A statistically significant decrease in comparison to control is denoted by *. n=12 for each concentration of IGF-I.

4.3.7 LDH release

As a further marker of cellular injury, LDH activity in cell supernatants was determined in all experiments. Representative results are shown in figure 4.7. No significant differences in LDH activity were detected in any of the preceding studies.

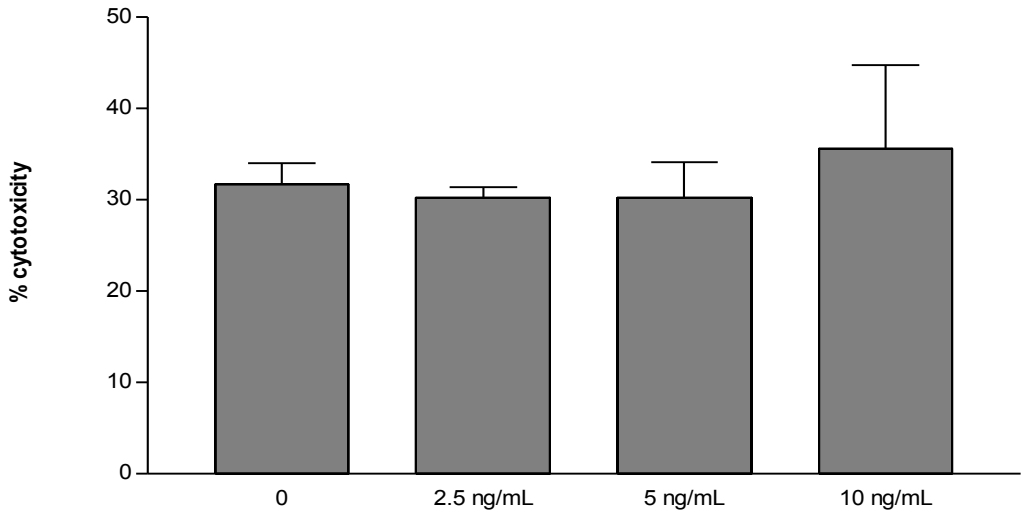


Figure 4.7: LDH release (corrected for cellular protein) from MG-63 cells following 24h incubation with 0, 2.5, 5 or 10 ng/mL IGF-I. n=6 for each concentration of IGF-I. No significant differences were demonstrated.

4.3.8 Effect of IGF-I on apoptosis in MG-63 cells: calpain activity

There was a significant increase in calpain activity when compared with controls after 24 h incubation with 5 ng/mL IGF-I ($p<0.01$) (figure 4.8).

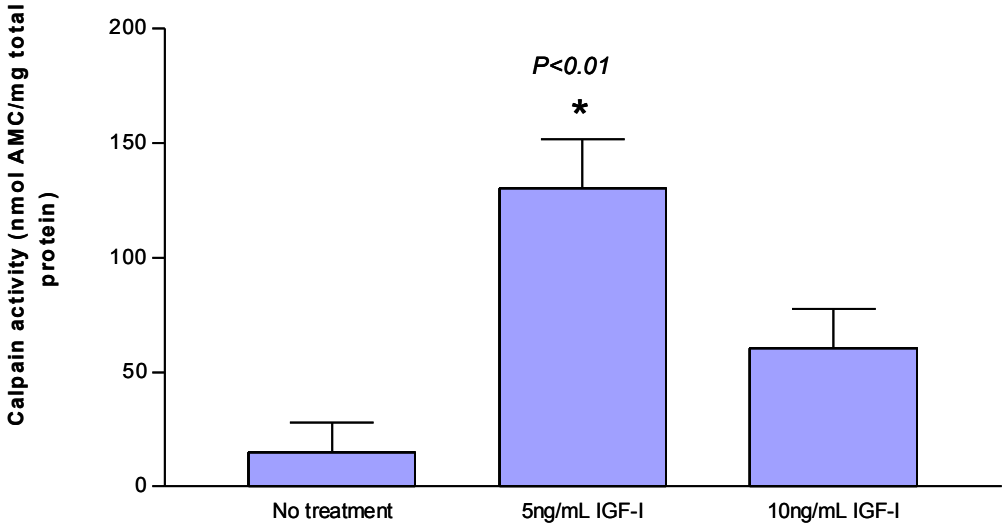


Figure 4.8: Calpain activity (corrected for cellular protein) in MG-63 cells following 24h incubation with 0, 5 or 10 ng/mL IGF-I. n=12 for each concentration of IGF-I. A statistically significant increase in comparison to control is denoted by *.

4.3.10 Fodrin breakdown

Western blotting for fodrin breakdown products in protein lysates from MG-63 incubated for 24h ± 10 ng/mL IGF-I showed an increase in calpain cleavage products in the presence of IGF-I, not demonstrable in the presence of the specific calpain inhibitor, calpeptin (figure 4.9).

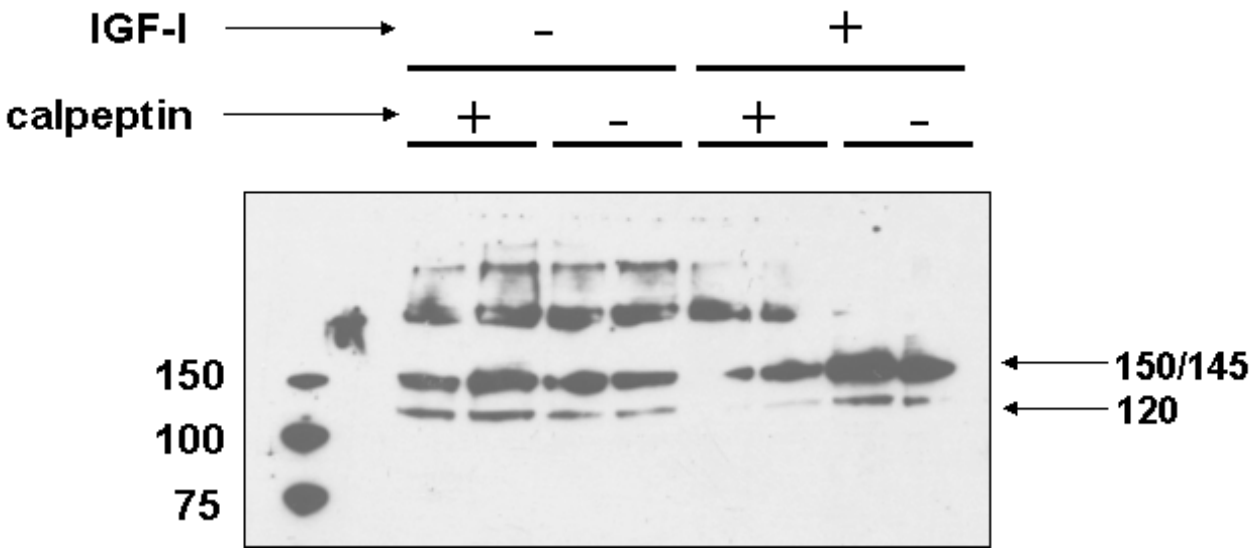
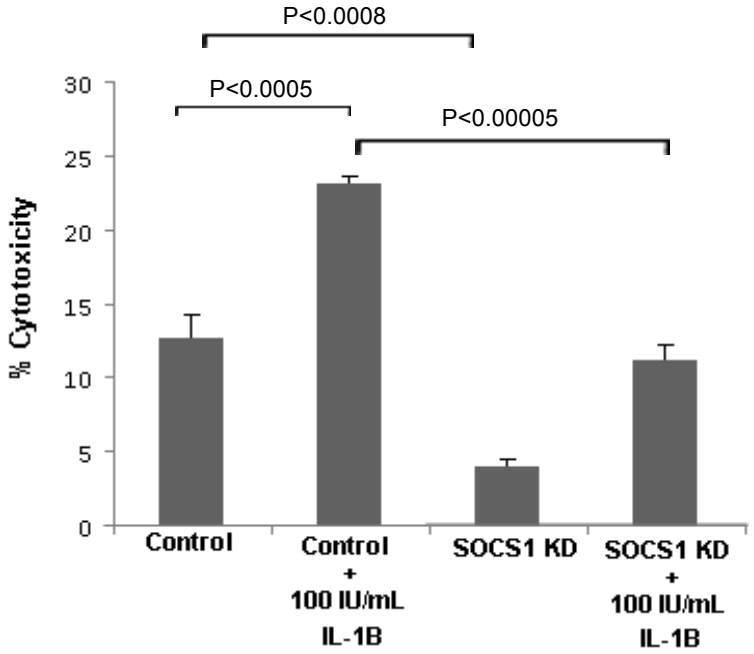


Figure 4.9. Western blot for fodrin breakdown products in MG-63 cells after 24 h incubation with and without 10 ng/mL IGF-I in the presence or absence of calpeptin, a specific calpain inhibitor. Caspase-3 cuts fodrin to 150 and 120kDa and calpain to 145kDa. There is an increase in calpain activity in response to IGF-I that is not seen in the presence of calpeptin.

4.3.13 SOCS knockdown differentially attenuates IL-1β induced necrosis in MG 63 cells

IL-1β treatment greatly elevated the degree of necrotic injury in the control group [% cytotoxicity, ±SD, n=3] (P<0.0005). Basal cytotoxicity was reduced by SOCS1 (P<0.0008) and SOCS3 (P<0.001) knockdown (KD), but was not affected by CIS KD. However, despite the lack of basal effect, IL-1β induced cytotoxicity was significantly reduced by CIS KD (P<0.001). SOCS3 KD afforded no protection from IL-1β induced cytotoxicity (figure 4.10 A-C).

A



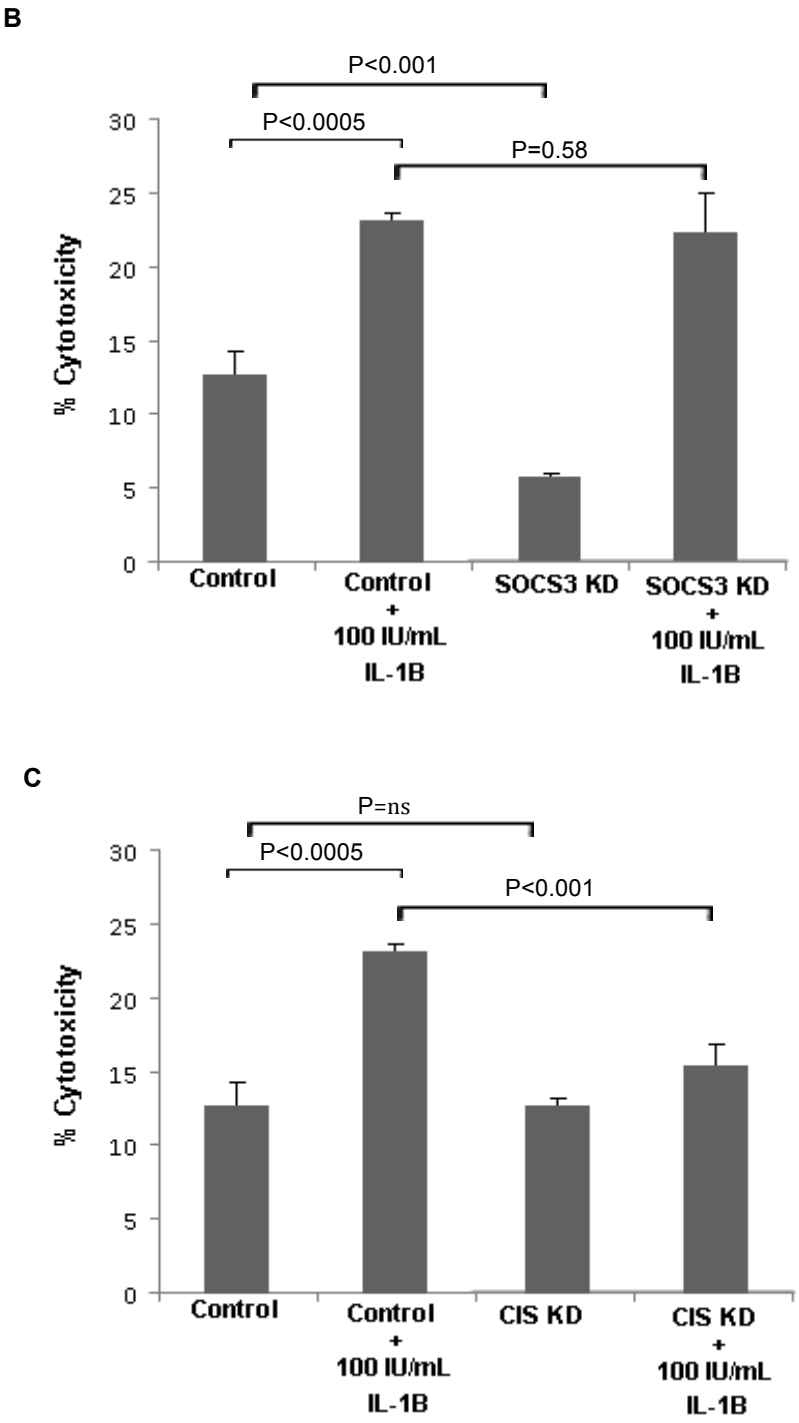
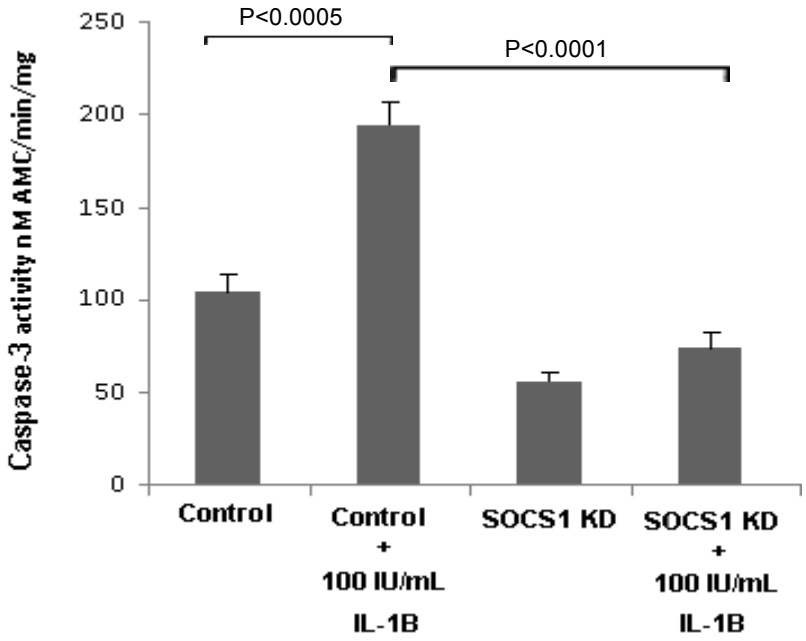


Figure 4.10: Cytotoxicity in human osteoblastic cells treated with IL-1β. IL-1β treatment greatly elevated the degree of necrotic injury in the control group [% cytotoxicity, ±SD, n=3] (P<0.0005). Basal cytotoxicity was reduced by SOCS1 (P<0.0008) and SOCS3 (P<0.001) knockdown (KD), but was not affected by CIS KD. Despite a lack of basal effect, IL-1β induced cytotoxicity was reduced by CIS KD (P<0.001). SOCS3 KD afforded no protection from IL-1β induced cytotoxicity SOCS 1 (A) and CIS (C), but not SOCS3 (B) knockdown resulted in significantly reduced necrotic injury.

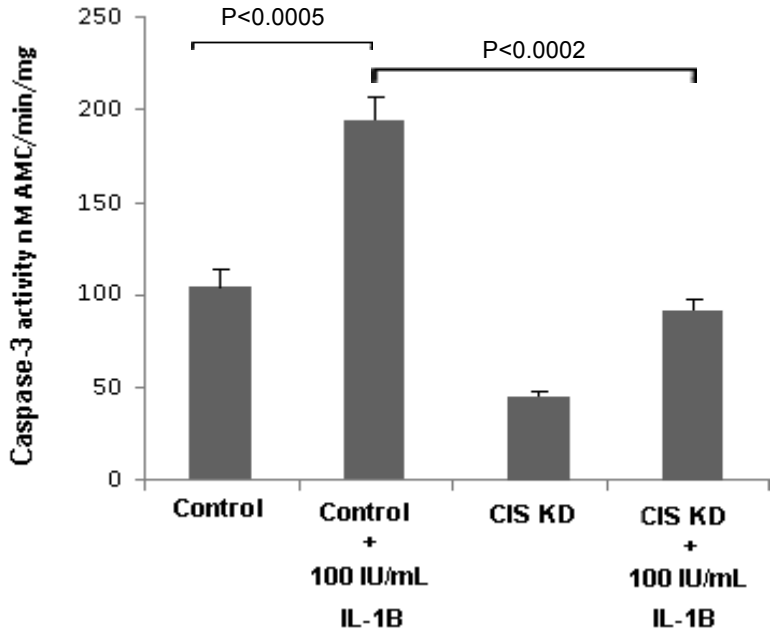
4.3.12 SOCS knockdown differentially attenuates IL-1β induced caspase-3 activity in MG 63 cells

IL-1β treatment greatly elevated caspase-3 activity in the control group C (nM AMC/min/mg protein, ±SD, n=3). Control 103.8 ± 9.8, Control + IL-1β 195.7 ± 11.9 (P<0.0005). SOCS knockdown (KD) significantly reduced caspase-3 activity in all groups (figure 4.11 A-C).

A



B



C

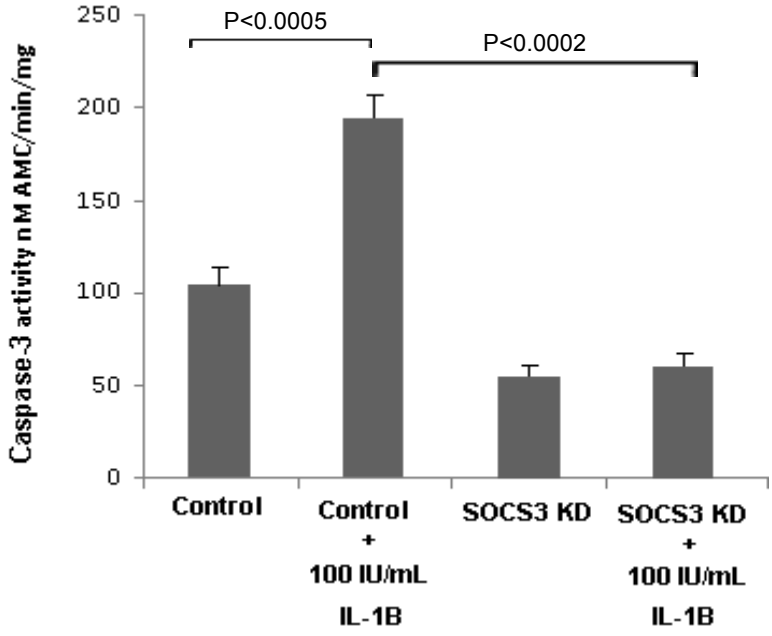
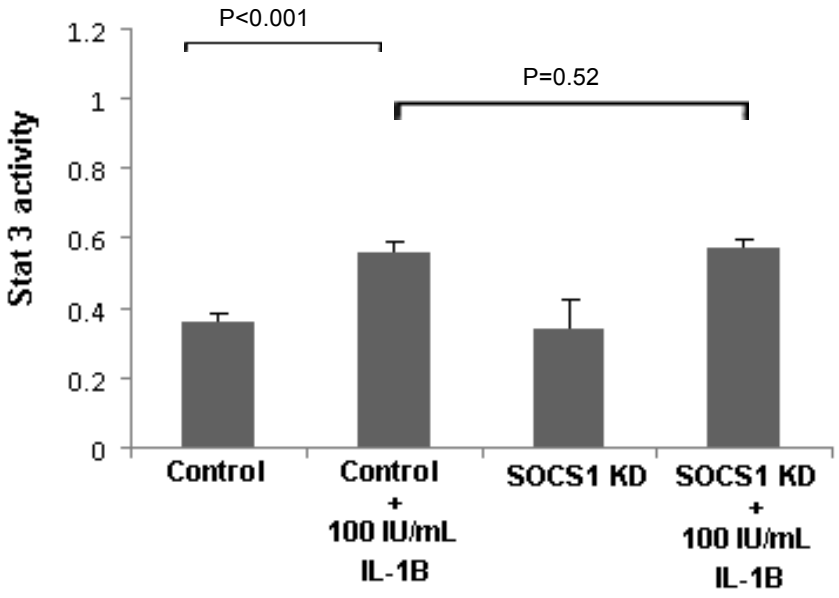


Figure 4.11: Caspase-3 activity in human osteoblastic cells treated with IL-1β. IL-1β treatment increased caspase-3 activity in the control group (nM AMC/min/mg protein, ±SD, n=3) (P<0.0005). SOCS 1 (A) (p<0.0001), SOCS 3 (B) (p<0.0002) and CIS (C) (p<0.0002) KD resulted in significantly reduced apoptotic injury after IL-1β.

4.3.13 SOCS knockdown differentially attenuates IL-1β induced STAT3 activity in MG 63 cells

A downstream effect of IL-Iβ was evident from STAT3 activation (absorbance/mg protein) in the control groups, C, 0.37 ± 0.02, Control + IL-1β 0.56 ± 0.03 (P<0.001) but interestingly CIS-Knockdown (KD) prevented IL-Iβ induced activation, whilst SOCS3-KD significantly elevated IL-Iβ induced STAT3 activity. STAT3 activity was similar in the SOCS1-KD group to control values (figure 4.12 A-C).

A



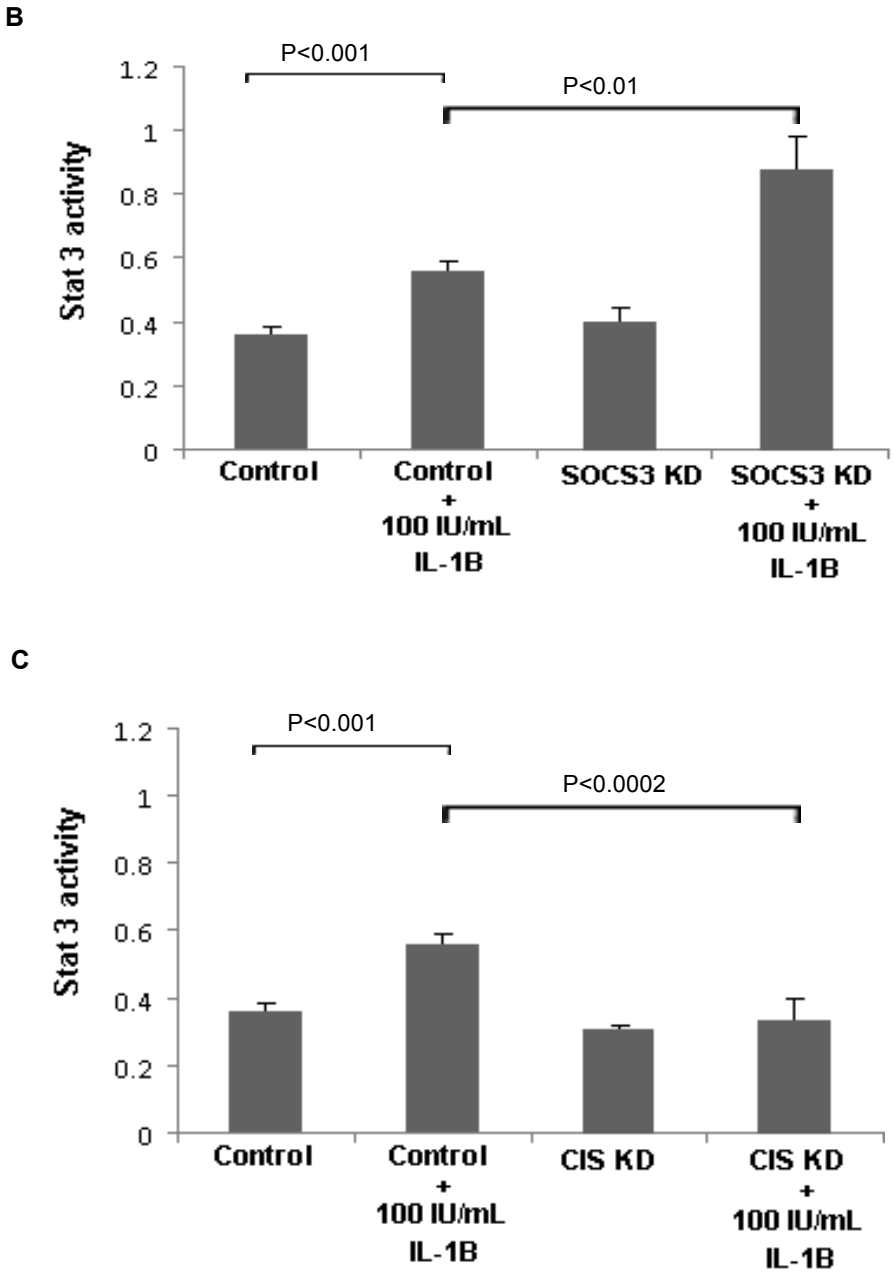


Figure 4.12: STAT3 activity in human osteoblastic cells treated with IL-1β. A downstream effect of IL-Iβ was evident from STAT3 activation in the control groups (p<0.001). STAT3 activity was similar in SOCS1 KD group (A) as control. SOCS 3 KD significantly increased STAT3 activity (B) (p<0.01) whilst CIS KD prevented IL-β induced STAT3 activation (C) (p<0002).

4.4 Discussion

Although osteoblast death is an important component of bone turnover in health and disease, the pathways that initiate and regulate the process are not yet fully understood. Although several growth factors, including FGF2, IL-6 and other gp130 cytokines may have a role in osteoblast survival *in vitro*, their relevance to bone remodelling *in vivo* is uncertain (Hill et al., 1997, Debiais et al., 2004, Li et al., 2008). Furthermore, the signal transduction pathways responsible for the coordination of osteoblast apoptosis remain unclear.

These studies confirm the dose dependent anti-apoptotic effect of IGF-I in human osteoblastic cells. This is consistent with previous studies (Hill et al., 1997, Gronowicz et al., 2000), as well as with it's identified cell survival role in other systems (Himpe et al., 2008, Annunziata et al., 2011). These observations might also help explain the consequences of the relative IGF-I deficiency or resistance seen in low turnover skeletal disorders, such as renal adynamic bone disorder and age associated osteoporosis (Hoyland and Picton, 1999, Perrini et al., 2010). As may be expected, a reduction in the activity of the key effector protease caspase-3 following IGF-I exposure appears constant. IGF-I is known to upregulate the calcium binding protein calbindin-D28k, which, in turn, binds and inhibits caspase-3 (Bellido et al., 2000). To date, the most clearly defined anti-apoptotic signal transduction pathways following ligand/IGF-IR binding are via PI3-kinase (DiGirolamo et al., 2007). PI3-kinase is associated with the tyrosine kinase receptor and is activated by various growth factors, including insulin, PDGF, the IGF family and nerve growth factor (NGF). The fungal metabolite Wortmannin is a PI3-kinase inhibitor that was originally shown to inhibit the ability of NGF to prevent the apoptosis of rat phaeochromocytoma cells following serum starvation (Yao and Cooper, 1995). However, it should be noted that although wortmannin and other PI3-kinase inhibitors such as LY 294002 have provided helpful insights into PI3-kinase biology, they suffer from a lack of specificity that mandates caution when interpreting *in vitro* mechanisms (Davies et al., 2000). Wortmannin also

inhibits other members of the PI3-kinase superfamily, including the protein kinases ataxia telangiectasia mutated, mammalian target of rapamycin (mTOR) and DNA dependent protein kinase (Chen et al., 2008). This lack of substrate specificity has also contributed to their failure to advance to clinical use in cancer therapy (Gupta et al., 2003). More recently, several novel PI3-kinase inhibitors differing in their isoform specificity have been developed. These include the pyridinylfuranopyrimidine PI-103 and the imidazopyridines PIK-75 and PIK-90 (Knight et al., 2006). PI3-kinase is now also known to play a role as a negative regulator of caspase-3 activation in other systems (Fujita et al., 1998, Schneider et al., 2008). The amelioration of the anti-apoptotic effect of IGF-I by PI3-kinase inhibition in the present studies suggests that this pathway is important for IGF-I functionality in osteoblastic cells. It is therefore interesting to note that increased basal tyrosine phosphorylation, which would blunt the IGF-IR signalling through PI3-kinase, is seen in osteoporotic subjects (Perrini et al., 2008). PI3-kinase activates the protein Akt/PKB which, in turn, phosphorylates and inactivates several pro-apoptotic proteins, including Bad and other members of the Bcl-2 family (Peruzzi et al., 1999, Bai et al., 1999). In its non-phosphorylated state, Bad locates at the mitochondrial membrane and interacts with Bcl-2, preventing its anti-apoptotic function. Akt/PKB also prevents the progression of the caspase cascade through the phosphorylation of caspase-9 (Kermer et al., 2000, Chen et al., 2011). The significant reduction in caspase-9 activity following IGF-I exposure demonstrated in these studies is consistent with this mechanistic observation. In addition, the concomitant decrease in caspase-8 activity is compatible with previous observations that the extrinsic pathway is active during cell death in MG-63 cells (Jilka et al., 1998, Lin et al., 2008) as well as other human osteoblastic cells (Brama et al., 2011). Caspase-8 can also activate the mitochondrial pathway via the bcl-2 protein Bid, so multiple pathways and pathway interactions are likely to be in operation (Li et al., 1998). Akt/PKB may also inhibit apoptosis through the activation of NF-κB (Chang et al., 2003). Akt phosphorylates I-κB, facilitating the translocation of NF-κB to the nucleus where it initiates the transcription of cell survival genes (Karin and Delhase, 2000,

Madrid et al., 2000). NF-κB was originally thought to be pro-apoptotic, as many cytokines that reduce cell survival, such as TNFα, induce it's activation (Beg and Baldwin, 1994). However, it is clear that NF-κB can prevent apoptosis, whilst it's inhibition may potentiate cell death (Wang et al., 1996).

The cysteine protease calpain was originally described as a mediator of necrotic cell death, though a role in caspase independent apoptosis and autophagy is now widely accepted (Wang, 2000, Harwood et al., 2005, Demarchi and Schneider, 2007). The significance of the IGF-I induced increase in calpain activity in the present studies is unclear, although two putative explanations can be offered. Firstly, calpain shares many common substrates with caspase-3, so the reduction in caspase-3 activity may leave a surfeit of substrate (in this study, the synthetic fluorogenic peptide N-succinyl-Leu-Leu-Val-Tyr-AMC) available to calpain, with a consequent increase in apparent activity (Harwood et al., 2003). Secondly, ligand binding to IGF-IR leads, possibly via Akt/PKB, to a rapid activation of voltage dependent calcium channels (Blair et al., 1999). A potential downstream effect of this could be an increase in calcium-dependent calpain activity. There is evidence that calpain plays a role in the proliferation and differentiation of osteoblastic cells (Kashiwagi et al., 2011) and it therefore possible that the preservation of these activities, rather than a cell survival function, dominates the calpain response to IGF-I in this context. However, the enhancement of fodrin breakdown would suggest that the calpain is active at the cell membrane and therefore involved as an effector of injurious signalling. It has been shown that calpain can negatively regulate p53 dependent apoptosis (Atencio et al., 2000, Benetti et al., 2001) and that it can degrade NF-κB inhibitors, with the resultant activation of NF-κB dependent survival pathways (Han et al., 1999, Demarchi et al., 2005). This is thought to play a role in the resistance to anti-tumour therapy (Mlynarczuk-Bialy et al., 2006). Accordingly, calpain activity may be increased by IGF-I in these osteoblastic cells in order to fulfil an anti-apoptotic function. The increase in fodrin breakdown may be explained as a manifestation of the positive effect of both IGF-I and calpain on normal

cell growth, homeostasis and repair. Alternatively, calpain may increase to promote cell death following IGF-I exposure, but be effectively counterbalanced by the promotion of other anti-apoptotic pathways, including those utilising alternative proteases, such as the caspases.

SOCS proteins have been recognised for their role in the processing and regulation of apoptotic and anti-apoptotic signals. This may be protective, such as the amelioration by SOCS3 of IL-1β induced cell injury in pancreatic ß-cells (Karlsen et al., 2001, Bruun et al., 2009); or produce a reduction in cell viability, such as seen with SOCS1 and SOCS3 overexpression in embryonic stem cells (Duval et al., 2000). The effect of IGF-I on SOCS gene expression in osteoblastic cells demonstrated in section 3.3.1 is of interest, as reports of the utility of IGF-I for inducing SOCS expression are relatively scarce. The downstream role SOCS proteins may play in the regulation of IGF-I functionality is currently unknown, but could potentially include an influence on apoptotic signalling and therefore cell survival. While it is possible that conventional pathways downstream of IGF-IR, such as PI3-kinase, may also be influenced by SOCS (Himpe and Kooijman, 2009), there is also evidence that IGF-I may additionally signal through JAK/STAT and be more directly susceptible to SOCS regulation. For example, SOCS inhibit IGF-I induced STAT3 activation (Dey et al., 2000, Zong et al., 2000). There are several putative mechanisms for this STAT activation through IGF-IR. Firstly, after activation by IGF-IR, JAKS could directly phosphorylate STAT. Secondly; activated IGF-IR could associate with JAK to phosphorylate STAT, with a STAT-binding site generated by activated IGF-IR or JAK. Thirdly, JAKS could provide STAT recruitment sites within the receptor complex. The negative regulation of STAT3 by SOCS3 has been further investigated in the context of IGF-I mediated neuronal survival (Yadav et al., 2005). Of note is that IGF-I has also been shown to regulate survival through PI3-kinase in these cells, underlining the presence of multiple survival signals downstream of IGF-IR (Kenchappa et al., 2004). Although the present studies in osteoblastic cells did not demonstrate that the overexpression of SOCS1 or SOCS3

could regulate the anti-apoptotic effect of IGF-I, as measured by either caspase-3 activity or DNA fragmentation (data shown in appendix 3), this is not the case in other systems such as neuronal cells. In neuroblastoma cells, SOCS3 was found to co-localise and associate with IGF-IR after IGF-I stimulation, thereby inhibiting STAT3 activation. SOCS3 overexpression reduced their sensitivity to the anti-apoptotic effects of IGF-I, with an 80% decrease in cell survival following IGF-I stimulation (Yadav et al., 2005). However, when primary cortical neuronal cells were made to overexpress SOCS3, there was no demonstrable difference in cell survival, as was the case in MG-63 osteoblastic cells.

IL-1β was one of the first non-JAK/STAT cytokines shown to upregulate SOCS genes, particularly SOCS3. IL-1β is pro-apoptotic for osteoblastic cells (Tsuboi et al., 1999) and other cell types, such as pancreatic β cells (Vanderford, 2010). The latter appears, at least partially, under the control of SOCS3 (Karlsen et al., 2004). The data presented in chapter 3 demonstrated the ability of IL-1β to induce SOCS1 and SOCS3 expression in osteoblastic cells (chapter 3.3.1) and for SOCS1 and SOCS3 overexpression to enhance both basal and IL-1β stimulated IL-6 release (chapter 3.3.3-4). The functional implications of these observations are unknown, but could have potential relevance to the regulation of cell survival. The increased IL-1β stimulated OPG release in the setting of SOCS3 overexpression (chapter 3.3.8) is potentially interesting, as it could provide a potential counter regulatory mechanism to block TRAIL mediated cell death (Pantouli et al., 2005). In the present studies, IL-1β consistently and significantly increased the degree of both necrotic and apoptotic cell death in osteoblastic cells, as measured by LDH release and activity of caspase-3 respectively. Caspase-3 was chosen for these studies, as it is a key effector caspase in both extrinsic and intrinsic pathways involving caspases 8 and 9 respectively. The SOCS knockdown results collectively indicate that SOCS have a role in the regulation of this process and that it may be mediated by different SOCS proteins depending on the type of cell death involved. All three of the SOCS studied appeared involved in the regulation of the

apoptotic protease cascade, with knockdown of SOCS1, SOCS3 and CIS all significantly reducing basal caspase-3 activity. However, whereas CIS knockdown failed to reduce basal necrosis, unlike SOCS1 and SOCS3 knockdown, it did reduce IL-1β induced cytotoxicity. Furthermore, SOCS3 knockdown did not affect IL-1β induced necrosis. So, in this system, SOCS3 would appear to have a pro-apoptotic and anti-necrotic role, while CIS differentially influences cytokine induced, but not basal, necrosis. Both of these are almost certainly oversimplifications. In addition, SOCS3 knockdown significantly elevated IL-1β induced STAT3 activity, suggesting that although there is evidence in other cell systems for SOC3 interaction with JAK independent pathways such as MAPK/Ras pathways following IL-1β stimulation, it is JAK/STAT signalling that dominates in this particular context. There are precedents for these observations; for example, overexpression of SOCS1 or SOCS3 in LIF stimulated embryonic stem cells leads to a reduction in cell viability (Duval et al., 2000). LIF is analogous to IL-1β, in that its effects on cell proliferation, differentiation and apoptotic signalling are mediated differentially by both JAK/STAT and MAPK/Ras pathways, although almost certainly with a degree of synergy (Kummer et al., 1997, Burdon et al., 2002). In this regard, it is interesting that CIS knockdown had the opposite effect to SOCS3, significantly inhibiting IL-1β induced STAT3 activation. This again underlines the intricacies and interdependencies necessary to maintain a balance between cytokine signalling and cytokine functioning (Forrai et al., 2006).

CHAPTER 5

Final discussion and conclusions

The studies presented in this thesis describe several aspects of the function of human osteoblasts. In the intervening years since the experimental work was undertaken this cell lineage has come under increasing scrutiny, not only for its role in skeletal health and disease, but also for the relevance of its systemic interactions in other contexts. This expanding body of knowledge has helped to explain many historical observations concerning bone and its biology and has already offered several potential targets for novel therapeutic interventions.

Osteoblasts are derived from mesenchymal stem cells that reach bone via vascular channels associated with remodelling sites. Once they arrive at the remodelling surface, they progress through pre-osteoblastic, matrix producing and, eventually, osteocytic stages. The sequential expression of several important transcription factors, including runt-related transcription factor 2 (Runx2), formerly Cbfa-1, and osterix, are now well described throughout this growth and differentiation (Komori et al., 1997) (Nakashima et al., 2002). The significance of the various endocrine, autocrine and paracrine factors that influence osteoblastic development, including IGFs, PTH and BMPs has continued to be elucidated (Qin et al., 2003, Zuo et al., 2012). For example, the action of PTH and BMPs is now known to be closely associated with the activation of the recently described Wnt signalling pathway (Westendorf et al., 2004, Monroe et al., 2012). Mature osteoblasts produce the various regulators of matrix mineralisation, such as osteocalcin, and express RANK-L for osteoclast recruitment (Mellis et al., 2011). The fate of these cells is to either terminally differentiate into osteocytes or to die through apoptosis (Manolagas, 2000). Osteocytes are the most abundant cell in bone and their death following skeletal micro-damage is thought to be the key event in the initiation of subsequent osteoclastic bone resorption (Zhao et al., 2002, Kurata et al., 2006, Cardoso et al., 2009). Dying cells can promote osteoclast differentiation through the release of apoptotic bodies that express M-CSF and RANK-L (Kogianni et al., 2008). Such observations have confirmed that osteoblast viability is a fundamental tenet of bone homeostasis. Furthermore, the ability to resist apoptosis may be a key

determinant of whether a matrix producing osteoblast is able to progress towards an osteocytic phenotype (Verborgt et al., 2002). This progression was previously viewed as a passive process, proceeding as individual cells were enveloped and encased by osteoid (Franz-Odendaal et al., 2006). However, it is now seen as active and regulated, with protection from apoptosis possibly afforded by autophagy, the process where lysosomal degradation recycles cellular products (Xia et al., 2010, Bonewald, 2011). In addition, the different forms of cell death may be important; for example, the promotion of necrosis with diphtheria toxin following forced osteocytic expression of the toxin receptor in mice causes osteoclast activation (Tatsumi et al., 2007). The death of osteocytes is now recognised in disorders associated with skeletal instability, including osteoporosis, with consequent impairment of the ability to sense micro-damage and initiate repair (Weinstein et al., 2000, Kitase et al., 2010, Bonewald, 2011). Osteocyte apoptosis can be promoted by treatment with corticosteroids, as well as by oestrogen withdrawal; the latter mediated by IL-1 and TNF-α (Emerton et al., 2010). Known inhibitors of osteocyte apoptosis include oestrogen, bisphosphonates, calcitonin and calbindin-D28k (Bonewald, 2007). However, although resisting apoptosis may help prevent bone loss in some circumstances, it may be detrimental in others where apoptosis is necessary to initiate the normal housekeeping repair of the micro-damage caused by the large mechanical loads continuously placed upon the skeleton. This means that the regulation of apoptotic processes in osteoblastic cells has developed into a key focus of bone related research, giving several of the observations in this thesis particular resonance and potential for future exploration.

The isolated mature osteoblast cannot simultaneously perform all the functions necessary to advance bone formation, so subpopulations of cells at various differentiation stages, including osteoprogenitor cells, matrix secreting mature osteoblasts and osteocytes must be maintained within the skeletal microenvironment in order to provide the functional heterogeneity required for bone generation (Aubin, 2001). This means that, although it can provide valuable insights, the utility of cell

culture based *in vitro* research is quite restricted in its capacity to reflect the overall phenotypic diversity present *in vivo.* The experiments described in this thesis were performed in osteosarcoma cells originally derived from malignant bone tumours and therefore subject to all the inherent limitations of such an approach. To ameliorate potential inconsistencies to some extent, many of the studies were carried out simultaneously in both MG-63 and SaOS-2 osteosarcoma cells. Such cells have multiple verifiable osteoblastic characteristics, but, almost by definition, are prone to exhibit abnormal cellular function, particularly with respect to their proliferation kinetics and production of osteoid (Scheven et al., 2002, Pautke et al., 2004, Shapira and Halabi, 2009). MG-63 populations, in particular, can be phenotypically heterogeneous, with both mature and immature osteoblastic features represented (Clover and Gowen, 1994). In addition, contact inhibition is often impaired, so they may not be restricted to a monolayer in culture (Pautke et al., 2004). However, osteosarcoma cells are ubiquitous and valuable in bone cell research, principally because cultures of primary osteoblasts are extremely difficult to initiate and maintain. Such primary cultures are derived from healthy donor tissue, but quite marked variation is usual between cells from different subjects (Katzburg et al., 1999). In addition, purification techniques are complicated and can lead to phenotypic inconsistencies (Clover and Gowen, 1994). Most importantly from a practical perspective, primary cells can be maintained for only 3-4 passages before they experience significant drift in their osteoblastic character.

The developing appreciation of the complexity of skeletal biology, particularly with respect to the initiation and regulation of bone formation and resorption, has helped inform a reassessment of many common bone disorders, including renal osteodystrophy (Sprague, 2010). Furthermore, it has begun to explain the apparent mismatch between the heterogeneous clinicopathological manifestations of this (and other) disorders and the relatively limited pathophysiological rationalisation afforded through the conventional framework of abnormal vitamin D and/or PTH metabolism. Although the skeleton may appear a static tissue or organ, its biology is in fact

extremely intricate and vibrant at the cellular level. The concept of bone remodelling, the closely regulated process of osteoclastic resorption and osteoblastic formation, originated four decades ago and our current refined, if incomplete, understanding of the process has formed the basis of the treatments for osteoporosis in widespread clinical use (Frost, 1969, Marie and Kassem, 2011). The term basic multicellular unit (BMU) is traditionally used to describe the spatial relationships of osteoclastic and osteoblastic subpopulations within a remodelling cavity. However, this is now known to be part of a specialised vascular structure, designated the bone remodelling compartment (BRC) (Hauge et al., 2001). It is the BRC that is responsible for translating micro-damage into coupled osteoclast and osteoblast activity through the signalling of the osteocyte network (Eriksen, 2010). The enclosure of regulatory factors within a structure that is distinct from the rest of bone allows control of the process without intrusion from the many growth factors present in the nearby marrow space. This regulation is fundamental, as changes in the balance between resorption and formation will have rapid deleterious consequences through either net bone loss or gain, both of which will impact on bone strength. Unfortunately, this spatial relationship also provides an ideal location for the seeding of malignant metastases (Mundy, 1997). Of note, bisphosphonates are now widely deployed to reduce the number of skeletal secondaries in several solid organ cancers as well as multiple myeloma. This effect has been attributed to the inhibition of angiogenesis, but it may simply reflect the reduced number of BRCs and amount of bone accessible to the tumour following the use of these agents (Polascik, 2009).

Low bone turnover states are characterised by a decrease in the number of BRCs, while high turnover states are characterised by an increase. The normal remodelling cycle lasts approximately 200 days, but can be reduced to 100 days in hyperparathyroidism, thyrotoxicosis and other high turnover states. Conversely, it may exceed 1,000 days in adynamic bone disorder, hypothyroidism and other low turnover states (including after treatment with a bisphosphonate) (Eriksen et al., 1984b, Eriksen,

2010). Osteoblasts are active for longer, with osteoclastic resorption of a remodelling cavity, or lacuna, occurring over a period of 30-40 days, with osteoblastic formation replacing this over approximately 150 days (Eriksen et al., 1984a, Eriksen et al., 1984b). In many disorders, including osteoporosis, osteoblastic refilling of the lacuna is impaired, leading to net bone loss (Eriksen et al., 1990). The mechanisms underlying cell coupling during this process remain only partly understood, although our comprehension has improved greatly over the last decade. Prior to this the prevailing view was that the release of growth factors, like IGF-I and other cytokines, embedded within the bone matrix during the osteoclastic phase maintained equilibrium by exerting control over subsequent formation (Mohan and Baylink, 1996). However, it is now recognised that osteoblastic bone formation can advance without preceding osteoclastic resorptive activity (Karsdal et al., 2007).

Unfortunately, this quiet revolution in our understanding of bone biology over the last ten years has, on the whole, bypassed the clinical renal community. The majority of clinicians continue to think from the narrow perspective of surrogate biological markers, such as serum PTH, and are generally supported in doing so by national and international guidelines, despite the paucity of evidence that underpins them. Although the latest KDIGO clinical practice guidelines have generally been accepted and adopted internationally (including in the UK) they contain only two 1A graded pieces of evidence, and both of these relate to aspects of paediatric, rather than adult, practice (Moe, 2009) (Steddon and Sharples, 2011). Abnormal skeletal structure and function are virtually ubiquitous in CKD and progress as GFR deteriorates, becoming especially prevalent in patients dependent on dialysis (Goldsmith and Cunningham, 2011). Furthermore, extraskeletal calcification, particularly vascular, is now firmly established as a detrimental consequence of CKD, with growing evidence of significant interplay between the skeleton and the circulation (Tomlinson and Cunningham, 2009). In general, PTH and other markers in clinical use are poor at supporting decision-making in relation to skeletal turnover and integrity. It is easy to overlook the fact that PTH is

primarily representative of parathyroid activity, not bone remodelling (Qi et al., 1995). The current recommendation of PTH measurement every three months will allow assessment of its concentration for 0.003% of the patient's year, which is clearly divorced from any meaningful assessment of the state of a patients' skeletal health (Garrett, 2012). It is interesting that the clinical biomarker in current use with the closest relationship to bone turnover is generally underappreciated and therefore underutilised. Bone derived alkaline phosphatase is a relatively effective marker of osteoblastic activity and is associated with both bone mineral density and fracture risk in dialysis patients (Haarhaus et al., 2009, Drechsler et al., 2011). However, its use is not routine, perhaps reflecting concerns regarding cross-reaction with liver-derived alkaline phosphatase.

Over the years, bone histomorphometry has provided a useful insight into bone physiology, particularly with respect to remodelling (Kulak and Dempster, 2010). Information acquired using tetracycline double labelling as a time marker have been extremely valuable, because it demonstrates the cellular activity of osteoclasts and osteoblasts and bone formation (Ott, 2009). However, the histomorphometrical analysis of bone biopsy material is now rarely undertaken in clinical practice, further dissociating clinicians from the consideration of skeletal health as an end-point of clinical decisions. The cellular processes unfolding at the level of the skeleton, such as those presented in this thesis, as well as the far-reaching systemic consequences of these, are currently poorly conceptualized. This is despite the significant increase in prevalence of low turnover, or adynamic, bone disorder; a syndrome that takes its definition from a decrease in osteoblast and osteoclast numbers and reduced bone formation (Frazao and Martins, 2009). This increase in adynamic bone has been driven by multiple factors, including an ageing CKD population, the prevalence of diabetes related CKD and, perhaps more controversially, higher calcium loading as part of current treatment strategies (Andress, 2008). Whatever its cause, this increase mandates a broader clinical appreciation and understanding of underlying bone physiology and

pathophysiology.

This is given additional importance when the systemic consequences of CKD-MBD, including, vascular calcification and excess cardiovascular morbidity and mortality, are taken into consideration. Osteoblasts appear to regulate serum phosphate through the expression of factors such as phosphate-regulating gene with homologies to endopeptidases on the X chromosome (Phex), dentix matrix protein 1 (DMP-1) and FGF-23 (Thompson et al., 2002, Feng et al., 2006, Liu et al., 2006). Phex and DMP-1 both act to downregulate FGF-23 expression, which facilitates renal phosphate reabsorption and the maintenance of normal bone mineralisation (Feng et al., 2009). In the absence of either Phex or DMP-1, FGF-23 is elevated in both the osteoblast and the circulation, leading to excess renal phosphate wasting and, eventually, osteomalacia and rickets (Amatschek et al., 2010, Rowe, 2012). Such observations have led to the suggestion that the osteoblast network should be considered as a form of endocrine system, given that it has important remote targets including the kidney (Feng et al., 2006, Bonewald, 2011). Osteoblast derived FGF-23 may have wider-reaching importance than the control of renal phosphate handling (Martin et al., 2012). Its concentration is elevated in patients with CKD and continues to rise as GFR deteriorates (Fliser et al., 2007), an increase that is strongly associated with both cardiovascular disease and vascular calcification (Gutierrez, 2010, Bernheim and Benchetrit, 2011). It remains to be seen how FGF-23 interacts with tissues other than the kidney, but further clarity around these lines of communication should provide important insights, not only into disorders of phosphate homeostasis, but also the relationship of cardiovascular disease to CKD. In addition, there is increasing evidence for close interaction, and perhaps common lineage, between vascular endothelial cells and osteoblasts; for example, endothelial cells can promote differentiation of mesenchymal stem cells towards an osteoblastic phenotype (Kaigler et al., 2003). Both endothelin and vascular endothelial derived growth factor (VEGF) appear involved in this cardiovascular and skeletal cross talk (Brandi and Collin-Osdoby, 2006).

Osteoblasts express the VEGF receptor and can also express VEGF itself during differentiation (Tombran-Tink and Barnstable, 2004).

There has long been speculation that a component, or more likely components of the uraemic environment could impede, or disturb aspects of normal cellular function as kidney dysfunction progresses (Wills and Jenkins, 1976, Massry, 1977). For bone, it has been inferred from the abnormal serum concentrations of many growth factors in CKD patients, such as the demonstrable excess of the key resorptive cytokine IL-6, that such aberrations have the potential to disrupt the remodelling cycle (Herbelin et al., 1991). The initial studies presented in this thesis aimed to explore the hypothesis that uraemia might exert an influence on the local regulation of bone remodelling through altered osteoblastic release of either IL-6, OPG, or both. The most important findings were that haemodialysis patients had significantly higher concentrations of IL-6 than healthy subjects and that incubation of human osteoblastic cells with untreated sera from healthy individuals significantly raised osteoblastic IL-6 production above that induced by sera from uraemic subjects. Furthermore, treatment with dialysis did not restore the capacity of uraemic serum to augment IL-6 release. In contrast, osteoblastic OPG production was no different after incubation with healthy or uraemic serum. These findings support the premise that non-dialyzable constituents of uraemic serum might influence bone turnover through modified osteoblastic cytokine signalling and therefore contribute to the dysregulation of bone turnover characteristic of osteodystrophy. In addition, apparent removal by charcoal stripping, but not heat treatment or dialysis, suggests that candidate molecules are more complex, perhaps hydrophobic; for example steroids, than those susceptible to simple heat denaturation or removal across standard haemodialysis membranes. Indeed, the fact that the bone and mineral complications of kidney disease are more prevalent, more severe and more difficult to treat in dialysis patients emphasizes the known limitations of dialysis therapies in the correction of many aspects of the uraemic milieu.

The uraemic state is a multi-faceted disorder, meaning that uraemic serum is an unpredictable experimental instrument that is susceptible to multiple confounding factors; including concurrent inflammation, differing concentrations of circulating hormones as well as the possible effects of co-existing co-morbid conditions. Not only did this have to be taken into consideration during the interpretation of results, but it also guided subsequent experiments in chapters 3 and 4 toward exploration of the cellular regulation of osteoblastic cytokine signalling, rather than toward attempts to identify those factors in uraemic setting that may interfere with it. The considerable heterogeneity of uraemia means that pooled uraemic serum is not likely to replicate the complicated and often constantly changing metabolic features of advanced CKD. It will also not allow meaningful representation of patient groups classified according to the severity of their renal failure or the modality of their dialysis treatment. For example, it is known that peritoneal dialysis is a risk factor for low turnover bone disease (Andress, 2008). With hindsight, the uraemic serum experiments could have been structured to include comparisons between sera obtained from patients treated with the different dialysis modalities, as well as from patients with advanced CKD who had not yet commenced dialysis treatment. Uraemic serum is also likely to be affected by the patient's drug treatments. Many drugs have a prolonged half-life in dialysis patients and could be present in considerable excess in serum. The effect of these drugs, as well as their retained metabolites, on *in vitro* systems is unknown, but could be significant. Furthermore, in many cases the drug regimens will include active vitamin D sterols, the receptor for which is expressed on human osteoblastic cells. Even within dialysis modality, it would have been possible, and perhaps more appropriate, to separate patients according clinical estimates of bone turnover, using PTH concentration, alkaline phosphatase and concurrent treatments as a guide. Such an approach has been undertaken by other investigators, including for studies involving SOCS, where differential expression of SOCS mRNA has been found according to dialysis modality; for example, significantly higher CIS expression in lymphocytes from pre-dialysis and

peritoneal dialysis patients, but not from haemodialysis patients (Rastmanesh et al., 2009).

At the time the data presented was obtained, the growing body of evidence for the importance of cytokine signalling in bone health meant that the relatively recently described SOCS family of cytokine regulators appeared a novel and interesting group to investigate further. Although our understanding of SOCS in many cell systems has advanced significantly since their discovery fifteen years ago, the study of their function within osteoblasts, and skeletal tissue in general, remains in its relative infancy. Indeed, the present studies were the first to demonstrate their presence and inducibility in human osteoblastic cells. It is interesting to note that SOCS1 is now believed to undertake an important regulatory role in the late stages of osteoblast differentiation, given the importance now attributed to cell maturation through matrix-producing and osteocytic stages, particularly in light of renewed interest in the latter as a regulatory cell for remodelling (Abe et al., 2006).

It is also interesting that SOCS have progressively come under scrutiny in the uraemic state, particularly with respect to their association with growth hormone resistance, poor erythropoietin response and, more recently, a potential role in CKD progression (Rabkin et al., 2005, Tan and Rabkin, 2005, Liu et al., 2011). The initial work presented here with conventional RT-PCR showed promise, but results were disappointingly inconsistent. Subsequent fluorescence-based kinetic 'real-time' PCR significantly improved reproducibility and enabled the collection of more robust quantitative data. Unfortunately, the detection of SOCS protein proved extremely difficult due to the paucity of high-quality commercially available SOCS antibodies at the time. This was an experience shared with other laboratories with an interest in this area. In terms of mRNA, SOCS were found to be both constitutively expressed and inducible to different, and in some cases, surprising degrees by osteotropic cytokines and growth factors. In general, the sequential pattern of expression was consistent with a negative feedback

function, presumably acting to regulate uncontrolled and potentially harmful over-stimulation. With hindsight, and given contemporary understanding of the complexities of SOCS function, this is an over-simplification of the relationship between relative SOCS expression and feedback inhibition. In many systems the effect of SOCS has been shown to be highly sensitive, with very small differences in expression leading to powerful inhibition of JAK/STAT and other interdependent pathways. The effect of IGF-I on osteoblastic SOCS1 and CIS gene expression is of particular interest. Reports of the effect of IGF-I, traditionally a 'non-JAK/STAT' cytokine, on SOCS expression remain scarce (Ahmed and Farquharson, 2010). The departure from a strict JAK/STAT paradigm for SOCS functionality is also demonstrated by the increased SOCS3 and CIS expression following osteoblastic stimulation with LPS. At the time, this finding was unexpected, but it is interesting to note that since these data were obtained, SOCS3 has become recognized as a potent regulator of LPS in many cell systems, particularly the CNS(Ramgolam and Markovic-Plese, 2011, Strebovsky et al., 2012). IGF-I induced SOCS expression also provides an important reminder of the limitations of *in vitro* data when extrapolated to whole systems or organisms. In transfection models, SOCS1 and SOCS3 appear the stronger inhibitors of IGF-I, but in transgenic animals deletion of SOC2 causes IGF-I induced gigantism, while CIS over-expression causes growth retardation (Alexander and Hilton, 2004).

Beyond inducible expression, a functional role for SOCS was suggested by enhanced IL-6 release following SOCS1 and SOCS3 over-expression, as well as the enhanced IL-1β stimulated OPG release following SOCS3 overexpression. The finding of intact gp130/STAT3 signalling in cells transfected with SOCS3 and producing significant amounts of IL-6 supports the involvement of other non-JAK/STAT pathways, as has now been demonstrated in many non-osteoblastic cell systems (Cassatella et al., 1999, Mansell et al., 2006). In fact, balanced cytokine signalling is likely to depend on complex cross talk between numerous intracellular signals, including SOCS, JAK/STAT and others pathways, including Ras/MAPK. The isolated study of SOCS functionality is

therefore likely to raise as many questions as it answers and further studies would need to acknowledge and explore these interdependencies from the outset.

Subsequent experiments utilised apoptotic and necrotic cell death as functional end points to determine the role, if any, of SOCS in this crucial component of bone remodelling. The anti-apoptotic effect of IGF-I in osteoblastic cells was confirmed, underscoring the importance of relative IGF-I resistance to low turnover skeletal disorders such as renal adynamic bone disorder (Cao et al., 2007). A reduction in DNA fragmentation as well as protease activity, including caspase-3 were consistent following IGF-I exposure, although a potential lack of specificity of protease substrates should be acknowledged in all the caspase assays presented. It would also have been interesting and highly desirable to have explored apoptotic end points in more detail with techniques such as viability staining, fluorescence microscopy and quantitation of annexin V binding using flow cytometry had the time and techniques been available.

SOCS have been increasingly recognized for their role in the processing of apoptotic and survival signals (Sutherland et al., 2007, Yan et al., 2008, Horndasch and Culig, 2011a). In the present studies, SOCS overexpression did not demonstrably affect osteoblast survival and it would be interesting to repeat this and further explore the reasons underpinning it; for example, through examination of SOCS-IGF-IR interaction and STAT activation under these circumstances. SOCS gene knock down suggested that cytokine induced cell loss is mediated differentially depending on the type of cell death involved. SOCS1, SOCS3 and CIS all appeared relevant to the protease cascade, with reduced basal caspase-3 activity. However, whereas CIS knockdown failed to reduce basal necrosis, unlike SOCS1 and SOCS3 knockdown, it did reduce IL-1β induced cytotoxicity. Furthermore, SOCS3 knockdown did not affect cytokine-induced necrosis. It would be interesting to explore these observations further in the context of the new knowledge concerning the importance of the different types of osteoblastic cell death and their potential relationship to the initiation of osteoclastic

bone resorption (Tatsumi et al., 2007, Xia et al., 2010, Bonewald, 2011). As compelling as these results are, the possibility of off-target effects of SOCS siRNA not seen with control siRNA should be acknowledged. Such effects occur when an siRNA is processed by the RNA silencing complex, causing downregulation of unintended bystander targets with consequent alterations in the expression of many genes.

The data presented in this thesis add to our knowledge surrounding the cells and growth factors involved in the bone remodelling process. Our understanding of osteoblast and osteoclast signalling and its regulation is the foundation for the development of therapies able to influence bone mass and integrity. Until quite recently, these efforts have primarily been focused on the development of drugs that can prevent bone loss, usually by targeting bone resorption. Examples of such 'anti-catabolic' agents include oestrogens, selective oestrogen receptor modulators (SERMS), bisphosphonates and, more recently, RANK-L inhibitors (Riggs and Parfitt, 2005). However, the expanding overview of osteoblast biology has started to encourage the development of 'anabolic' agents that aim to increase bone formation. Such anabolic therapies may increase osteoblastic bone formation by promoting osteoblast differentiation or preventing their apoptosis (Khosla et al., 2008). The data presented here reaffirm the potent anti-apoptotic property of IGF-I for osteoblastic cells, a property which are likely to be important for the maintenance of normal bone mass (Giustina et al., 2008). In addition, considerable interest remains in the potential for this growth factor to be used as an anabolic agent. The concentrations of growth hormone, IGF-I and certain IGF binding proteins (IGFBP) are altered in both osteoporosis and ageing (Yamaguchi et al., 2006, Amin et al., 2007) In addition, reduced serum IGF-I levels are associated with decreased bone strength, while ageing appears to produce receptor mediated relative skeletal resistance to IGF-I (Giustina et al., 2008). In animal models of osteoporosis, administration of IGF-I promotes bone formation and ameliorates bone loss (Fowlkes et al., 2006). PTH is currently the only approved anabolic therapy for osteoporosis and exerts its action, at least in part, through the

enhanced local production of IGF-I (Jilka, 2007). This therapeutic use of PTH is based on the observation that intermittent, rather than sustained PTH administration advances bone formation over bone resorption (Compston, 2007). In this situation it promotes osteoblastic cell proliferation and extends their longevity through an anti-apoptotic effect. It also inhibits the Wnt antagonist sclerostin to enhance Wnt signalling pathways (Westendorf et al., 2004, Kramer et al., 2010). An alternative approach would be to stimulate endogenous PTH secretion using calcium-sensing receptor antagonists, or calcilytics (Arey et al., 2005, Kumar et al., 2010). The near future is likely a new generation of therapeutic agents that exert their effect through the osteoblast (Marie and Kassem, 2011). For example, canonical Wnt signalling has become a key drug target. Activation of the canonical Wnt/β-catenin pathway promotes osteoblast proliferation and differentiation at the expense of adipocyte differentiation (Bodine and Komm, 2006). Consequently, activation of the Wnt signalling with glycogen synthase kinase 3 inhibitors produces enhanced bone formation, density and strength in osteopenic mice (Clement-Lacroix et al., 2005, Kulkarni et al., 2006). However, given concerns regarding the implications of Wnt signalling in cancer development, indirect targeting through the inhibition of Wnt antagonists currently shows the most promise (Enders, 2009). One such approach is the inhibition of the osteoblastic protein sclerostin, a negative modulator of bone formation (Poole et al., 2005). Systemic injections of a monoclonal antibody to sclerostin increase bone formation, bone mass and strength in osteopenic animals and to lead to an increase in markers of bone formation in postmenopausal women (Li et al., 2009, Paszty et al., 2010, Padhi et al., 2011).

Collectively, the studies presented in this thesis provide several insights into osteoblastic cytokine signalling and its regulation under both normal and uraemic conditions. Importantly, they contribute to the relatively small number of studies concerning the presence and function of the SOCS family in human osteoblastic cells, including potential involvement in aspects of cell survival. These findings add to the

body of knowledge regarding the coupling of osteoblastic bone formation and osteoclastic resorption within the remodelling cycle in both healthy and uraemic bone.

The last decade has seen impressive advances in our understanding of local and systemic biology of cells of osteoblastic lineage. The next will hopefully see the benefits of this new knowledge realized through the development of meaningful diagnostic and therapeutic advances that transfer to the clinical setting. Ultimately, one would hope that all clinicians will soon have the tools available to stimulate a more sophisticated understanding of the complexity, function and importance of bone tissue and to assist them to successfully preserve the skeletal and cardiovascular health of their patients.

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Appendices

Appendix 1 Supplementary data chapter 2

Appendix 1.1 Standard curves for IL-6 and OPG DuoSet ELISA Development kits

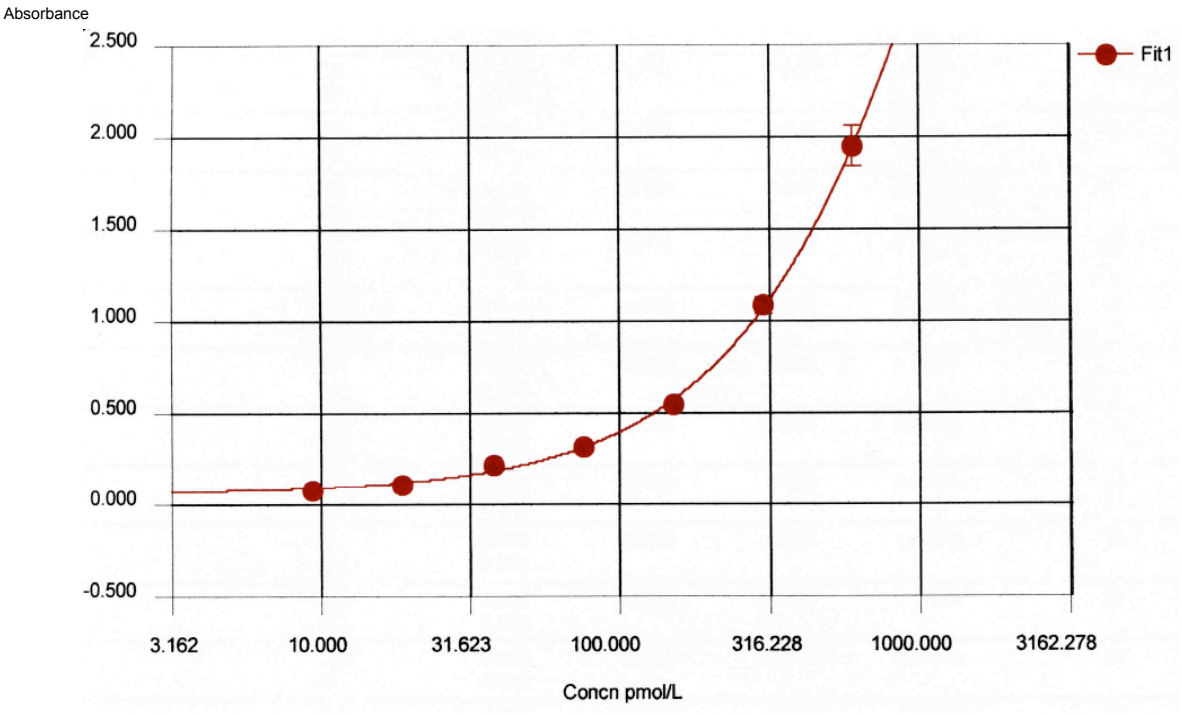

Figure A.1.1 Typical standard curve for IL-6 Duoset ELISA (R&D Systems, Abingdon, UK). The relevant sample values were found in the sensitive part of the response curve (above 300pmo/L).

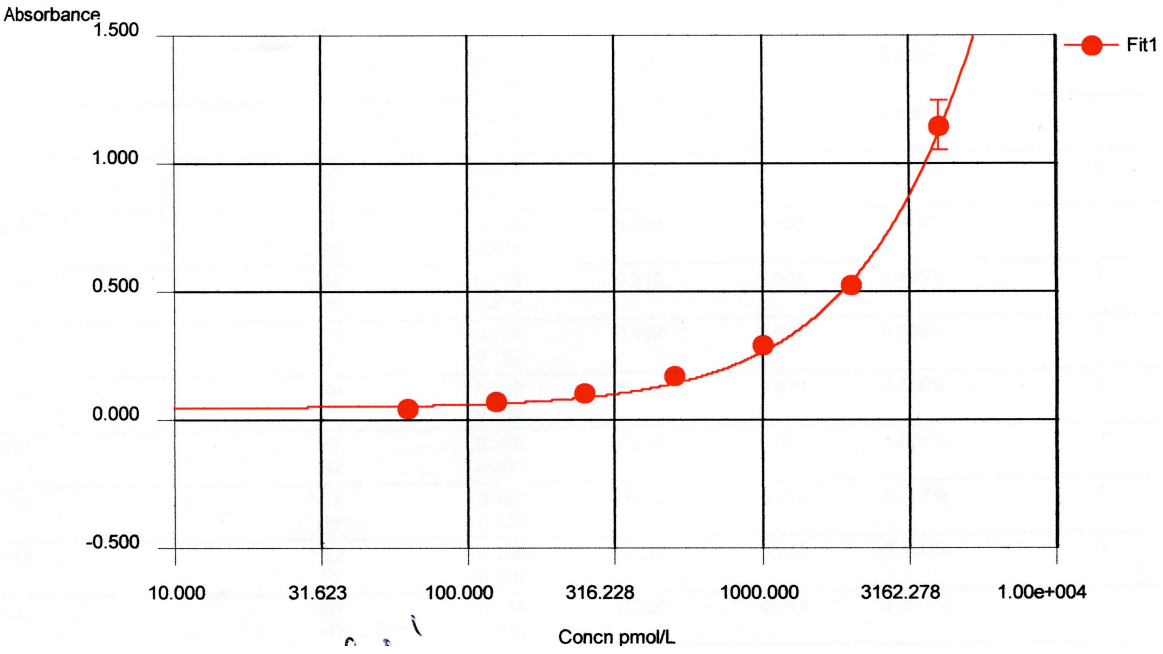

Figure A.1.2 Typical standard curve for OPG Duoset ELISA (R&D Systems, Abingdon, UK). The relevant sample values were found in the sensitive part of the response curve (above 500pmo/L).

Appendix 2 Preliminary data chapter 3

Appendix 2.1 Verification of MG-63 cytokine response

Effect of IL-1ß stimulation on IL-6 mRNA expression

Preliminary experiments were aimed at confirming the previous observation that IL-1ß stimulation augments IL-6 production in MG-63 cells. After harvesting and counting, MG-63 cells were plated at a density of 0.5 x 10^6 cells per well of a 6-well plate in 3 mL medium and incubated at 37ºC for 24h in a humidified incubator with an air to CO_2 mix of 19:1. The cells were then serum starved for 12h, before incubation with either 100 IU/mL IL-1ß in serum free media or serum free media alone. Cells were harvested at times 0, 15, 30, 60, 120 and 240 min, with subsequent mRNA extraction, cDNA synthesis and PCR amplification using IL-6 specific primers (forward primer 5'-CTGCTCCTGGTGTTGCCT-3', reverse 5'-CCTGCAGCCACTGGTTCT-3', 35 cycles of 93ºC for 60s, 60ºC for 60s and 72º for 60s, followed by a further 10min at 72ºC for final primer extension). The integrity of RNA for each specimen was verified using human GAPDH primers. Results confirmed the IL-6 response of MG-63 cells. Representative results are shown in figure A.2.1.

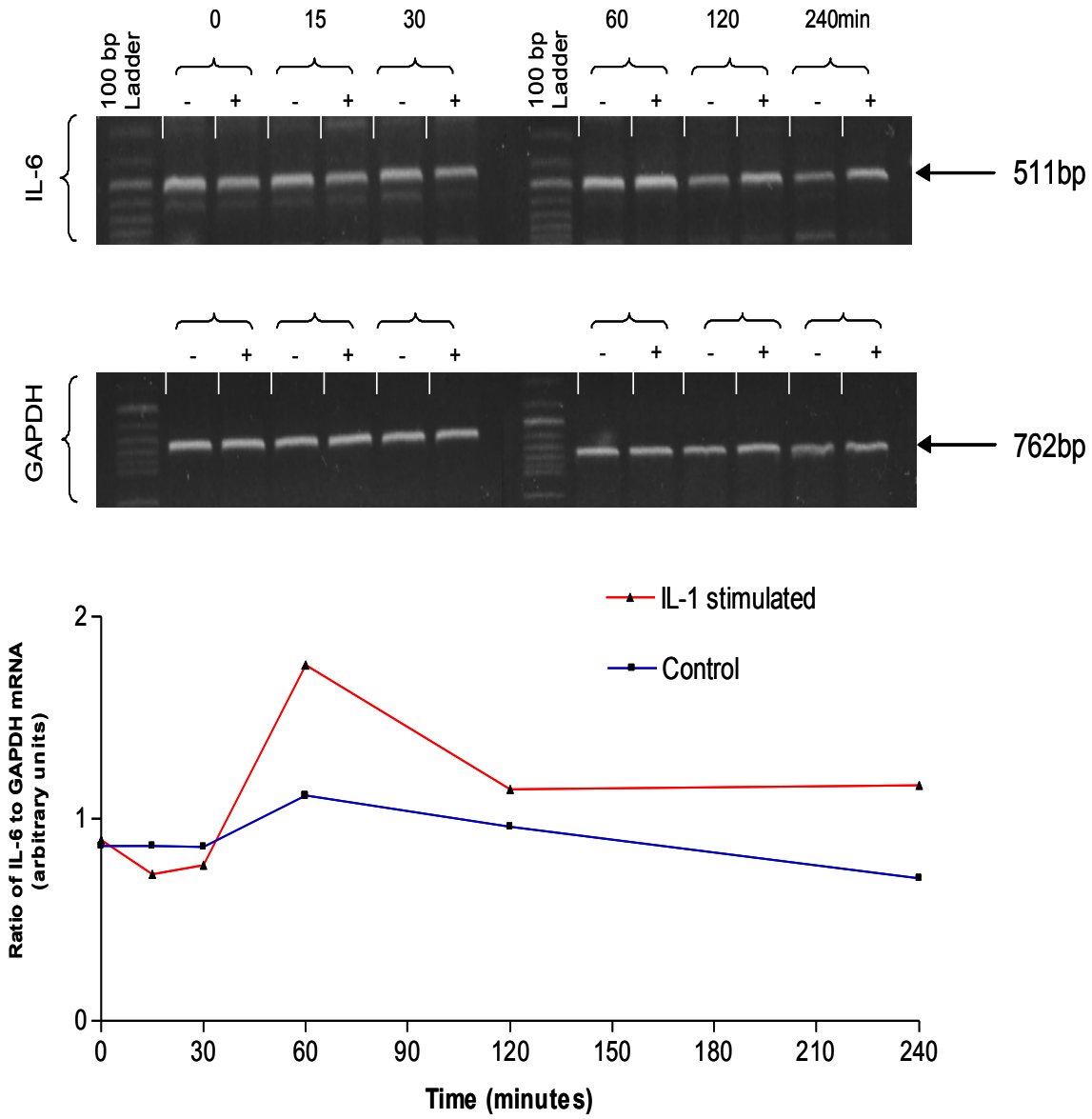

Figure A.2.1: Effect of IL-1ß stimulation on IL-6 mRNA expression in MG-63 cells. Gel lanes marked with a plus (+) contain products from IL-1ß stimulated cells, while those marked with a minus (-) contain (unstimulated) controls. The X-Y plot shows the result of densitometric analysis, with IL-6 mRNA production increasing above controls in the IL-1ß stimulated cells after 60 min and remaining elevated thereafter. n=1 for each time point.

Appendix 2.2 Optimisation of SOCS PCR conditions

Linearity of cycle number

Optimisation of the RT-PCR procedure was performed with respect to the number of amplification cycles to ensure a linear relationship between the amount of RNA template and the optical density of the PCR band generated. Results are shown in figure A.2.2. GAPDH and SOCS2 remain linear throughout. SOCS3 is linear between cycles 25 to 30 and plateaus thereafter. CIS is linear between cycles 28 and 35. In subsequent amplifications 28 cycles were used for SOCS2 and SOCS3 and 30 for CIS. Twenty-eight or 30 cycles were used for GAPDH, depending on the (simultaneously amplified) amplicon of interest.

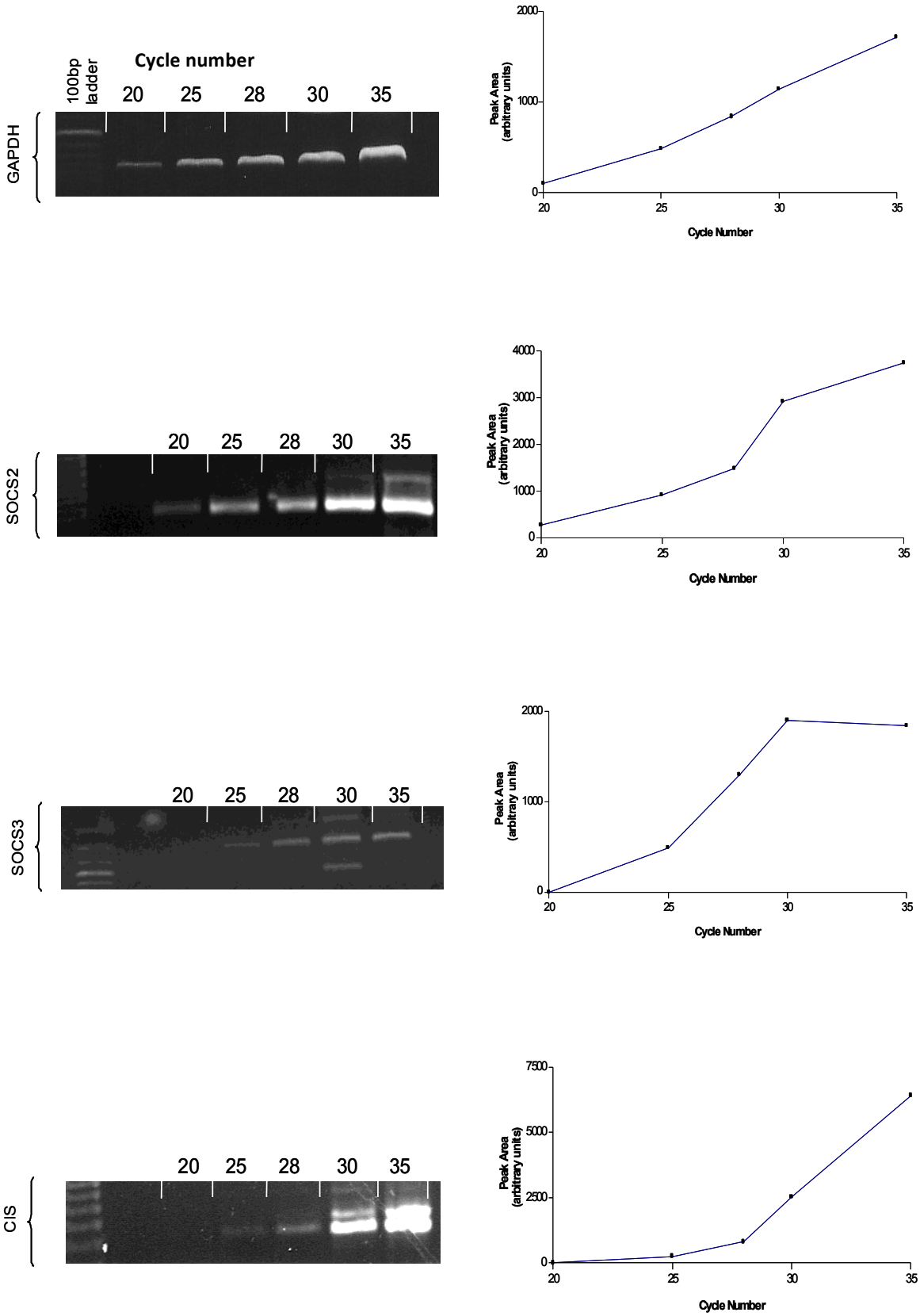

Figure A.2.2: Optimisation of SOCS PCR conditions: linearity of cycle number. In order to ensure a linear relationship between amount of RNA template and optical density of the PCR band generated, optimisation of the RT-PCR procedure was performed with respect to the number of amplification cycles.

Effect of total RNA amounts

The effect of different total RNA amounts on SOCS detection was investigated by varying the starting concentration of RNA template. This was achieved by increasing the volume of cDNA (0, 1, 2, 3 and 4 µL) used in PCR amplification (total RNA input was kept constant in all first strand reactions). All amplifications were performed using the optimum cycle number determined above. For GAPDH, SOCS3 and CIS the relationship between the amount of starting cDNA and amount of PCR product is approximately linear. For SOCS2 the relationship is no longer linear above 2µL of starting product. These results are shown in figure A.2.3. In subsequent reactions 2 µL of cDNA were used for SOCS3 and CIS and 1 µL for SOCS2. One or 2 µL of cDNA were used for GAPDH, depending on the (simultaneously amplified) amplicon of interest

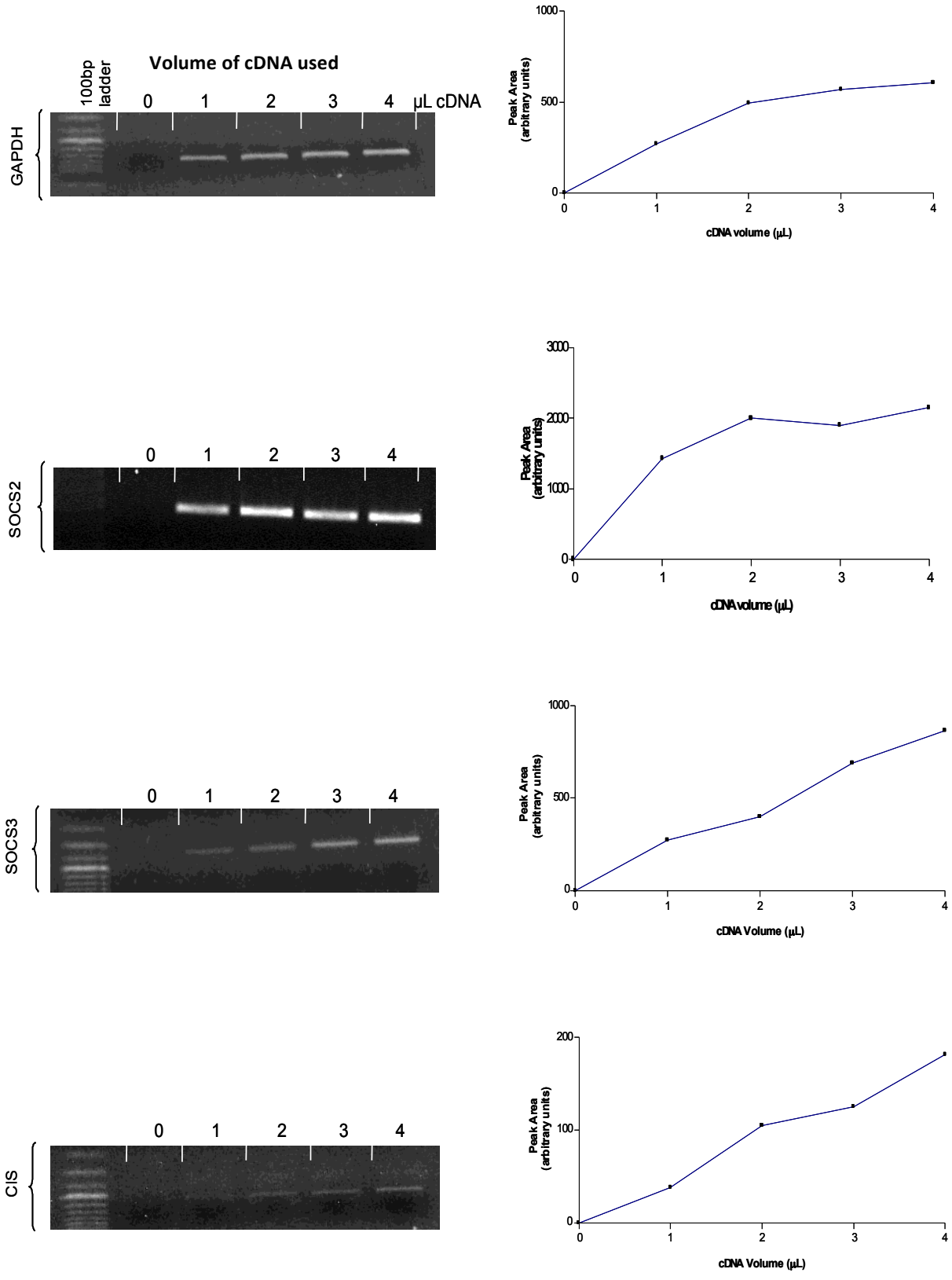

Figure A.2.3: Optimisation of SOCS PCR conditions: effect of total RNA amount. The effect of different total RNA amounts on SOCS detection was investigated by varying the starting concentration of RNA template. This was achieved by increasing the volume of cDNA (0, 1, 2, 3 and 4 µL) used in PCR amplification.

Appendix 2.3 Effect of serum supplementation on SOCS3 expression

To investigate the influence of serum supplementation of media on basal SOCS3 expression, MG-63 cells were grown under the following conditions for 4h: serum free media, charcoal stripped 5% FBS or 5% FBS. Charcoal stripping removes many growth factors and cytokines that could potentially influence SOCS expression. Results are shown in figure A.2.4. No differences were seen in basal SOCS3 mRNA expression following 4h exposure to either serum free, serum supplemented, or charcoal stripped serum supplemented media. This implies that the effect of media as a potential confounding factor could be discarded in subsequent experiments.

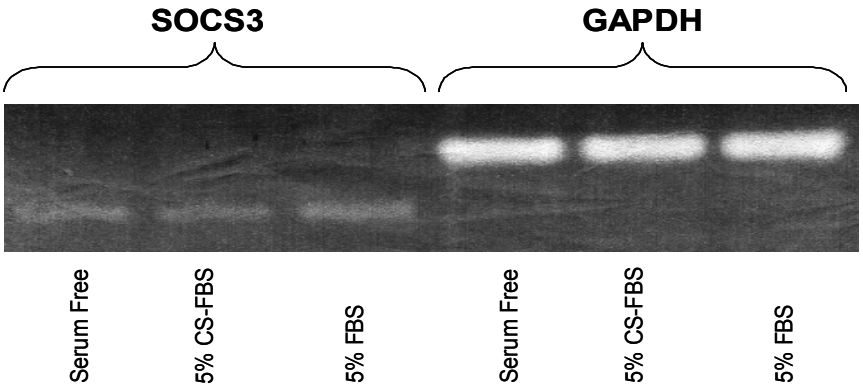

Figure A.2.4: Effect of media with and without serum supplementation on SOCS3 expression. No differences were seen in basal SOCS3 mRNA expression following 4 hours exposure to either serum free, serum supplemented, or charcoal stripped serum supplemented media.

Appendix 2.4 Regulation of SOCS expression by cytokines: stimulation of MG-63 cells with IL-1ß and IGF-I

Following the initial detection of SOCS mRNA in the MG-63 cell line, experiments were designed to examine any regulation of expression of SOCS mRNA by the cytokines IL-1ß and IGF-I. After being harvested and counted, MG-63 cells were plated at a density of 0.5 x 10^6 cells per well of a 6-well plate in 3 mL medium and incubated at 37ºC for 24h. The cells were then growth arrested in serum starved media for 12 hours, before stimulation with IL-1ß (100 IU/mL) or IGF-I (10 ng/mL) for time points between 0 and 24h. An additional untreated sample was collected at each time point. mRNA was isolated from the cells followed by cDNA synthesis and PCR amplification with SOCS specific primers. The integrity of RNA for each specimen was verified using human GAPDH primers.

Results for IL-1ß are shown in figures A.2.5 (SOCS2) and A.2.6 (SOCS3) and for IGF-I in figure A.2.7 (SOCS2 and 3). Due to difficulties experienced in cross experiment comparison, representative results are shown. Up to 60min following IL-1ß stimulation there were no differences in SOCS2 expression between control and stimulated groups. After 60min there was a modest rise in expression in the stimulated arm, this was most pronounced at 24h, and 30min following stimulation with IL-1ß there was a rise in SOCS3 expression in both the control and stimulated groups. This was more pronounced in the stimulated group and persists until the end of the experimental period. The reason for the increase in the unstimulated group is unclear, but could indicate that there is a certain amount of basal variability in SOCS expression, perhaps in response to autocrine factors.

No consistent pattern of expression was seen in either SOCS2 or SOCS3 following stimulation of MG-63 cells with IGF-I. Results for CIS mRNA expression in response to either IL-1ß or IGF-I were inconsistent and therefore difficult to interpret.

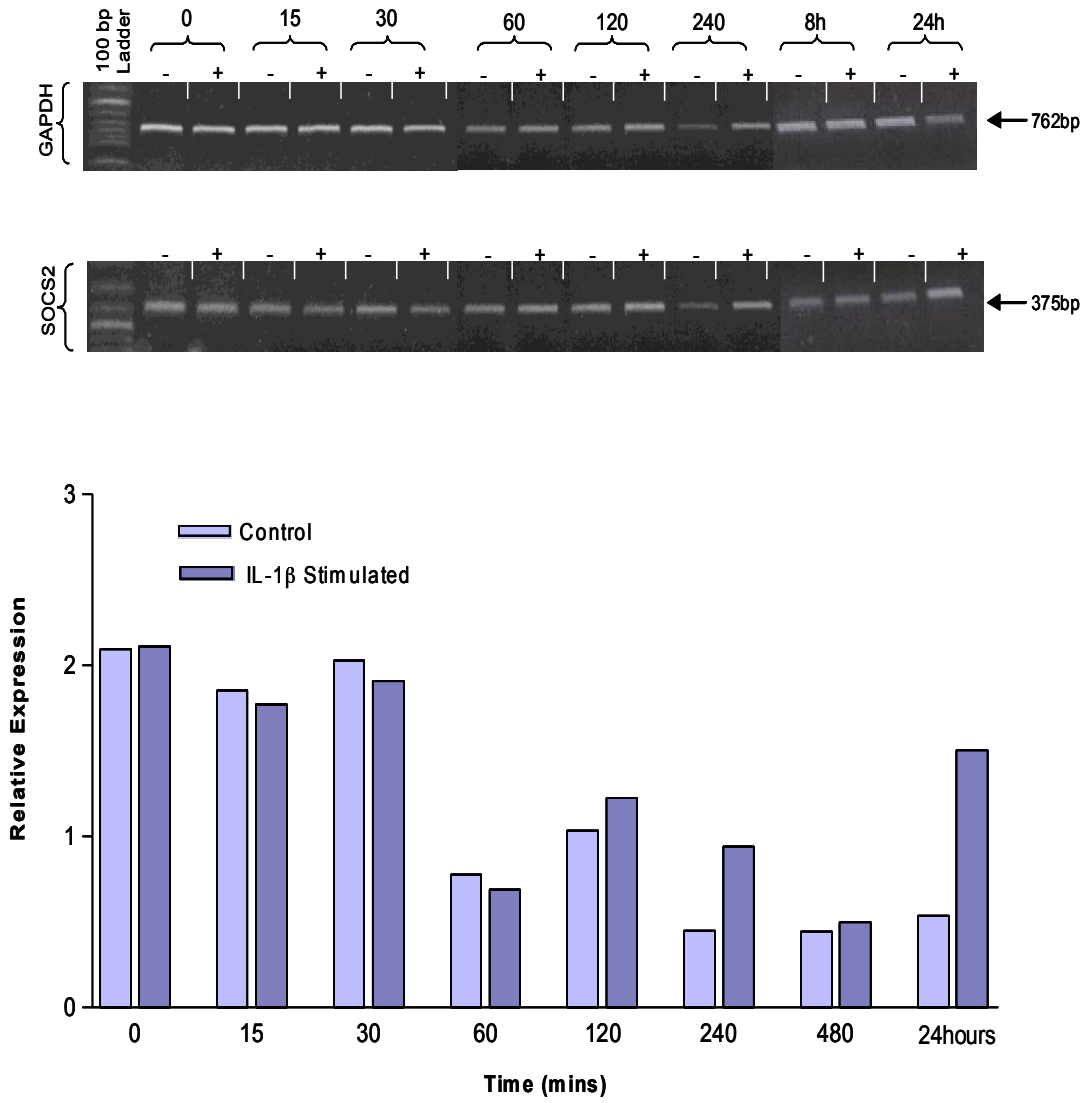

Figure A.2.5: SOCS2 expression following stimulation of MG-63 cells with IL-1ß (100 IU/mL). Up to 60min there are no differences between control and stimulated groups. After 60min there is a modest increase in SOCS2 expression in the stimulated cells with respect to controls, though expression in both groups has fallen compared to baseline. The difference in expression is most pronounced at 24h.

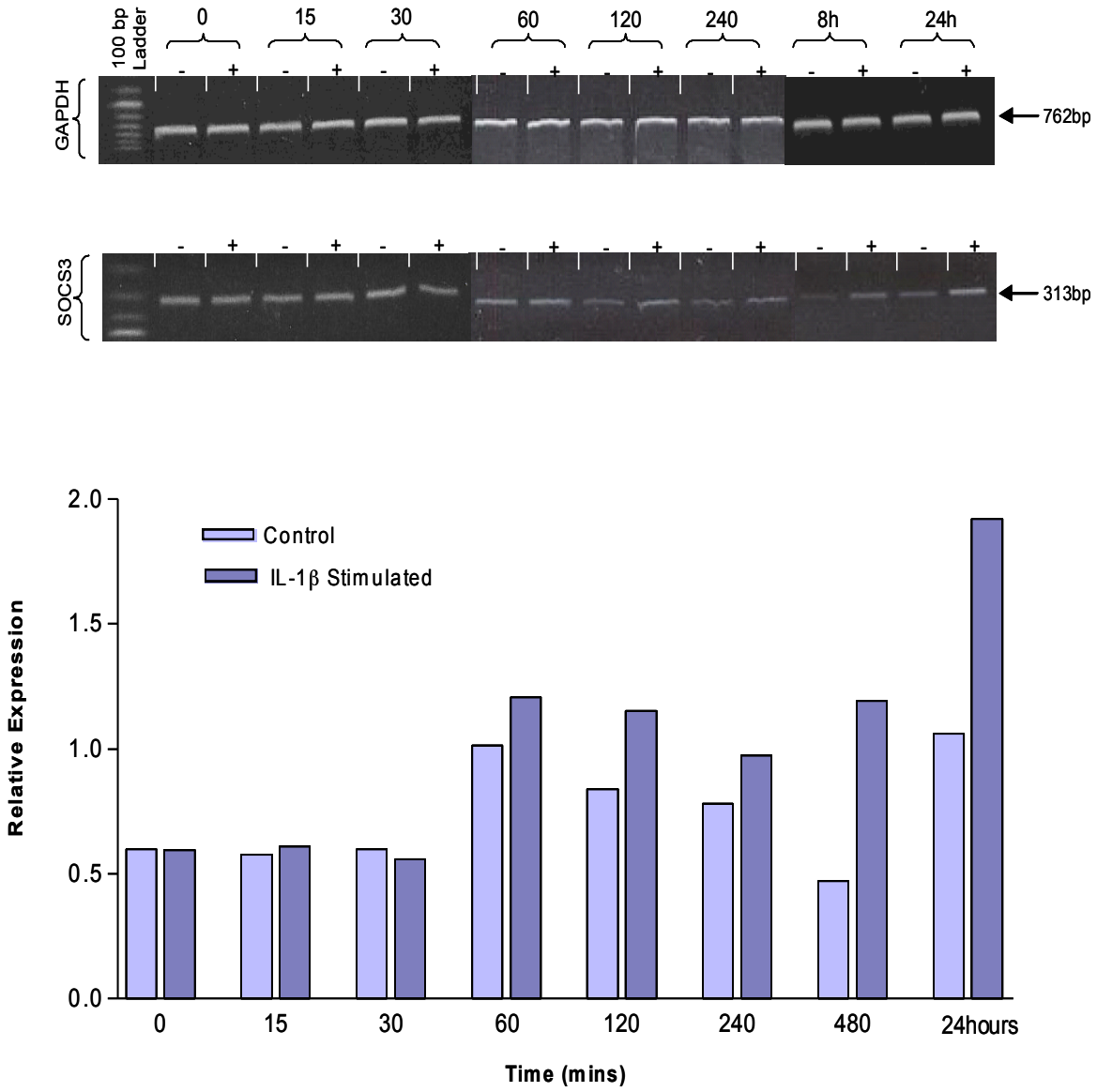

Figure A.2.6: SOCS3 expression following stimulation of MG-63 cells with IL-1ß (100 IU/mL). After 30min there is a rise in SOCS3 expression in both the control and stimulated groups. This is more pronounced in the stimulated group and persists until the end of the experiment.

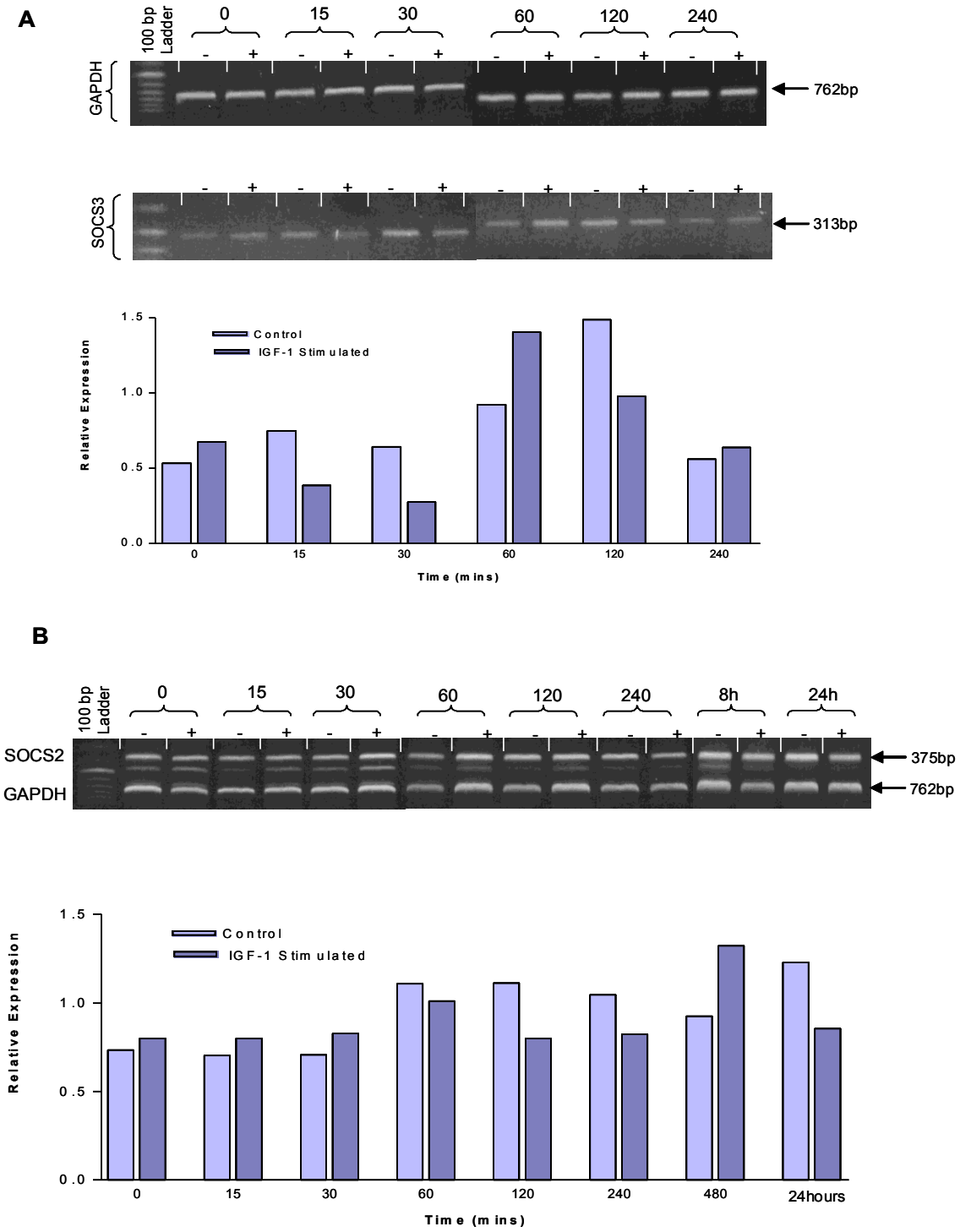

Figure A.2.7: SOCS3 (A) and SOCS2 (B) expression following stimulation of MG-63 cells with IGF-I (10ng/mL). The SOCS2 PCRs in B are run duplex with GAPDH. In these representative experiments and repeats, no consistent pattern of expression was seen following stimulation.

Appendix 3 Supplementary data chapter 4

Appendix 3.1 Effect of SOCS1 and SOCS3 overexpression on caspase-3 activity and DNA fragmentation with and without treatment with IGF-I

In these studies, IGF-I has no effect on caspase-3 activity (figure A.3.1a) and DNA fragmentation (figure A.3.1b), respectively, in the presence of empty vector. This should have acted as a positive control. In the absence of such an effect, no conclusions can be drawn from the lack of effect following over-expression of SOCS-1 and SOCS-3. It is interesting to note the much higher basal caspase activity and cell death in these experiments. Such variability is a feature of biological assays, but might also indicate that the cells were undergoing unusually high basal apoptosis for unspecified reasons during this experiment; e.g. the cells had reached over-confluence.

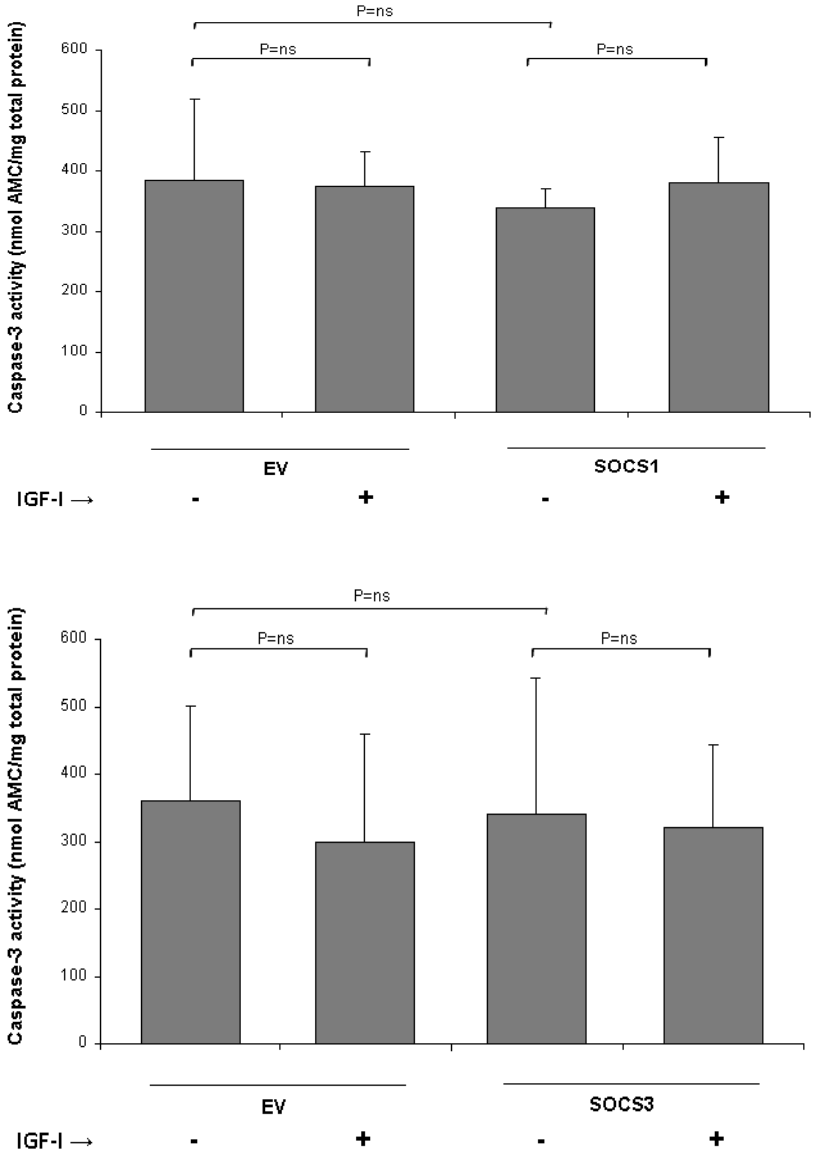

Figure A.3.1a Caspase-3 activity in cells overexpressing SOCS1 and SOCS3 compared with empty vector (EV) controls, with and without 24h incubation with 10ng/mL IGF-I. n=6 for each group. No statistically significant differences were demonstrated.

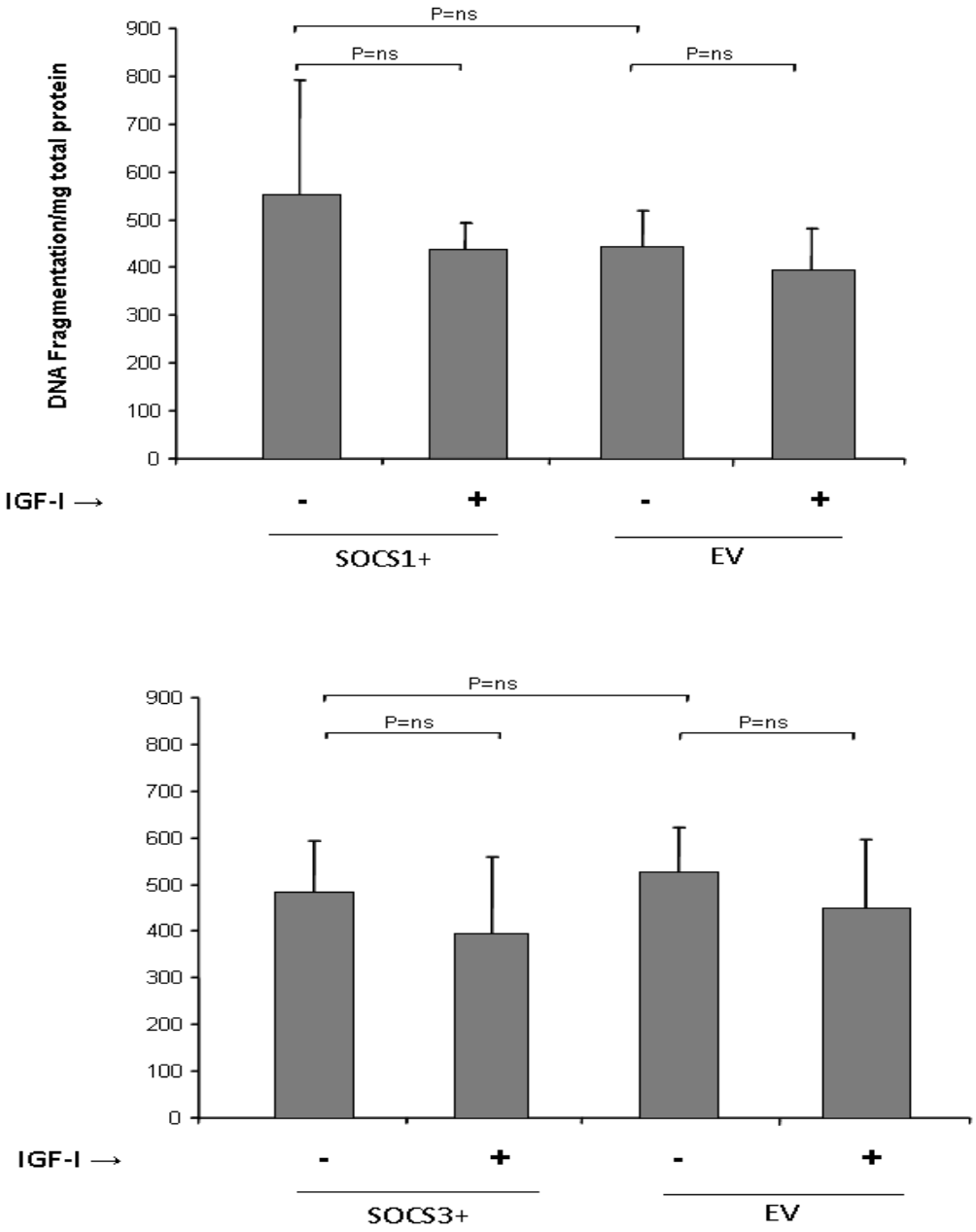

Figure A.3.1b DNA fragmentation in cells overexpressing SOCS1 and SOCS3 compared with empty vector (EV) controls, with and without 24h incubation with 10ng/mL IGF-I. n=6 for each group. No statistically significant differences were demonstrated.

Declaration

This thesis is my own work, with collaborative work fully acknowledged. It has not previously been presented to another institution for a higher degree.

‘There is nothing so fatal to character than half finished tasks’

David Lloyd George