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ABBREVIATIONS

1,25(OH) ₂ D ₃	1,25 dihydroxyvitamin D ₃
A2P	L-ascorbic acid 2-phosphate
AAA	ATPases associated with a variety of activities
ALP	alkaline phosphatase
aPKC	atypical protein kinase C
ATF	activating transcription factor
αΜΕΜ	minimum essential medium, alpha
BM	bone marrow
BMD	bone mineral density
BMP	bone morphogenetic protein
BMU	basic multicellular unit
bp	base pair
BSA	bovine serum albumin
BSP	bone sialoprotein
C/EBP	CAAT/enhancer-binding protein
cAMP	cyclic adenosine monophosphate
CAR	Coxsackie-adenovirus receptor
Cath K	cathepsin K
CBP	CREB binding protein
CCL2	monocyte chemotactic protein 1
CFU-GM	granulocyte macrophage colony forming unit
CHI3L1	chitinase 3-like 1
Col-I/II/X	type I/II/X collagen
CREB	cAMP response element binding
CTx	C-telopeptide of collagen crosslinks
DCt	delta Ct
DKK1	dickkopf 1
DMEM	Dulbecco's modified Eagle's medium
DMP1	dentin matrix protein 1
Dpd	deoxypyridolines
Dsh	dishevelled
ER	oestrogen receptor

ERK	extracellular signal-regulated kinase
FBS	foetal bovine serum
FEO	familial expansile osteolysis
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FPPS	farnesyl pyrophosphate synthase
Fra	fos-related antigen
GATA6	GATA binding protein 6
GCOS	GeneChip operating software
GFP	green fluorescent protein
gla	γ-carboxylic acid
GM-CSF	granulocyte macrophage colony stimulating factor
GO	gene ontology
GSK	glycogen synthase kinase
HOB	human osteoblasts
hr	hour
IBMPFD	inclusion body myopathy, Paget's disease and frontotemporal dementia
IGF	insulin-like growth factor
Ihh	Indian hedgehog
IKK	IkB kinase
IL	interleukin
IL-1R	Interleukin 1 receptor
I-Smad	inhibitory Smad
ΙκΒ	inhibitor of NFkB
JAK	Janus kinase
JNK	jun N-terminal kinase
KEGG	Kyoto encyclopaedia for genes and genomes
LRP	low-density lipoprotein receptor-related protein
M gene	matrix gene
МАРК	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
MEF2	myocyte-enhancer factor 2
MEM	minimum essential medium
MEPE	matrix extracellular phosphoglycoprotein

MGB	minor groove binder		
MGP	matrix gla protein		
min	minute		
MMP	matrix metalloproteinase		
MOI	multiplicity of infection		
MRFs	myogenic regulatory factors		
MV	measles virus		
N gene	nucleocapsid gene		
NCoR	nuclear receptor corepressor		
NFAT	nuclear factor of activated T cells		
ΝΓκΒ	nuclear factor kappa B		
NGFR	nerve growth factor receptor		
NIBSC	National Institute of Biological Standards and Control		
NTC	no template control		
NTx	N-telopeptide of collagen crosslinks		
OB	osteoblast		
OC	osteocalcin		
ONPG	o-nitrophenyl-β-D-galactopyranoside		
OPG	osteoprotegerin		
Osx	osterix		
P1CP	carboxy-terminal propeptide of type I collagen		
P1NP	amino-terminal propeptide of type I collagen		
p62	sequestosome 1		
PB1	phox and bem1p-1		
PBMC	peripheral blood mononuclear cells		
PBS	phosphate buffered saline		
PCOT2	principal coordinates and Hotelling's T squared		
PCR	polymerase chain reaction		
PDGF	platelet-derived growth factor		
pfu	plaque-forming units		
PGD ₂	prostaglandin D ₂		
PGE ₂	prostaglandin E ₂		
PI3K	phosphatidylinositol-3 kinase		
ΡΡΑRγ	peroxisome proliferator-activated receptor γ		

PTGDS	prostaglandin D2 synthase, brain isoform		
РТН	parathyroid hormone		
PTHrP	parathyroid hormone related protein		
Pyd	pyridinolines		
RANK	receptor activator of nuclear factor kB		
RANKL	RANK ligand		
RGS	regulator of G-protein signalling		
RIN	RNA integrity number		
RIP	receptor interacting protein		
RMA	robust multichip analysis		
R-Smad	receptor-activated Smad		
RT-PCR	reverse transcription polymerase chain reaction		
S	second		
SAFE	significance analysis of function and expression		
SERM	selective oestrogen receptor modulators		
sFRP	secreted frizzled-related protein		
SGK1	serum/glucocorticoid regulated kinase 1		
SIBLING	small integrin-binding ligand, N-glycosylated proteins		
SMRT	silencing mediator of retinoid and thyroid receptors		
SOST	sclerostin		
SQSTM1	sequestosome 1		
SSPE	subacute sclerosing panencephalitis		
STAT	signal transducers and activators of transcription		
TBS	tris-buffered saline		
TBS-T	tris-buffered saline with Tween20		
TFGβ	transforming growth factor β		
TNF	tumour necrosis factor		
TNFR1	TNFa receptor		
TRADD	TNFR1-associated via death domain		
TRAF6	TNF receptor-associated factor 6		
TRAP	tartrate resistant acid phosphatase		
UBA	ubiquitin associated		
VCP	valosin-containing protein		
VDR	vitamin D receptor		

WIF	Wnt inhibitory factor
WISP	WNT1 inducible signalling pathway protein

Selected	amino	acid	abbreviations

D	aspartic acid
G	glycine
K	lysine
L	leucine
Р	proline
R	arginine
Х	truncation mutation/stop codon

CHAPTER 1: INTRODUCTION Part A. BONE BIOLOGY

The skeleton is an important organ that serves a number of purposes. Firstly, it has a structural function, providing protection for vital organs, and sites for muscle attachment. It is also important in mineral homeostasis, being a major storage depot for minerals, particularly calcium, which is important in many cellular functions. Finally, the skeleton contains the bone marrow, the site of haematopoiesis in the body. Bone is a dynamic tissue that responds to a variety of mechanical and biological stimuli. These responses are mediated by the bone cells: mesenchymal osteoblasts and osteocytes which form and maintain the bone, and the haematopoietic-derived osteoclasts which break down or resorb bone.

1.1 Macrostructure of bone

The human skeleton can be subdivided into two parts, the axial skeleton, which includes the skull, vertebral column and rib cage, and is particularly important for protecting vital organs, and the appendicular skeleton which includes the limbs. The axial skeleton is composed mainly of flat bones, such as the skull and scapula, while the appendicular skeleton contains long bones such as the femur, tibia and radius. Long bones consist of a hollow tube called the diaphysis, with flared ends known as epiphyses as depicted in Figure 1.1. There are two types of bone, cortical bone, which is very hard and dense, and trabecular or spongy bone which has a more variable and porous structure. All bone is made up of osteons, which are composed of an organic matrix, primarily composed of type I collagen, and mineralised with calcium salts that form hydroxyapatite crystals. The outside of the bone is covered in a fibrous sheet known as the periosteum. The periosteum covers all outer surfaces of the bone apart from at joints, where articular cartilage is present, and contains blood vessels, nerves and bone cells. The inner surface of the cortical bone is covered by the endosteum, a similar membranous sheath that is in direct contact with the bone marrow.

1.1.1 Cortical bone

Cortical bone makes up the outer layer of all bones, and some bones such as the ribs are formed entirely from cortical bone. Approximately 80% of the adult skeleton is cortical bone. The osteons in cortical bone are known as Haversian systems, and contain cylindrical structures, composed of concentric lamellae, with a channel down the centre containing blood vessels and nerves.



Figure 1.1: Macrostructure of a human femur The main features of a long bone are shown. Modified from Martini *et al.* 2003 [1].

1.1.2 Trabecular bone

Trabecular bone is found in the ends of long bones, and in the vertebrae. The proportion of trabecular bone varies considerably between different skeletal sites, comprising around 75% of vertebrae, 50% of the ends of long bones and 5% of the radial diaphysis [2]. Trabecular bone is made up of a lattice-like three dimensional structure resulting in a much higher surface area than cortical bone, and the gaps contain bone marrow.

1.2 Bone formation and modelling

New bone formation is known as modelling, and occurs during childhood growth, or as a response to mechanical stimuli. During bone modelling, bone formation by osteoblasts can

occur without preceding osteoclast bone resorption, so these processes are not coupled tightly as they are during bone remodelling.

1.2.1 Intramembranous ossification

Development of the bones of the skull, and parts of the clavicle occurs via a process known as intramembranous ossification. Elongation of the long bones within the periosteum occurs via a similar, but developmentally distinct process. Intramembranous ossification involves the direct differentiation of mesenchymal cells into osteoblasts. In the skull the sutures supply cell populations which migrate to bone surfaces and differentiate into osteoblasts. The bone expands from primary ossification centres which eventually meet at the sutures [3].



Figure 1.2: Formation and growth of long bones by endochondral ossification

(A) Mesenchymal condensation leads to the development of a cartilage model. (B) Capillaries invade the perichondrium surrounding the future diaphysis transforming it into the periosteum, which triggers chondrocyte differentiation (C), causing chondrocyte hypertrophy and apoptosis associated with mineralisation of the cartilage matrix in the diaphysis. (D and E) Vascular invasion allows the migration of osteoblast precursors that deposit bone on the degraded matrix. Chondrogenesis at the ends of the long bone establishes growth plates. (F) Secondary centres of ossification develop later in foetal life. (G and H) Growth plates serve as a continuous source of cartilage conversion to bone, promoting linear growth. (I and J) Long bones cease growth at the end of puberty, and growth plates are replaced by bone. Articular cartilage remains. Modified from Karaplis, 2002 [3].

1.2.2 Endochondral ossification

In many parts of the skeleton, bone is formed by endochondral ossification, a process that involves synthesis of a cartilage template before bone formation can occur (Figure 1.2). Mesenchymal condensations differentiate into chondrocytes that rapidly produce cartilage structures resembling the final shape of the bone. Chondrocytes in the centre or diaphysial region of the structure eventually become hypertrophic and undergo apoptosis, causing cartilage mineralisation, and allowing vascular invasion. This allows infiltration of cells which differentiate into osteoblasts, and form bone in the degraded cartilage matrix. Later in development, secondary ossification centres form in the epiphysial regions of the bone, leaving the growth plates in between as a site of chondrogenesis. This allows continual formation of a cartilage template for bone elongation until the end of puberty when the growth of long bones ceases, and the growth plate is replaced by bone [3].

1.3 Bone remodelling

In the adult skeleton bone is continually turned over in a process known as remodelling. The skeleton is completely replaced every 11 years, although bone turnover occurs much more quickly in the trabecular bone [4]. Remodelling is a coupled process where bone resorption is closely followed by bone formation, so in a healthy skeleton there is no net loss or gain of bone mass. Bone remodelling occurs in distinct areas by groups of sequentially acting cells known as the basic multicellular unit (BMU). At any one time there are millions of BMUs in the skeleton at different stages, and the duration of BMU activity may be up to eight months [5]. Bone remodelling originates at sites of microdamage, which is likely to involve signals originating from osteocytes, or resulting from osteocyte apoptosis. In cortical bone, remodelling probably occurs exclusively at sites of fatigued or damaged lamellar bone. In contrast, the rate of trabecular bone turnover is much higher than required to maintain the structural integrity of the skeleton, and is important for mineral homeostasis, so the location of many BMU sites is likely to be random [2].

There are four phases of the bone remodelling cycle: activation, resorption, reversal, and formation [2]. A diagram of bone remodelling in a BMU is shown in Figure 1.3. Activation involves recruitment of osteoclast precursors to a previously quiescent bone surface, and their differentiation into multinucleated cells. Resorption occurs once mature osteoclasts are activated by the actions of local cytokines produced by osteoblasts, a process that is regulated by other factors produced locally or systemically such as parathyroid hormone (PTH). Active

resorption by multinucleated osteoclasts lasts for about a week, followed by slower resorption involving mononuclear cells. The reversal phase lasts about a week and involves the recruitment of osteoblast precursors by coupling signals that are not well defined. Release of growth factors and cytokines such as transforming growth factor β (TGF β) and the insulinlike growth factors (IGF1 and IGF2) are likely to be important in this process. Direct signals from the osteoclast to the osteoblast precursors such as bi-directional ephrin signalling are also involved [6]. The formation phase involves two steps: osteoblasts deposit new bone matrix, then regulate its mineralisation. Compared to bone resorption, formation is a slower process that takes a number of months. Mineralisation is initiated about two weeks after bone formation and generally occurs at the same rate as bone formation, although bone continues to accumulate mineral long after formation has ceased. Once bone formation is completed, the majority of osteoblasts undergo apoptosis, however some become buried in the matrix during bone formation and differentiate into osteocytes, while others remain on the surface and become bone lining cells. The net result is the formation of a new osteon.



Figure 1.3: Bone remodelling in a BMU

Once a BMU is initiated, possibly in response to osteocyte apoptosis, bone lining cells retract and osteoclasts are formed and resorb bone. This is followed by a reversal phase, and recruitment of osteoblast precursors which differentiate and replace the resorbed bone with new matrix. Some osteoblasts are embedded in the newly formed bone and become osteocytes, while others become bone lining cells once bone formation is complete. The remainder undergo apoptosis. From roche.com.

1.4 Bone mass

Bones grow throughout childhood until their adult form is reached at the end of puberty. Peak bone mass and strength of the skeleton is attained during the third decade of life. Genetic factors are the most important determinants of peak bone mass in humans. Environmental factors also contribute, including nutrition and body weight, physical activity, and levels of various hormones [7]. Later in life, bone mass begins to decline. Women, in particular, lose bone rapidly after the menopause. There are a number of diseases that affect bone mass. Clinically, reduced bone mass is known as osteopaenia, and more severely reduced bone mass as osteoporosis. Conditions with excessively high bone density are known as osteopetrosis. In the clinic, bone mass can be estimated using bone mineral density (BMD) measurements performed using DEXA scans.

1.5 Bone matrix

Bone makes up the largest proportion of the body's connective tissue, and is unique in being mineralised. The organic, collagen-based matrix provides elasticity and flexibility while the mineral gives the tissue rigidity and strength. The most important and abundant component of the organic matrix is type I collagen, which comprises around 90% of bone protein content. Type I collagen is a triple helical molecule made up of two α 1 chains and an α 2 chain. With appropriate posttranslational modifications, the chains form a left-handed helix [8]. Fibrils form and assemble in a staggered fashion, and inter-molecular cross-links are added to increase the strength of the matrix. Type I collagen is produced as a propeptide, but the ends are rapidly cleaved producing the N-terminal and C-terminal propeptides of collagen (P1NP and P1CP) which can be used clinically as serum markers of bone formation. Additionally, the breakdown products of the inter-molecular collagen cross-links are good clinical markers of bone resorption that can be detected in serum or urine, and include pyridinolines (Pyd), deoxypyridinolines (Dpd), N-telopeptide of collagen cross-links (NTx) and C-telopeptide of collagen cross-links (CTx) [9].

Bone also contains approximately 10% non-collagenous proteins. Although some of these are exogenously derived, mainly from the serum, such as albumin, α_2 -HS-glycoprotein and various growth factors, the majority are synthesised by the osteoblasts [10]. These proteins can be broken down into a number of groups. The individual roles of many of these proteins are not well defined, but they are known to regulate mineralisation and modulate bone cell activity. The first group of non-collagenous proteins produced by osteoblasts are the

proteoglycans, which include biglycan and decorin. These proteins bind to collagen and are thought to be important for the integrity of connective tissue matrices, and for modulating TGF β activity. Biglycan knockout mice have decreased trabecular bone mass indicating that this protein acts as a positive regulator of bone formation [11].

There are also a number of important glycosylated proteins in the matrix. Alkaline phosphatase (bone-liver-kidney isozyme with bone-specific posttranslational modifications) is produced in high levels during bone formation, and serum levels are an accurate clinical marker of bone formation. The function of alkaline phosphatase in bone is controversial, but most is bound to the extracellular surface of osteoblasts, and it is thought to be important for breaking down inhibitors of mineralisation such as pyrophosphates. Mice lacking the alkaline phosphatase gene show an abnormal pattern of calcification [12]. Other glycosylated proteins in the bone matrix include osteonectin, which is the most abundant non-collagenous protein and has a positive role in bone formation, and tenascin C and W, factors that appear to regulate the organisation of the matrix and activity of cells [10].

The third group of non-collagenous proteins are involved in mediating cell attachment to the matrix and are known as small integrin-binding ligand, N-glycosylated proteins (SIBLINGs). These proteins include osteopontin, bone sialoprotein, dentin matrix protein (DMP1) and matrix extracellular phosphoglycoprotein (MEPE). All contain RGD (arginine-glycine-aspartic acid) motifs, the consensus sequence that binds to cell surface integrins. RGD motifs are present in a number of other matrix proteins including type I collagen, fibronectin and osteomodulin, although the reasons for the abundance of integrin binding proteins in the bone matrix is unclear. Osteopontin and bone sialoprotein are involved in anchoring osteoclasts to bone, however they have opposing effects on mineralisation; osteopontin inhibits mineralisation, while bone sialoprotein is able to initiate mineralisation *in vitro*.

The final group of non-collagenous proteins is made up of the three γ -carboxylic acidcontaining (gla) proteins. Matrix gla protein (MGP), and osteocalcin (or bone gla protein) which are produced by osteoblasts, and protein S, which is mainly produced in the liver, but in smaller quantities in osteogenic cells, contain gla residues formed by posttranslational modification by vitamin K-dependent γ -carboxylases. MGP is found in many connective tissues and is an inhibitor of mineralisation. Osteocalcin is expressed by mature osteoblasts and osteocytes, and osteocalcin deficiency in mice results in increased bone mineral density [13]. Recent evidence also suggests that osteocalcin in its uncarboxylated state acts as a hormone in energy metabolism, with knockout mice showing a phenotype similar to type 2 diabetes [14].

1.5.1 Mineralisation

The mineralisation of bone occurs after the organic matrix is deposited, and mineral accounts for 50-70% of the mass of adult bone [10]. The mineral crystals are similar in composition to geological hydroxyapatite, composed of calcium, phosphate and hydroxide, although they are much smaller, and contain numerous impurities [10]. Mineralisation begins in the gaps between collagen fibrils, and as the bone matures the crystals become larger and more pure. Nucleation of crystal formation is catalysed by osteoblastic cells, and requires accumulation of calcium and phosphate ions, proteins and acidic phospholipids as well as breakdown of factors that inhibit mineralisation (such as ATP and pyrophosphate). Crystal growth is restricted by the amount of space available in the collagen matrix, and is modulated by various non-collagenous proteins which can alter the size and shape of the crystals formed. Other ions such as magnesium, strontium and carbonate can also be incorporated into bone mineral. BMD correlates well with bone strength, and is used extensively in the clinic to predict fracture risk, however crystal size and composition is also important; for example, skeletal fluorosis causes formation of excessively large mineral crystals resulting in more brittle bones.

1.6 Bone cells

1.6.1 Osteocytes

Osteocytes are the most abundant cell in bone, although they have not been as well characterised as osteoblasts or osteoclasts. Osteocytes are terminally differentiated osteoblasts, and are located in lacunae within the bone matrix. They have processes that extend in all directions through channels in the matrix known as canaliculi which connect them to neighbouring osteocytes, and in some cases to cells on the bone surface, forming an extensive three dimensional network (Figure 1.4). Osteocytes communicate with other cells through connexin 43-containing gap junctions, which are essential for osteocyte activity and survival [15].

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Figure 1.4: Osteocyte cells and networks

Scanning electron microscopy images of isolated osteocytes after 5 minutes (A), 30 minutes (B) or 24 hours (C, D) in culture. Cells immediately begin to form cytoplasmic processes. The immunobeads used for separation have not been removed in C. Scale bars show 10 μ m. E shows the canalicular network in murine cortical bone. The canilicular system was filled with resin, and then mineral was removed. Note that the canaliculi connect the osteocyte lacunae with the bone surface at the bottom of the image. A-D reproduced from Nijweide *et al.* 2002 [16], E reproduced with permission from Bonewald, 2008 [17].

Osteocytes function primarily the bone's mechanostat. They sense changes in fluid movement through their lacunae, and respond by altering production of various bone active factors including nitric oxide, prostaglandin E_2 (PGE₂) and IGF1, or by undergoing apoptosis [18]. Osteocytes express a number of matrix proteins including osteocalcin. More osteocyte-specific genes include DMP1 and MEPE, as well as sclerostin (SOST), the Wnt signalling inhibitor thought to play an important role in osteocyte-mediated control of bone formation. Osteocytes also produce fibroblast growth factor (FGF) 23, a vital modulator of phosphate metabolism, which reduces both renal phosphate reabsorption and production of 1,25

dihydroxyvitamin D_3 (1,25(OH)₂ D_3). FGF23 expression is regulated by DMP1 and Phex, and deletion of these genes in mice or mutations in humans results in excessive FGF23 production by osteocytes leading to hypophosphataemic rickets.



Figure 1.5: The mesenchymal cell lineage

Osteoblasts differentiate from mesenchymal progenitor cells that also give rise to myocytes, under the control of MRFs and MEF2, to adipocytes under the control of C/EBP α , β and δ and PPAR γ , and to chondrocytes under the control of SOX5, -6 and -9 and STAT1. RUNX2 is essential for osteoblast differentiation and is also involved in chondrocyte maturation. Osterix (Osx) acts downstream of RUNX2 to induce formation of mature osteoblasts that express osteoblast markers, including osteocalcin (OC). Abbreviations: MRFs, myogenic regulatory factors (including MyoD, myogenin, myogenic factor 5 and myogenic regulatory factor 4); MEF2, myocyte-enhancer factor 2; C/EPB, CAAT/enhancer-binding protein; PPAR γ , peroxisome proliferator-activated receptor γ ; Col-I/II/X, type I/II/X collagen; STAT, signal transducers and activators of transcription; Ihh, Indian hedgehog; Fra, fos-related antigen; BSP, bone sialoprotein. Reproduced with permission from Harada and Rodan, 2003 [19].

1.6.2 Osteoblasts

Osteoblasts are the bone forming cells that are derived from mesenchymal stem cells. These progenitors can also differentiate into various other cell types including adipocytes, chondrocytes, fibroblasts and myoblasts (Figure 1.5). Osteoblasts form from osteoprogenitors, highly proliferative cells committed to the osteoblast lineage. Immature osteoprogenitors express the STRO-1 surface marker, and RUNX2. As they mature, osteoprogenitors begin to express type I collagen and alkaline phosphatase, as well as transiently expressing bone

sialoprotein. Preosteoblasts have limited proliferative capacity, but cannot produce functional bone matrix. They no longer express STRO-1, but have increased expression of alkaline phosphatase, and begin to express bone sialoprotein, osteopontin and osteocalcin.

Mature osteoblasts are post-proliferative cuboidal cells located at areas of bone formation that produce various matrix proteins in addition to selected cytokines, hormone receptors and growth factors [20]. They show increasing expression of *RUNX2*, bone sialoprotein and osteocalcin, and continue to express high levels of collagen type I. Osteoblastic cells can produce mineralised matrix *in vitro*; generally this requires a number of weeks culture in the presence of ascorbic acid and a source of phosphate. While the main function of the osteoblast is bone formation, they also play a vital role in the control of osteoclast differentiation and activity, which will be discussed later. Osteoblast differentiation is influenced by a number of important signalling cascades including the Wnt/ β -catenin pathway, bone morphogenetic protein (BMP) signalling, and a number of important transcription factors, including RUNX2, osterix, and DLX5 [15]. A schematic diagram showing the stages of osteoblast differentiation and some of the factors that are important in this process is shown in Figure 1.6.

1.6.2.1 Wnt signalling

Canonical Wnt signalling (or Wnt/ β -catenin signalling) is an important developmental pathway, that also has functions in adult cells. In the absence of Wnt signalling β -catenin is phosphorylated by glycogen synthase kinase (GSK)-3 β and targeted to the proteasome where it is degraded. The canonical pathway is activated when a Wnt molecule binds to its receptors Frizzled and low density lipoprotein receptor-related protein (LRP)5/6, inhibiting GSK-3 β , and allowing accumulation and nuclear translocation of β -catenin. In the nucleus, β -catenin binds to the Tcf/Lef transcription factors to induce transcription of target genes (Figure 1.7). The discovery that mutations in *LRP5* cause bone diseases in humans highlighted the importance of this signalling pathway in bone metabolism. Inactivating mutations in *LRP5* cause recessive osteoporosis-pseudoglioma syndrome due to reduced Wnt signalling [21]. Conversely, specific mutations in the β -propeller domain of the protein cause a dominant high bone mass phenotype due to reduced binding of Wnt signalling inhibitors dickkopf 1 (DKK1) and SOST [22-25].

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Figure 1.6: Osteoblastic cell differentiation

This schematic model shows mesenchymal stem cell differentiation towards the osteoblastic lineage, and the effect of transcription factors on this process. Abbreviations: NFAT, nuclear factor of activated T cells; NFAT2 is also known as NFATc1; ATF, activating transcription factor. Modified with permission from Krause *et al.* 2008 [26].





Figure 1.7: Wnt/β-catenin signalling pathway

In the unliganded state (left) β -catenin is phosphorylated by GSK3 and degraded. When Wnt ligands bind their receptors (right), GSK3 is inactivated through mechanisms involving Axin, Frat-1, and Dishevelled (Dsh). β -catenin accumulates in the cytoplasm, then translocates to the nucleus where it binds to TCF/LEF, causing displacement of transcriptional corepressors (e.g., silencing mediator of retinoid and thyroid receptors, SMRT and nuclear receptor corepressor, NCoR) with transcriptional coactivators (e.g., p300 and cAMP response element–binding protein, CBP). Wnt signalling can be blocked by interactions of Wnt with inhibitory factors including Wnt inhibitory factor 1 (WIF-1) and the secreted frizzled-related proteins (sFRP), or the interaction of LRP5/6 with the Dkk/Kremen complex or SOST. Modified from Krishnan *et al.* 2006 [27].

During embryonic development, active Wnt signalling increases bone mass by renewal of stem cells, stimulation of preosteoblastic replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis [27]. β -catenin is vital for early osteoblastogenesis, and loss of β -catenin in mesenchymal cells in mice leads to the development of cartilage-only skeletons [28]. In contrast, excessive Wnt signalling (via stabilised β -catenin) prevents skeleton formation completely, probably because chondrocytes do not develop [28].

In the adult, β -catenin activity causes mesenchymal stem cells to enter an osteogenic pathway at the expense of adipocytes, and Wnt signalling can inhibit osteoclastogenesis by altering gene expression in osteoblasts [29, 30]. On the other hand, *in vitro* studies have shown that inhibition of Wnt signalling is required for terminal differentiation and mineralisation of osteoblasts, and inhibitors of Wnt signalling such as DKK1 and DKK2 are up-regulated during osteoblast differentiation [31-33]. There is also evidence that the β -catenin/LEF1 complex can interact with RUNX2, preventing RUNX2-induced transcription [34]. Overall, Wnt/ β -catenin signalling is necessary for bone modelling, and osteoblast formation, and appears to be a vital signal for commitment of osteoblast precursors.

1.6.2.2 BMP signalling

The bone morphogenetic proteins BMP2, BMP4, and BMP7 belonging to the TGF β superfamily are potent inducers of osteogenesis [35]. Binding of BMPs to the specific BMP receptor complex stimulates phosphorylation of the intracellular receptor Smads (R-Smads; Smad-1, -5, or -8), which bind to Smad-4 or other transcription factors to regulate gene expression. The BMPs also activate mitogen activated protein kinase (MAPK) signalling. BMP activity is regulated within the cell by the inhibitory Smads (I-Smads), and by extracellular antagonists including noggin and chordin [15]. The BMPs are important in embryogenesis, and BMP null mutant mice tend to show early embryonic lethality [36]. They are important in the adult skeleton too, inducing osteoblastic differentiation, expression of transcription factors crucial for osteogenesis including RUNX2, osterix and DLX5 and enhancing osteoblast activity [36]. BMPs have been shown to improve healing in fractures [37]. The importance of BMP signalling in bone formation is demonstrated in patients with the rare disorder fibrodysplasia ossificans progressiva, which is characterised by progressive ossification of soft tissue. This disease is caused by mutations in the *ACVR1* gene, a BMP

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receptor, which allow spontaneous BMP signalling without the presence of a ligand [38]. Injection of BMPs into soft tissue can also cause ectopic bone formation.

1.6.2.3 RUNX2

The transcription factor RUNX2 (also known as Cbfa1) is regarded as a 'master switch' gene in osteoblast formation. *Runx2* deficient mice die shortly after birth due to a complete lack of intramembranous and endochondral ossification [39]. RUNX2 induces transcription by binding to the RUNX consensus sequence (known as OSE2), which is found in all the major osteoblast-specific genes, including collagen type I alpha 1, osteopontin, bone sialoprotein and osteocalcin [40]. It also interacts with several other regulatory proteins to activate or suppress other genes important for osteoblast differentiation. However, down-regulation of *RUNX2* is necessary for terminal differentiation of osteoblasts, and mice over-expressing the gene are osteopaenic due to reduced numbers of mature osteoblasts [40]. RUNX2 activity is tightly controlled, and regulated by a large number of factors. Repressors and inhibitors include the tumour suppressor p53, HOXA2 which inhibits *RUNX2* expression, and STAT1 and SOX9 which reduce its activity. Co-activators include core binding factor $\beta1$ and C/EBP β , and RUNX2 functions synergistically with the BMP-responsive Smads.

1.6.2.4 Osterix

Osterix is an osteoblast-specific zinc finger transcription factor that functions downstream of RUNX2. *Osx*-deficient mice do not form bone and lack osteoblasts indicating the critical role of this transcription factor in osteoblast differentiation, although they do still express *Runx2* [41]. The mechanisms of action and regulation of osterix are not well understood. It directs the maturation of pre-osteoblasts, and can form a complex with NFATc1 resulting in type I collagen transcription. Similar to RUNX2, p53 represses osterix transcription [40].

1.6.2.5 Other transcription factors

There are a number of other transcription factors that play important roles in osteoblast differentiation, but are not absolutely critical for bone formation. ATF4 has a positive role in osteoblast development and can interact with RUNX2 to regulate osteocalcin transcription. The AP1 transcription factor is another important regulator of bone formation. AP1 is a heterodimer of proteins from the Fos and Jun families. Several homeobox proteins influence osteoblast differentiation. MSX2 promotes proliferation and differentiation of osteoprogenitors, but inhibits RUNX2 activity and osteoblast gene expression in more mature

osteoblasts, and is down-regulated during osteoblast differentiation [42]. DLX5, on the other hand, shows increased expression during osteoblast differentiation, and activates RUNX2, bone sialoprotein and osteocalcin expression [43]. The C/EBPs are also important transcription factors in osteoblasts, and loss of C/EBPs in mice results in decreased bone formation and osteopaenia. The main competing pathway for mesenchymal stem cells within the bone microenvironment is adipogenesis, so the master transcription factor of adipogenesis, PPAR γ has the ability to antagonise RUNX2.

1.6.3 Osteoclasts

Bone resorbing osteoclasts are giant multinucleated cells of haematopoietic origin that form by differentiation and fusion of monocyte precursors. Osteoclasts have a number of features that assist with identification *in vitro* and *in vivo*. They generally contain 3-20 nuclei, and express high levels of tartrate-resistant acid phosphatase (TRAP), which can be utilised to stain the cells. Active osteoclasts also attach to bone with a ruffled border, and contain fibrillar actin rings (Figure 1.8).



Figure 1.8: Transmission electron microscopy image of a primary rat osteoclast on bone The cell shows strong adherence, an extensive ruffled border, and partial matrix degradation between the sealing zones. Reproduced with permission from Ross, 2008 [44].

Osteoclastogenesis requires the activity of the cytokines macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) which are both produced by osteoblast precursors or stromal cells [45]. Both are membrane bound factors, meaning osteoclastogenesis *in vivo* requires cell-cell contact between the osteoclast precursors and stromal cells. There are also active soluble forms of both proteins produced by sheddase enzymes. Osteoclastogenesis can be stimulated *in vitro* by culturing osteoclast

precursors with soluble RANKL and M-CSF for one to two weeks. Alternatively they can be produced in bone marrow or co-culture environments where stromal or osteoblastic cells produce the RANKL and M-CSF required. M-CSF is the factor missing in the osteopetrotic *op/op* mice, and is important for proliferation, survival and differentiation of osteoclast precursors [46]. It binds to its receptor, c-Fms, on the osteoclast precursors, which recruits c-Src and phosphatidylinositol-3 kinase (PI3K), stimulating the Akt signalling pathway which promotes proliferation in osteoclast precursors, and survival.

RANKL is a member of the tumour necrosis factor (TNF) superfamily, and, in the presence of M-CSF is both necessary and sufficient for osteoclast differentiation and activity [47, 48]. Rankl deficient mice display severe osteopetrosis, and completely lack osteoclasts [49]. The discovery of RANKL as the elusive osteoclast differentiation factor was preceded by the discovery of its decoy receptor, osteoprotegerin (OPG). OPG is an unusual member of the TNF superfamily because it does not contain a transmembrane domain, but is secreted by cells, particularly osteoblasts, and binds strongly to RANKL, preventing its activity. Opg deficient mice develop severe osteoporosis due to increased osteoclast formation and activity [50, 51]. The receptor for RANKL, which is expressed on osteoclasts and their precursors is RANK. Rank knockout mice have a similar phenotype to Rankl knockout mice, involving severe osteopetrosis with no osteoclasts present [52]. RANKL binding to RANK causes receptor trimerisation, and stimulates signalling through nuclear factor kappa B (NF κ B), the three MAPK pathways and PI3K [53]. The adaptor protein TNF receptor-associated factor 6 (TRAF6) is important in this process. One of the transcription factors activated by this signalling is NFATc1, which is a major regulator of osteoclast differentiation. The AP1 complex made up of c-Fos or Fra1 and c-Jun is also important in osteoclast differentiation and is active upstream of NFATc1. A simplified diagram of RANKL/RANK/OPG control of osteoclastogenesis is shown in Figure 1.9.

There are a large number of factors that influence osteoclast formation and activity. Granulocyte macrophage colony stimulating factor (GM-CSF) and vascular endothelial growth factor can play similar roles to M-CSF in osteoclast formation, and compensation by these factors allows phenotype resolution in the *op/op* M-CSF deficient mice as they age. A number of pro-inflammatory cytokines enhance osteoclastogenesis and activity, particularly TNF α and interleukin (IL) 1, which can stimulate osteoclastogenesis *in vitro* in the absence of RANKL. Many other factors affect osteoclastogenesis, both directly by stimulating pathways

such as NF κ B that are important in the osteoclast, and indirectly through modulating RANKL and OPG expression in stromal cells.



Figure 1.9: Schematic showing the roles of RANKL and OPG in osteoclastogenesis RANKL expressed on the membrane of osteoblastic cells stimulates osteoclast differentiation and activity by binding and activating its receptor RANK on the osteoclast lineage cells. This stimulates various signalling cascades, in particular the NF κ B pathway. Osteoblasts also secrete OPG, which blocks the activity of RANKL, thereby inhibiting osteoclast differentiation and activity.

The main function of the osteoclast is bone resorption. This occurs by creation of a microenvironment suitable for bone degradation. The osteoclast attaches to bone using adhesion molecules called integrins, particularly $\alpha\nu\beta$ 3 integrin, which attaches to RGD motifs found in proteins incorporated into the bone. Attachment results in polarisation of the cell, and transport of acidified vesicles to the bone surface causing the formation of a ruffled border, a structure unique to osteoclasts. A 'sealing zone' forms around the acidified area where resorption is taking place. Osteoclasts produce an acidified environment, and secrete proteases in order to facilitate bone resorption. Within the cell, carbonic anhydrase converts carbon dioxide and water to a proton and bicarbonate, and the bicarbonate ions are exchanged for chloride at the non-resorbing surface to maintain intracellular pH. The extracellular

resorbing zone around the ruffled border is acidified to around pH 4.5 by the activity of proton pumps and chloride channels. This mobilises the mineral, exposing the organic component of the matrix, which is degraded by cathepsin K and other matrix metalloproteinases (MMPs) (Figure 1.10). The proton and chloride channels, cathepsin K, and the $\alpha\nu\beta3$ integrin are all vital for proper osteoclast activity, and loss of function of these genes in either human diseases or mouse models results in excess bone mass [45].



Figure 1.10: The mechanism of osteoclastic bone resorption The osteoclast adheres to bone through the integrin $\alpha\nu\beta3$, creating a sealing zone. Hydrochloric acid is produced by the combined actions of vacuolar proton pump, chloride channels, and a chloride-bicarbonate exchanger. Acid and acidic proteases such as cathepsin K (Cath K), MMP9 and MMP13 are secreted into the area enclosed by the sealing zone to facilitate resorption. Reproduced with permission from Ross, 2008 [44].

Osteoclasts have a fairly limited lifespan, and undergo apoptosis once their activity is complete. They do, however, have some impact on osteoblast recruitment and activity, before undergoing programmed cell death. While osteoblastic cells have long been known to produce factors necessary for osteoclastogenesis, the importance and mechanisms of osteoclast-osteoblast communication are less certain. However, the fact that osteoblast activity directly follows bone resorption within the BMU suggests that important coupling mechanisms exist, some of which have been identified and mentioned earlier.

1.7 Hormones and growth factors involved in bone metabolism

1.7.1 Parathyroid hormone and parathyroid hormone related protein

PTH is an 84 amino acid systemic hormone produced by the parathyroid gland. It plays a central role in calcium homeostasis through its actions in bone and kidney, and indirectly in the intestine via its influence on renal vitamin D hydroxylation. The main regulator of PTH excretion is extracellular calcium. Calcium sensing receptor-mediated signalling increases PTH secretion when calcium levels are low, and decreases PTH when calcium levels are high.

Increased phosphate or decreased 1,25(OH)₂D₃ levels also increase PTH levels. The overall effects of PTH are to increase serum calcium and decrease serum phosphate. Its effects are mediated mainly through the PTH/PTH related protein (PTHrP) receptor, a G protein-coupled receptor that, when activated, signals mainly via cyclic adenosine monophosphate (cAMP) production [54]. PTH has complex effects on bone, which vary depending on the site and mode of administration. PTH releases calcium stores into the serum by stimulating osteoclast differentiation and activity. Therefore, if PTH levels are chronically elevated like in hyperparathyroidism, or if it administered continuously, the effect on bone is catabolic. Increased osteoblast RANKL and M-CSF expression, and decreased OPG expression in response to PTH exposure cause this effect. Conversely, when PTH is administered intermittently it leads to increase osteoblast numbers *in vivo*, probably due to decreased apoptosis, and inhibition of adipogenesis, and it also stimulates osteoblast differentiation [54, 55]. Full length PTH or the N-terminal end of the peptide, PTH(1-34) (teriparatide), are the only anabolic drugs available as a treatment for osteoporosis [56].

PTHrP has sequence similarities with PTH, and can act through the same receptor, but appears to have different functions *in vivo*. PTHrP is expressed in a wide range of tissues and acts mainly in an autocrine and paracrine manner. It plays an important role in maintaining the growth plate during endochondral bone growth. Osteoblast-specific ablation of PTHrP expression results in osteopaenia in adult mice, suggesting that it positively regulates bone formation. Since it functions through the same receptor, many of the effects of PTHrP administration are similar to PTH administration [57].

1.7.2 Vitamin D

Vitamin D is a steroid pre-hormone synthesised in the skin during exposure to sunlight. It requires successive hydroxylations in the liver and kidney to be converted to its most biologically active form, $1,25(OH)_2D_3$. In combination with PTH, it maintains serum calcium concentrations by altering the intestinal absorption of calcium, and by mobilising calcium from the bone. It also plays important roles in the immune system, cancer prevention and cardiovascular health.

 $1,25(OH)_2D_3$ exerts its effects by binding to the vitamin D receptor (VDR) in the cytoplasm, which triggers nuclear translocation, and binding to the retinoic X receptor. This complex

binds to vitamin D response elements to modify transcription of $1,25(OH)_2D_3$ responsive genes. $1,25(OH)_2D_3$ mobilises calcium from the bone by up-regulating *RANKL* expression in osteoblasts, although it has other gene targets in osteoblasts including alkaline phosphatase, osteocalcin and osteopontin. It has an antiproliferative, and pro-differentiation effect on many cell types [58]. $1,25(OH)_2D_3$ is often used to stimulate osteoclastogenesis in *in vitro* bone marrow or co-culture systems, which produce very few osteoclasts in normal culture media. The actions of $1,25(OH)_2D_3$ on intestinal calcium absorption are critical for ensuring sufficient calcium in the blood for proper mineralisation of bone. $1,25(OH)_2D_3$ deficiency can cause rickets, the failure of or delayed mineralisation of newly formed bone at the growth plate, and osteomalacia, failure of mineralisation of newly formed osteoid at sites of bone turnover. Rickets can cause various bone deformities, and although these conditions are readily preventable, they are still fairly common, particularly in dark-skinned people living at extreme latitudes.

1.7.3 Oestrogen and sex hormones

Oestrogen is the major female sex hormone, which exerts its actions through the intracellular oestrogen receptors, ER α and ER β , that function in a similar manner to the VDR. These receptors are expressed in bone cells, albeit at much lower levels than in reproductive tissues. Oestrogen is important for maintenance of bone mass in both females and males. The majority of its actions reduce bone resorption, however it also has positive effects on bone formation [59]. The loss of oestrogen due to menopause or ovariectomy results in loss of both cortical and trabecular bone due to increased bone turnover [60]. Oestrogen up-regulates expression of TGFB, IGF1 and type I collagen in osteoblasts, and plays a role in cytokine production ensuring lowered expression of osteoclastogenic cytokines such as TNFa, IL-1 and IL-6, and increased OPG production. It also promotes osteogenesis by increasing osteoclast apoptosis, but inhibiting osteoblast apoptosis. Other direct effects of oestrogen on osteoclasts include inhibition of differentiation, and reduced cytokine sensitivity. Since hormone replacement therapy increases the risk of breast and uterine cancer, there has been great interest in developing selective oestrogen receptor modulators (SERMs). These compounds antagonise oestrogen in reproductive tissue, while acting as receptor agonists in other non-classical target tissues such as bone and the cardiovascular system [61]. One such compound, raloxifene, is used as a drug to treat post-menopausal osteoporosis. While the effects of oestrogen are the most important and most pronounced of the sex hormones with
respect to bone, progesterone and androgens have positive effects as well. The importance of androgens is highlighted in hypogonadal men who are prone to osteoporosis [62].

1.7.4 Glucocorticoids

The stress hormone cortisol stimulates differentiation and activity of osteoblasts and osteoclasts at physiological doses, however, when glucocorticoids are administered at therapeutic doses they have negative effects on bone. Common derivatives of cortisol such as prednisone or dexamethasone are used clinically to treat a wide range of inflammatory diseases including asthma, inflammatory bowel disease, and skin diseases. Histomorphometry in patients treated with high dose glucocorticoids indicates increased osteoblast and osteocyte apoptosis, decreased osteoblast function and increased BMU activation frequency. At physiological doses the *in vitro* effects on osteoblasts include decreased proliferation, increased differentiation, and increased apoptosis, as well as increased sensitivity to some anabolic hormones. At higher doses similar effects are seen on proliferation and apoptosis, but bone formation is inhibited. Glucocorticoids modify gene expression in osteoblasts including increasing RANKL and decreasing OPG expression, decreasing collagen and osteocalcin production, and increasing alkaline phosphatase and bone sialoprotein expression. Indirect effects of these hormones that also affect bone metabolism include increased PTH secretion and sensitivity, inhibition of sex hormone synthesis and reduced calcium and phosphate absorption in the intestine [63].

1.7.5 Transforming growth factor β

The TGF β family contains four isoforms, with TGF β 1 being predominantly expressed in bone. It is incorporated into the bone matrix, and the quantity in bone can change, for example bones from ovariectomised or 1,25(OH)₂D₃-deficient rats have less TGF β than healthy rats. TGF β is considered a potential osteoclast-osteoblast coupling factor within the BMU, and has chemotactic properties for a number of cell types including osteoblast precursors. TGF β has variable effects on osteoblast proliferation depending on differentiation stage, but stimulates matrix formation, and inhibits mineralisation. It induces expression of collagen and a number of other matrix proteins, while repressing expression of several proteases and osteocalcin. *In vivo* TGF β induces bone formation if injected near bone (but not ectopically like BMPs). It signals through ubiquitously expressed TGF β receptors, of which there are three types. Many of its effects are mediated by phosphorylation of R-Smads -2 and -3 which combine with

Smad-4 to stimulate transcription. TGF β is unique among growth factors in that it is secreted as a latent form that requires activation by proteolytic cleavage [64].

1.7.6 Other growth factors

The insulin-like growth factors IGF1 and IGF2 are important anabolic autocrine/paracrine factors in bone. These 7 kDa polypeptides circulate at high concentrations in the serum, and are produced by bone cells and incorporated into the bone matrix. Like TGF β , the amount of IGF1 and IGF2 incorporated into the matrix varies according to external factors. Expression is modulated by many factors, for example, IGF1 expression is up-regulated by PTH, oestrogen and mechanical loading, and down-regulated by glucocorticoids. The effects of IGFs on osteoblasts include increased proliferation and collagen synthesis, and increased function of mature osteoblasts. Although they stimulate osteoclast formation and activity, the overall effect is anabolic, demonstrated by increased bone mineral density in mice over-expressing IGF1 in osteoblasts. The effects of IGFs are modulated by the IGF binding protein family, which contains six members. All IGF binding proteins can bind and sequester IGFs preventing their activity, however some of these proteins facilitate increased IGF activity once they have undergone appropriate posttranslational modification.

The fibroblast growth factors also affect bone metabolism. There are 23 FGFs, but FGF1 and FGF2 are the most widely expressed and best characterised. The main FGF receptors are FGFR1-3 and activating mutations in these receptors demonstrate the importance of appropriate FGF action during skeletal development: mutations in FGFR3 cause achondroplasia, a form of dwarfism, and mutations in all the receptors cause a number of diseases characterised by craniofacial defects [65]. The *in vitro* effects of FGFs include increased osteoblast proliferation and apoptosis, and reduced expression of differentiation markers, as well as increased TGF β and decreased IGF1 expression. FGFs are also capable of stimulating osteoclastogenesis and bone resorption, at least partly as a result of increased PGE₂ production which stimulates RANKL expression. *In vivo* FGFs increase bone formation, and *Fgf2* knockout mice have decreased trabecular bone mass [65]. FGF2 is likely to be important in fracture repair.

Platelet derived growth factor (PDGF) also plays a role in bone metabolism, increasing osteoblast proliferation, but inhibiting differentiation. It also increases osteoclast resorption, and appears to assist fracture repair [66].

1.8 Common disorders of bone metabolism

1.8.1 Osteoporosis

Osteoporosis is a condition characterised by low bone mass and deteriorating bone microarchitecture, resulting in an increased risk of fragility fracture, and is a major public health issue. The World Health Organisation defines osteoporosis as BMD more than 2.5 standard deviations below the young adult mean. Epidemiological data suggest that one in two women, and one in five men over 50 will have an osteoporotic fracture in their remaining lifetime [67]. Hip fractures are particularly problematic, as they require hospitalisation, and are associated with increased mortality and significant morbidity. Vertebral fractures are also common, although they are often asymptomatic, so do not have such dramatic effects on quality of life.

Osteoporosis occurs as a consequence of inadequate bone accumulation during young adult life (low peak bone mass), or as a result of accelerated bone loss. Bone loss due to ageing is normal, and occurs particularly markedly in women in the first five years after menopause due to oestrogen deficiency. At this stage women lose about 3% of their trabecular spinal bone mass annually, slowing to a more generalised bone loss at similar rates to men (about 0.5% per year) [7]. Other factors that cause age-related bone loss include increased PTH levels, osteoblast senescence and decreased physical activity. Major risk factors for osteoporosis and fractures include personal or family history of fragility fractures, low body weight, smoking and long-term glucocorticoid therapy [68].

There are a number of treatments that can reduce bone loss in osteoporosis. Nutrition is important to maintain bone mass, so adequate intake of calcium and protein, and sun exposure to ensure adequate vitamin D levels are important, and supplementation may be necessary. Calcium and vitamin D supplementation result in modest slowing of bone loss, and possibly reduce fracture risk [69], although they are not considered sufficient treatment for severely osteoporotic patients. Most treatments available for osteoporosis are antiresorptive therapies that inhibit osteoclast activity and bone destruction, but have little or no anabolic effect, meaning they halt bone loss but do not restore bone that has already been lost. The major class of antiresorptive drugs is the bisphosphonates. Other drugs that may be used are calcitonin, the SERM raloxifene, and strontium ranelate. Another promising antiresorptive is a RANKL monoclonal antibody, denosumab, which has recently completed phase three clinical trials. The only anabolic drugs currently available for osteoporosis treatment are PTH preparations, however these drugs are very expensive and require daily injections, so are only recommended in patients with severe osteoporosis and very high fracture risk.

1.8.2 Hyperparathyroidism

A number of disorders can cause chronic increases in circulating PTH, potentially causing bone loss. PTH-induced bone loss is distinct from oestrogen-deficiency related bone loss because it occurs mainly in the cortical appendicular skeleton rather than the axial trabecular components [70]. Primary hyperparathyroidism is a common cause of hypercalcaemia, and is most commonly caused by adenoma of the parathyroid gland leading to excessive PTH secretion [71]. Bone disease associated with this condition can be corrected by removing the affected parathyroid tissue. Secondary hyperparathyroidism results from hypocalcaemia which is most commonly caused by renal failure or vitamin D deficiency [70]. Tertiary hyperparathyroidism is characterised by hypercalcaemia, and is caused by autonomous parathyroid hyperfunction after prolonged secondary hyperparathyroidism [70].

1.8.3 Cancer and bone

There are a number of neoplasms of bone cells, although they are rare, and generally more common in children. Benign tumours include nonossifying fibroma, osteochondroma and giant cell tumour of bone. The most common primary malignancy of the skeleton is osteosarcoma, and there are other rarer sarcomas [72]. Multiple myeloma is an incurable malignancy of B cells in the bone marrow that generally affects middle aged patients, and has a very poor prognosis. Multiple myeloma bone disease occurs in 70-80% of patients, and is associated with lytic lesions that can cause severe bone pain and fractures [73]. Multiple myeloma cells produce a number of factors that stimulate increased osteoclast activity both directly and indirectly, including RANKL, macrophage inflammatory protein 1a and IL-6. They also produce factors that inhibit osteoblast activity, in particular by inhibiting the Wnt signalling pathway with factors like DKK1 and sFRP-2, as well as IL-3 and IL-7, which also inhibit osteoblast differentiation [73, 74]. The increased bone resorption releases growth factors from the bone that contribute to survival and growth of the cancer. High-dose bisphosphonates are used to treat multiple myeloma and other lytic bone diseases, and are effective at reducing skeletal complications, in addition to increasing myeloma cell apoptosis nearby. However, they do not improve survival rates, and skeletal complications are still common in patients receiving this treatment.

Metastatic bone tumours are 25 times more common than primary bone malignancies, and bone is the third most common site for solid tumour metastasis [72, 75]. Bone metastases are particularly prevalent in breast cancer, and prostate, lung and renal cancers also frequently metastasise to bone. Cancer cells disrupt bone cell coupling resulting in the formation of lesions which can be osteolytic or osteoblastic. Osteolysis is common in breast cancer metastases, and tumour-derived PTHrP is often an important factor driving osteoclastogenesis, along with other cytokines. Similar to multiple myeloma, inhibition of Wnt signalling with factors like DKK1 can reduce osteoblast activity, and release of growth factors from the skeleton can perpetuate the disease [75, 76]. Osteoblastic lesions are particularly characteristic of prostate cancer metastases, and a major mediator of this is endothelin 1, which appears to stimulate Wnt signalling [75, 77].

Part B. PAGET'S DISEASE OF BONE

Paget's disease of bone is the second most common bone disease after osteoporosis and is characterised by focal lesions where excessive and disordered bone remodelling occurs. It is named after James Paget, who first thoroughly described the disease in 1877 [78]. Paget described five cases, and cited three further cases from the literature, all of whom suffered pain and increasing deformity, particularly in the legs, and two subsequently died from osteosarcoma (Figure 1.11). He concluded that the disease was probably a result of chronic inflammation, and named it osteitis deformants.



Figure 1.11: Sketch of a patient with Paget's disease This is one of the original patients described by James Paget. The left leg shows considerable bowing as a result of disease activity. From Paget, 1877 [78].

1.9 Epidemiology

Collecting epidemiological data on Paget's disease has been problematic as it is often asymptomatic. Studies have used stored radiographs or autopsy data. Paget's disease of bone is rare in people under the age of 40, and prevalence increases with age. The disease is most common in Europe, North America and Australasia [79]. England has the highest rates of

disease in Europe, and there are different levels of prevalence around the country. Prevalence among hospital patients aged 55 years and over in the Lancashire region ranged from 6.3 to 8.3%, while the nationwide average was about 5% (Figure 1.12) [80]. There appears to be no relationship between prevalence and environmental factors such as industrialisation or latitude. In New Zealand the prevalence is thought to be about 3% in the population over 55 years of age [79, 81].



Figure 1.12: Variable prevalence of Paget's disease in the UK Age- and sex-standardised prevalence rates (%) of Paget's disease among hospital patients aged over 55 years in 31 British towns showing markedly increased prevalence in Lancashire (shaded). Prevalence was determined based on surveys of stored radiographs performed in 1974. Reproduced with permission from Barker *et al.* 1980 [80].

Paget's disease mainly affects white people, although it is seen in a significant proportion of blacks in the USA, South Africa and Jamaica, but is less frequent in black African populations. It is extremely rare in Nordic countries, the Arab Middle East, China and Japan [4]. Paget's disease is generally noted to be slightly more common in men than women [4, 79].

The prevalence and severity of the Paget's disease appears to be decreasing. A study of patients in Auckland showed that while the number of referrals increased between 1973 and 1993, the average age at diagnosis increased by nine years. People born later tended to have less extensive disease, and the number of patients presenting with very high alkaline phosphatase levels decreased [82]. An extension of the New Zealand study through to 2002 showed the severity of disease has continued to decrease, while referral numbers are also declining [83]. Additionally, there are also data suggesting declining disease prevalence in the UK and Europe, and deaths attributed to Paget's disease in the UK and USA have decreased over time [84, 85]. A recent study in New Zealand families with Paget's disease and sequestosome 1 (*SQSTM1*) mutations indicated that of offspring who inherited the mutation, many had no indication of disease (in their 40s and 50s) and those who had developed the disease were diagnosed at a later age, and had less severe disease than their parents [86].

There is evidence of Paget's disease dating as far back as AD 900, with skeletons of Anglo-Saxon origin showing classic features of the disease [4]. Paget described 23 patients without having access to current diagnostic tools, suggesting that it was not uncommon in London at the time. It is not possible, however, to study changes in prevalence over a long period due to large changes in life expectancy.

1.10 Clinical features

Paget's disease of bone can cause gross deformities, or result in serious complications, but in some patients is asymptomatic. The most common symptom is pain. This can be caused by the lesion itself, or complications associated with lesions such as osteoarthritis, fracture or neurological compression syndromes. Pain originating from the lesion is likely to be due to increased vascularity in pagetic bone or local deformity. Patients may experience 'warmth over bone' which is likely to be due to increased vascularity, and increased skin temperature is often associated with sites of bone pain.

The abnormal and accelerated bone remodelling that occurs in Paget's disease does not always retain the correct shape of the bone, or respond to mechanical stimuli as it should. This can result in bone deformities, which can be severe. Thickening of bone, particularly the long bones is fairly common, and bowing of limbs is also observed (Figure 1.11). Skull and jaw deformities occur, and can cause dental and cosmetic problems. Paget's disease in the skull and spine can cause neurological compression syndromes that may require surgery to correct. Hearing loss is a common complication, and although progression can be halted by treatment, hearing is seldom restored. Pathological fractures are fairly common complications of Paget's disease, with the convex face of deformed limbs being particularly susceptible to pseudofractures (Figure 1.13(c)). Osteosarcoma is a rare but serious complication with a very poor prognosis, which occurs in up to 1% of patients. Osteosarcoma is otherwise very uncommon in older people.

The most common sites of disease are the pelvis, lumbar spine and femur, one or more of these sites is affected in 75% of cases [4]. Osteoarthritis is common in patients with Paget's disease, and epidemiological studies have shown that patients with Paget's disease are 3.1 times more likely to require a hip replacement for osteoarthritis, and 1.7 times more likely to require a knee replacement than age matched controls [87].

1.11 Diagnosis and treatment

Elevated serum alkaline phosphatase is common in patients with Paget's disease, and levels decrease with effective treatment making it the most widely used biochemical marker for monitoring the disease [88, 89]. Urinary markers of bone resorption such as NTx and CTx or Dpd also tend to be elevated with active disease. Pagetic lesions are generally evident on radiographs, and bone scans effectively identify areas of increased bone turnover characteristic of pagetic lesions (Figure 1.13). Radiographic evidence of a characteristic pagetic lesion is required for diagnosis.

Effective treatment for Paget's disease was not available until the early 1970s when calcitonin, a peptide hormone involved in calcium homeostasis, was introduced as a therapy [4]. Porcine, human and salmon calcitonins have all been used as therapeutics, with salmon calcitonin proving to be over ten times more potent that the human form. Calcitonin reduces osteoclast activity very rapidly after administration, and prevents osteoclast formation. It is administered subcutaneously, is only partially effective and the effects are not sustained, meaning it must be administered indefinitely on at least a weekly basis to ensure long term disease control. For these reasons, it is now rarely used.



Figure 1.13: Imaging of Paget's disease

The main class of drugs now used to treat Paget's disease is the bisphosphonates, which were introduced in the 1970s, and over the last 10 years much more potent analogues have become available. Bisphosphonates are stable pyrophosphate analogues which bind to bone mineral, and their chemical structure is shown in Figure 1.14. While bisphosphonates are toxic to all cells in sufficiently high doses, their affinity for bone mineral ensures that they are generally only internalised by resorbing osteoclasts. Nitrogen-containing bisphosphonates (such as pamidronate and zoledronate) exert their effects by inhibiting the enzyme farnesyl pyrophosphate synthase (FPPS), a key enzyme in the mevalonate pathway. FPPS catalyses the synthesis of isoprenoid chains which are the substrate used for prenylation of various small GTPases in their active state (causing inappropriate downstream signalling) resulting in impaired osteoclast function, and eventually apoptosis [90]. Bisphosphonates bind to sites of active

⁽a) Scintiscan of a patient showing lesions in the left distal humerus, left proximal tibia, right distal tibia and L5 vertebra. (b) Radiograph of a hand showing enlargement of the fourth proximal phalanx (arrow). The images to the right show the affected bone on the right hand, and the left hand for comparison. (c) Fissure fracture associated with a pagetic lesion (arrow). Images provided by Tim Cundy.

bone turnover, making them particularly effective drugs for focal conditions of accelerated turnover like Paget's disease and bone metastases. There is some evidence that bisphosphonates also have a positive effect on bone formation from both human data and animal models [91, 92]. Bisphosphonates affect osteoblast behaviour *in vitro*, although these effects vary depending on the specific drug, and the culture conditions used. Bisphosphonates appear to protect osteoblasts and osteocytes from apoptosis, decrease osteoblast proliferation, but stimulate differentiation, and increase OPG production [93-96].



Figure 1.14: Bisphosphonate structure

General chemical structure of the bisphosphonates (top), and of some specific bisphosphonates that have been used for the treatment of Paget's disease. Etidronate was the original bisphosphonate available for treatment of Paget's disease, while pamidronate, risedronate and zoledronate are more potent nitrogen-containing analogues.

Etidronate was the first bisphosphonate to be used clinically. Subsequently developed analogues are much more potent and have fewer side effects. Intravenous pamidronate, oral risedronate, and oral alendronate among others have been used for effective treatment of Paget's disease. Zoledronate is the newest bisphosphonate, and is very effective. In a study comparing the efficacy of zoledronate to oral risedronate in patients with active disease, a

single 5 mg infusion of zoledronate resulted in normalisation of alkaline phosphatase levels in 89% of patients 6 months after treatment, while risedronate treatment returned alkaline phosphatase levels to normal in only 58% of patients [97]. A two year follow up study showed that 98% of the patients treated with zoledronate had maintained normalised alkaline phosphatase levels, compared with 57% of the patients treated with risedronate [98]. Bisphosphonates tend to have minimal side effects, although gastrointestinal irritation is relatively common with oral delivery, and zoledronate infusions can cause fever and flu-like symptoms soon after treatment. Recently there have been reports of osteonecrosis of the jaw associated with bisphosphonate use, generally in conjunction with invasive dental procedures. The incidence of this complication with the dosages used in Paget's disease is extremely low, but the higher doses used in cancer patients are associated with a much greater risk [99, 100].

Bisphosphonate treatment of Paget's disease decreases bone turnover and vascularity in pagetic sites, which can often reduce pain, and may result in healing of lytic lesions and restoration of normal bone histology [101]. Treatment with bisphosphonates is recommended for symptomatic disease, for example, patients experiencing pain, or neurological compression syndromes [102]. Bisphosphonates are also often administered before joint replacement surgery to reduce the amount of bleeding during surgery. While some physicians do not treat non-symptomatic disease [102], most suggest providing treatment for patients with lytic lesions in long bones, lesions at sites that are likely to lead to neurological complications, arthritis, or deformity, and in individuals with involvement of the skull that could compromise hearing [103].

While antiresorptive therapy is generally very effective in the management of Paget's disease, other treatments may be required. Pain as a result of deformity and osteoarthritis may not resolve as a result of therapy, and other medication or treatments are often required for adequate symptom control. Surgical intervention is often required for effective disease management. Joint replacement surgery for osteoarthritis is the most common procedure, but surgery to correct neurological compression syndromes, deformity and fractures is required in some cases where other intervention is not effective.



Figure 1.15: Radiographic progression of Paget's disease These images of a tibia affected by Paget's disease show progression of the pagetic lesion. The earlier image shows a lytic front (arrow). Nine years later the lesion is sclerotic, and has progressed further down the bone resulting in bowing of the limb. Images provided by Kaye Ibbertson.

1.12 Pathophysiology

Pagetic lesions are areas of localised high bone turnover characterised by overactive osteoclast activity followed by accelerated and disordered bone formation. There may be up to ten times more remodelling in pagetic sites than in normal bone [4, 104]. Osteoclasts are more numerous, larger and more highly nucleated than in normal bone, containing up to 100 nuclei per cell. Similar osteoclast abnormalities are seen in other diseases of high bone List of research project topics and materials

turnover such as hyperparathyroidism. Electron microscopy of pagetic osteoclasts has shown the presence of nuclear and cytoplasmic inclusions that are made up of microfilaments, either randomly distributed, or arranged in a paracrystalline array [105, 106]. These inclusions resemble paramyxoviral nucleocapsids, which has led to much debate about a possible role for paramyxoviruses in the aetiology of Paget's disease (see section 1.13.2). Pagetic lesions begin with lytic areas caused by high bone resorption which can be visualised on radiographs. These lesions gradually advance along long bones at a rate of about 1 cm per year. Eventually, however, the bone lost by osteoclast over-activity is replaced with a mixture of lamellar and woven bone formed by large numbers of osteoblasts (Figure 1.15). In general, bone remodelling remains coupled in Paget's disease, and not all patients present with lytic lesions, especially those with less severe disease.



Figure 1.16: Scanning electron micrographs of normal and pagetic trabecular bone Both samples were taken from biopsies of the iliac crest. The normal bone (left) shows well-preserved trabecular plates and marrow spaces, whereas the pagetic bone (right) has completely lost this architectural appearance. Extensive pitting of the pagetic bone is apparent caused by dramatically increased osteoclastic bone resorption. From Siris and Roodman, 2006 [107].

The osteoblasts in Paget's disease are often considered to be normal [107], and while nuclear or cytoplasmic inclusions have not been found in these cells, there are descriptions of intranuclear abnormalities, similar to those described in other diseases of high bone turnover [4]. Osteoblasts are certainly more numerous, and lay down collagen in a haphazard fashion resulting in the formation of woven bone (which is normally only seen in the adult skeleton during fracture repair). This process causes the development of a sclerotic lesion. Compared to normal, lamellar bone, woven bone is poorly organised as it is made up of randomly-oriented collagen fibres. It also has a lower and more variable mineral content, and more numerous osteocyte lacunae with less organised canaliculi [4, 104]. The disordered microarchitecture in pagetic bone is depicted in Figure 1.16. Because pagetic bone is less organised than lamellar bone, it is more flexible, and less resistant to deformation. The lack of organisation means that the same amount of collagen takes up more space, so balanced bone resorption and formation can still lead to an increase in tissue area and expansion of the bones

involved. Distribution of non-collagenous proteins may also be altered in pagetic lesions [108]. Mineralisation is generally normal and can occur at an increased rate in pagetic bone to match the higher rate of bone formation [104]. Marrow fibrosis is a common observation, and increased vascularity in the marrow cavities is thought to contribute to pain and 'warmth over bone' experienced by some patients.

A number of *in vitro* studies have identified molecular differences between cultured cells from pagetic bone and those from normal bone. Pagetic bone marrow is hypersensitive to 1,25(OH)₂D₃, with osteoclasts forming at concentrations 10-100 times lower than normal [109-111]. While VDR levels do not appear to be altered in pagetic cells, binding affinity is increased, possibly mediated by increased expression of TAF_{II}-17, a co-activator of the VDR [111, 112]. 1,25(OH)₂D₃ hypersensitivity is also likely to be at least partially caused by hyper-responsivity to RANKL, which has been demonstrated in both bone marrow and peripheral blood mononuclear cells (PBMC) from patients with Paget's disease [113, 114]. Studies using semi-quantitative PCR suggest that *RANKL* expression levels are increased in pagetic osteoblast precursors [113, 115].

The proinflammatory cytokine IL-6 may play a role in the increased bone turnover in pagetic bone lesions. IL-6 levels are increased in pagetic lesions, in both osteoclastic and osteoblastic cells, and in serum from patients with Paget's disease [116-118], although other studies have found no changes in serum levels of the protein [119, 120]. IL-6 neutralising antibodies block 1,25(OH)₂D₃ and RANKL hypersensitivity in pagetic bone marrow cultures [113, 116], and partially block the pro-osteoclastogenic effect of pagetic serum [119].

There is also some evidence that pagetic osteoblasts are altered at the molecular level. A two dimensional electrophoresis study demonstrated altered levels of some secreted proteins in cultures of pagetic osteoblasts [121]. In a co-culture study, cultures of pagetic marrow enriched for immature CD34-positive haematopoietic cells showed similar granulocyte macrophage colony forming unit (CFU-GM) formation to controls. Conversely, CD34-depleted pagetic marrow was capable of stimulating increased CFU-GM formation in both pagetic and normal CD34-enriched marrow suggesting a role for the stromal cells in this effect [109]. Another recent co-culture study suggested pagetic lesion and osteosarcomaderived osteoblasts favoured increased osteoclastogenesis and resorption in normal PBMCs [115].

1.13 Aetiology

The aetiology of Paget's disease is not understood, but epidemiological data strongly suggest that both genetic and environmental factors are involved. Identification of possible environmental factors that may be involved in the disease has been difficult, and some, such as pet ownership, which appear to be associated with Paget's disease in some studies, have not been replicated in others [122-124].

1.13.1 Genetics

While most cases appear to be sporadic, a genetic predisposition to Paget's disease can be inherited in an autosomal dominant fashion. About 15% of patients are reported to have an affected relative [125], although given that the disease is often asymptomatic, the proportion of patients with familial disease may be much higher. Linkage studies have identified seven loci (PBD1-7), however most have not been robustly replicated in independent cohorts [126]. Further screening of the PDB3 locus at 5q35 resulted in the identification of mutations in the *SQSTM1* gene (encoding sequestosome 1, also known as p62) [127-130]. Approximately 20 different mutations have now been identified in the *SQSTM1* gene, all localised to exons 7 and 8 which make up the C-terminal region of the protein. Most mutations are located in or near the ubiquitin associated (UBA) domain, and those that have been tested all interfere with the ability of the protein to bind to ubiquitin [131, 132]. Many are substitution mutations that alter one amino acid, however there are also a number of truncations that involve the deletion of most or all of the UBA domain [129, 133].

SQSTM1 mutations have been identified at varying frequencies in families in Europe, North America and Australasia, as well as in sporadic cases of Paget's disease in some studies. A recent meta analysis suggests that *SQSTM1* mutations are found in 28.8% of families with the disease, and in 6.1% of sporadic cases [134]. Patients with *SQSTM1* mutations tend to have more extensive and severe disease than those without a known germline mutation [135]. There is not always a clear genotype-phenotype correlation, although a study involving a large number of families showed that patients with truncation mutations tended to have more bones affected than those with substitution mutations [136]. A number of patients have been identified with homozygous mutations in the *SQSTM1* gene. While one of these patients had more severe disease than other family members, others were not more severely affected than patients with the same mutation in only one allele [137, 138]. The *SQSTM1* mutations also

appear to have incomplete penetrance, suggesting that other factors are important in the development of the clinical condition [139].

SQSTM1 mutations only account for up to a third of families with Paget's disease, suggesting further genetic determinants exist. The most promising locus appears to be 10p13. Linkage to this locus accounted for the genetic susceptibility in most families of British descent with Paget's disease who did not have *SQSTM1* mutations [140]. There is also evidence that polymorphisms in the OPG gene, *TNFRSF11B*, increase susceptibility to Paget's disease in British and Belgian cohorts, although interestingly, in the later study this association was only observed in females [141, 142].

1.13.1.1 SQSTM1 gene function

SQSTM1 or p62 is a ubiquitously expressed adaptor protein found in the cytoplasm and nucleus. It has been implicated in diverse cellular activities including control of NFkB signalling, modulation of potassium channels, control of transcriptional activation [143], autophagy [144], sequestering ubiquitinated proteins [145] and inhibition of extracellular signal-regulated kinase (ERK) MAPK signalling [146]. The domain structure and selected binding partners of SQSTM1 are shown in Figure 1.17(a). The PB1 (Phox and Bem1p-1) domain binds to PB1 domains on other proteins, including other SQSTM1 molecules, and the atypical protein kinase Cs (aPKCs) which are promiscuous kinases involved in various signal cascades. The ZZ domain is a modified zinc finger which binds to the TNFa signalling adaptor, receptor interacting protein (RIP). SQSTM1 also contains a domain that binds to TRAF6, which is an E3 ubiquitin ligase involved in NFkB signal transduction. The UBA domain binds to polyubiquitin chains. Ubiquitin is an 8.5 kDa protein that is attached to lysine (K) residues on other proteins by the actions of a series of ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes. Further ubiquitin molecules can then be attached. When this occurs via the K48 (or K29) residue the ubiquitinated protein is targeted to the proteasome for degradation. K63-attached ubiquitin chains are generally involved in other processes including endocytosis and DNA repair, and K63-linked ubiquitination of TRAF6 is involved in the activation of NF κ B signalling [147].



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(a) Schematic diagram showing the domain structure of SQSTM1, indicating some of the known binding partners for various domains. (b) SQSTM1 (labelled p62) acts as a scaffold to assist in the transduction of NF κ B signals. When a ligand binds to its receptor (such as the TNF α receptor TNFR1, the IL-1 receptor IL-1R or the nerve growth factor receptors NGFR and TrkA) p62 links RIP and/or TRAF6 with aPKC. This leads to phosphorylation and activation of I κ B kinase (IKK), which in turn phosphorylates inhibitor of NF κ B (I κ B) targeting it for degradation. NF κ B is then released from the complex, and translocates to the nucleus to activate transcription of target genes. Abbreviations: TRADD, TNFR1-associated via death domain. Modified from Geetha and Wooten, 2002 [143].

While not all the functions of SQSTM1 are well understood, its role as a scaffold in NF κ B signalling is certainly relevant to osteoclastogenesis and bone biology. SQSTM1 forms a complex with TRAF6 or RIP and an aPKC to facilitate NF κ B signalling stimulated by factors such as TNF α , nerve growth factor, IL-1, and RANKL (Figure 1.17(b)) [143]. This complex allows optimal and sustained activation of NF κ B [148]. *Sqstm1* knockout mice are grossly normal, and young mice do not have an overt bone phenotype, suggesting it is not critical for NF κ B signalling. However the knockout mice show reduced osteoclastogenesis compared to wild-type mice *in vivo* in response to PTHrP, and reduced RANKL-induced osteoclastogenesis in bone marrow cultures [149]. In addition, aged mice show increased BMD consistent with defective osteoclastogenesis [148].

SQSTM1 may be involved in degradation of proteins via the ubiquitin-proteasome system. The UBA domain binds to both K48- and K63-linked ubiquitin chains, which appears to extend the half-life of the protein [150, 151]. SQSTM1 can bind to the proteasome, and may play a role as a shuttling factor to transport proteins to the proteasome [151, 152]. SQSTM1 is capable of forming aggregates, and has been colocalised to protein aggregates in Alzheimer's disease and Huntington's disease, Lewy bodies in Parkinson's disease and Mallory bodies which form in steatohepatitis [145]. These aggregates are believed to be protective and promote cell survival, and SQSTM1 knockout mice develop Alzheimer's disease-like neurodegeneration as they age [151, 153].

SQSTM1 also plays a role in other mechanisms of protein and organelle degradation. Longlived cytoplasmic proteins, protein aggregates, and damaged organelles are degraded by a process known as autophagy. This involves an area of cytoplasm being encapsulated as a membrane-bound autophagosome, which can then merge with endosomes or lysosomes to initiate degradation [144]. Recent research suggests that SQSTM1 plays a role in targeting polyubiquitinated proteins and aggregates to the autophagy machinery, facilitated by its ability to bind the LC3 proteins that are necessary for autophagosome formation [154, 155]. The LC3 binding region was mapped to amino acids 321-342 which is retained in all the pagetic mutations [155].

Recent work has led to some insights into the mechanisms by which the *SQSTM1* mutations found in patients with Paget's disease may affect bone turnover. Presence of the mutations appears to potentiate NF κ B signalling: when wild-type SQSTM1 is over-expressed, basal

levels and activation of NF κ B signalling by RANKL are reduced, however this is not the case when the mutants are over-expressed [133]. The reason for this apparent gain of function is not clear, however, mutant SQSTM1 does not aggregate in the same manner as the wild-type protein [156]. There are also a number of recent reports of mouse models expressing mutant *SQSTM1* [157-160]. Some develop focal lytic lesions, and bone marrow cultures from the mice expressing the mutant protein show increased osteoclastogenesis. Despite this recent progress, it is not clear how the molecular changes in cell signalling caused by mutant *SQSTM1* predispose patients to developing focal bone disease, and the mouse models reported do not recapitulate all features of Paget's disease.

1.13.2 Paramyxoviruses and Paget's disease

A number of studies suggest that viruses are associated with Paget's disease and could be one of the non-genetic factors contributing its development. In the 1970s, inclusions were observed in the nuclei of osteoclasts from pagetic patients [105, 106], which were later suggested to be of paramyxoviral origin. Examples of these inclusions are shown in Figure 1.18. Nuclear inclusions are also found in brain cells from patients with subacute sclerosing panencephalitis (SSPE), a fatal condition caused by a long-term measles virus infection of the brain (Figure 1.18) [161]. A number of publications report detection of mRNA or protein from measles virus [162-168], canine distemper virus [169-171] and respiratory syncytial virus [172-174] in samples from patients with Paget's disease, but generally not in controls. Other groups, however, have repeatedly failed to detect viral RNA or antigens [175-180].

Further evidence for a viral aetiology of Paget's disease is the demonstration that infection of osteoclasts with paramyxoviruses or measles virus nucleocapsid (N) protein produces Pagetlike changes in these cells [181-183]. Mice expressing the measles N gene under the control of the *Trap* promoter develop a pagetic phenotype that worsens with age [184]. These mice display increased osteoclast and osteoblast numbers, and some develop lesions in their vertebrae containing disorganised trabeculae and woven bone similar to a pagetic lesion.

The role of paramyxoviruses in Paget's disease is still controversial. While nuclear and cytoplasmic inclusions are a feature of some pagetic osteoclasts, they are not specific to this disease. Similar inclusions have also been found in osteoclasts or macrophages in other conditions not attributed to viral infection including osteopetrosis [185]. In the past, measles

virus has been implicated in other conditions, such as inflammatory bowel disease [186-188], but these links are not currently thought to be aetiologically important [189, 190].



Figure 1.18: Transmission electron microscopy images of typical inclusions in nuclei of pagetic osteoclasts (A) Low magnification of a multinucleated osteoclast with inclusions identified in one nucleus (arrow). Bar represents 20 μ m. (B) Detail of nuclear inclusion from A. Bar represents 0.1 μ m. (C and E) Low and high magnification images of inclusions in a nucleus of a pagetic osteoclast. (D and F) Inclusions in a brain cell of a patient with SSPE. Note the difference in organization of the structures and the "stiffness" of the pagetic inclusions compared with the undulating appearance of the measles virus nucleocapsids in SSPE. The micrograph pairs in C and D (bar represents 0.5 μ m) and E and F (bar represents 0.1 μ m) were taken using the same microscope, magnification and settings. Reproduced with permission from Helfrich *et al.* 2000 [178].

1.14 Genetic disorders similar to Paget's disease

There are a number of other metabolic bone diseases that show some phenotypic similarities to Paget's disease. Understanding the genetics and aetiology of these diseases may help to elucidate the disease processes occurring in Paget's disease.

1.14.1 Familial expansile osteolysis and other diseases caused by *RANK* mutations

Familial expansile osteolysis (FEO) was first described in 1988 in a large family from Northern Ireland [191]. It was inherited in an autosomal dominant fashion. The first presentation tended to be hearing loss, with bone pain and deformity generally apparent by the second decade of life. Both focal and generalised skeletal abnormalities were observed, however focal lesions commonly developed at previously unaffected sites [191, 192]. Lesions were mainly confined to the limbs, and rarely found in the axial skeleton (Figure 1.19).

Nuclear inclusion bodies were identified in osteoclasts from affected bone, like those in Paget's disease [192]. Genome-wide screening in this family led to the identification of an 18 base pair (bp) duplication (84dup18) in the *TNFSF11A* gene which encodes RANK [193]. This involved duplication of six amino acids of the RANK signal peptide, and promoter-reporter assays showed that this mutation resulted in increased constitutive NF κ B signalling [193]. This result combined with the autosomal dominant mode of inheritance suggests an activating mutation. Antiresorptive therapy such as bisphosphonates can be effective at reducing biochemical markers of bone turnover and bone pain, but it is not known whether treatment can prevent complications [194].



Figure 1.19: Photograph of a patient suffering from familial expansile osteolysis Severe deformity is apparent in the lower leg and his right femur is also involved. Reproduced with permission from Osterberg *et al.* 1988 [191]. Two other conditions show extensive phenotypic overlap with FEO. Expansile skeletal hyperphosphatasia was described in an Australian family [195]. Mutation screening identified a 15 bp duplication in the *TNFRSF11A* gene (84dup15) similar to the FEO mutation [196]. Early-onset familial Paget's disease was identified in a Japanese family [197]. This disease was caused by another duplication in the signal peptide on the *TNFRSF11A* gene, 75dup27 [193]. A mouse model carrying this mutation has recently been developed, and the heterozygote mimics the phenotype of the human syndrome [198].

1.14.2 Idiopathic hyperphosphatasia

Idiopathic hyperphosphatasia is a rare autosomal recessive condition characterised by generalised high bone turnover which results in skeletal deformity, bone expansion, bone pain, and high risk of pathological fractures. A large family of Iraqi origin residing in Auckland have three children with idiopathic hyperphosphatasia of intermediate severity. They suffered from difficulty walking from around five years of age, and the two older siblings were wheelchair bound by the age of 15. Two suffered long bone fractures, and progressive deafness was noted from the age of eight. Serum alkaline phosphatase and urinary NTx levels were markedly elevated in these patients [199]. A bone biopsy from the youngest patient showed an unusual appearance, with parallel trabecular plates and almost no crosslinking trabeculae.

Idiopathic hyperphosphatasia is caused by OPG deficiency. This was identified in two apparently unrelated Navajo patients from the USA who were homozygous for deletion of the *TNFRSF11B* gene which encodes OPG [196]. The Auckland family had a 3 bp deletion in exon 3 of the *TNFRSF11B* gene which resulted in loss of a conserved aspartate residue [199]. This mutation was shown to lower the activity of OPG in *in vitro* assays, as well as reducing OPG secretion [199, 200]. The International Hyperphosphatasia Collaborative Group identified a number of other *OPG* mutations associated with idiopathic hyperphosphatasia, although not all families in the study had mutations in the *TNFRSF11B* gene [201]. Mutations affecting the cysteine residues that were predicted to impair RANKL binding, and a large deletion, were associated with severe forms of the disease (deformity developing by 18 months of age). Other missense mutations towards the N-terminal end of the protein were associated with intermediate severity, while a mutation in the C-terminal end of the protein, which is not essential for activity *in vitro*, exhibited a milder phenotype. There are some reports of improvements in the symptoms of idiopathic hyperphosphatasia with antiresorptive List of research project topics and materials

treatment [194, 202], and recombinant OPG has also been used successfully [203], although it is not available for routine clinical practice.

1.14.3 Inclusion body myopathy, Paget's disease and frontotemporal dementia

Inclusion body myopathy, Paget's disease and frontotemporal dementia (IBMPFD) is a rare autosomal dominant disease that was identified in a series of families in the USA [204, 205]. Myopathy is the most common feature, occurring in 90% of patients. The Paget's disease component was present in 43% of patients with a mean onset age of 42 years, and typical pagetic lesions involving the spine, skull and pelvis. Dementia developed with 37% penetrance at a mean age of 54 years.

IBMPFD is caused by mutations in the valosin-containing protein (VCP) gene [206]. VCP is a member of the type II AAA ATPases (ATPases Associated with a variety of Activities) and is a highly conserved protein that is expressed ubiquitously at high levels [207]. It is involved in a variety of cellular processes, including control of membrane fusion, cell cycle regulation, stress responses, apoptosis, lymphocyte activation, transcriptional activation, endoplasmic reticulum associated degradation and protein degradation [207]. Most of these activities appear to be associated with the role of VCP as a chaperone for the ubiquitin-proteasome pathway. The mutant protein retains its ATPase activity, and mice haploinsufficient for Vcp have no phenotype suggesting a dominant negative or gain of function effect [208, 209]. In vitro studies suggest that mutant VCP has impaired ability to facilitate endoplasmic reticulumassociated degradation which results in accumulation of ubiquitinated proteins [209]. Mice expressing the mutant protein in muscle cells exhibit myopathy, and ubiquitin-containing protein inclusions [210], while patients show inclusion bodies in brain, bone cells and muscle, similar to the viral-like inclusions observed in classical Paget's disease. The mechanism by which VCP mutations cause IBMPFD is not well understood, but there are similarities with SOSTM1 mutations in that both are involved in the ubiquitin-proteasome pathway, and mutations are dominant but result in only partial penetrance with respect to Paget's disease. Mutations in VCP have not been found in classical Paget's disease, and there is no evidence of genetic association with this gene [211].

Part C. AIM

1.15 Aims of this study

While the clinical features of Paget's disease have been thoroughly characterised, and effective treatment is available, the cell biology and aetiology of the disease are still not well understood. This study sought to investigate the role of osteoblasts in Paget's disease. In order to do this we have collected bone samples and bone marrow from patients with Paget's disease and controls, cultured cells from the samples, and collected RNA. Pagetic tissue was compared to normal tissue from both pagetic patients and non-pagetic individuals. These samples have been used to investigate changes in gene expression in pagetic osteoblasts and bone marrow using microarray analysis and real time PCR. We wished to investigate the consequences of some of the most significant changes in gene expression in pagetic cells on bone cell biology. In particular, the effects of over-expressing keratin 18 in osteoblasts have been examined.

The role of genetic and environmental factors in the aetiology of Paget's disease is still unclear. Further aims of this study included examining the potential role of measles virus and somatic mutations in *SQSTM1* in Paget's disease, and investigating the effects of *SQSTM1* mutations in osteoblastic cells.

CHAPTER 2: METHODS

2.1 Materials

2.1.1 Cell culture

All cell culture media were purchased from Invitrogen. Media were made from powder using ultrapure water, and supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin (Invitrogen) and 0.22% sodium bicarbonate (Sigma-Aldrich). All media were sterilised by passing through a 0.2 μ M filter (Sartorius) before use, as were all other solutions made from non-sterile ingredients used in tissue culture. OptiMEM and ascorbic acid-free minimum essential medium alpha (α MEM) were purchased in liquid form. Foetal bovine serum (FBS) was purchased from Invitrogen, and each batch was tested in various cells to ensure that there was minimal inter-batch variability. Trypsin-EDTA was purchased from Invitrogen, Ficoll-Paque Plus and [H³]-thymidine were from Amersham Biosciences, bovine serum albumin (BSA) was from ICPbio, and heparin was from CP Pharmaceuticals Ltd. The transfection reagent Fugene6 was from Roche, and GeneJammer was from Stratagene. All other chemicals used for tissue culture were purchased from Sigma-Aldrich unless otherwise stated, and were of a tissue culture tested grade.

T75 tissue culture flasks, and 12- and 48-well plates were purchased from Corning. All other tissue culture plates were Greiner Bio-one, and T175 flasks were from BD. All plasticware used for tissue culture had been sterilised by gamma irradiation.

Recombinant proteins and drugs used in tissue culture included IL-6, soluble IL-6 receptor, Dkk1 and PDGF from R&D Systems, $TNF\alpha$, $TGF\beta$ and dexamethasone from Sigma, and $1,25(OH)_2D_3$ from Merck. Isolation of lactoferrin from skim milk was performed by Nutrition and Bioactives, Fonterra Innovation, Fonterra Co-operative Group Ltd in Palmerston North, New Zealand.

Alkaline phosphatase staining was performed using Sigma FAST BCIP/NBT tablets, and TRAP staining was performed using the leukocyte acid phosphatase kit (both Sigma). Cell viability staining was performed using the live/dead cell viability assay (Invitrogen), and DAPI nuclear stain was also from Invitrogen. The Dual-Glo Luciferase Assay System and reporter lysis buffer were from Promega.

Animals were obtained from the Vernon Jansen Unit on site, and were euthanized humanely before tissues were collected.

2.1.2 Molecular biology

The RNeasy Mini Kit, RNase-free DNase Kit, QIAquick Gel Extraction Kit and Plasmid Mega Kit were from Qiagen. The PCR product purification kit, High Pure Plasmid Isolation Kit, and random primers were from Roche. The site-directed mutagenesis kit was from Stratagene. All PCR primers were purchased desalted and lyophilised from Sigma-Genosys. Primers used for site-directed mutagenesis were purified using polyacrylamide gel electrophoresis. All other reagents used for RT-PCR and cloning, including agarose and plasmid vectors were from Invitrogen. The EZrTth RNA PCR kit used for measles virus detection was from Perkin Elmer. Real time PCR primer-probesets, master mix, low density arrays and plates were all purchased from Applied Biosystems. Microarrays were purchased from Affymetrix. The Adeno-X Maxi Purification Kit and Adeno-X Titre Kit were from Clontech.

2.1.3 Protein detection

The antibodies, proteins and reagents used for ELISAs were all from R&D Systems. The protease inhibitor cocktail used for harvesting cell lysates was from Sigma. Antibodies were purchased from Abcam (keratin 18), Invitrogen (Xpress and His tags), Roche (His tag) and Santa Cruz (total ERK1/2). Precast gels for western blotting were purchased from Biorad, protein ladders were from Invitrogen, and the nitrocellulose membrane and ECL reagent were from GE Healthcare. The DC protein assay kit was also from Biorad, and the Luminex kit was from Millipore.

2.1.4 Solutions

- Phosphate buffered saline (PBS): 137 mM NaCl, 7.9 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH 7.4
- TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8
- HTNG lysis buffer: 50 mM HEPES, 150 mM NaCl, 1% Triton, 10% glycerol, 1.5 mM MgCl₂ 1 mM EGTA

- 5x protein loading buffer: 12% SDS, 250 mM Tris, 8 mM EDTA, 15% glycerol, 0.4% bromophenol blue, 713 mM β -mercaptoethanol, pH 6.9
- Western blot running buffer: 1.9 mM glycine, 250 mM Tris, 2% SDS, pH 8.9
- Western blot transfer buffer: 100 mM glycine, 12 mM Tris, 20% methanol, pH 8.3
- Tris-buffered saline (TBS): 20 mM Tris, 150 mM NaCl, pH 7.5
- 2x ONPG substrate: 1.33 mg/mL ONPG (o-nitrophenyl-β-D-galactopyranoside), 2 mM MgCl₂, 100 mM β-mercaptoethanol in 200 mM sodium phosphate buffer, pH 7.3

2.2 Cell culture

All sterile tissue culture work was performed in a class two tissue culture cabinet. Cells were grown in a hydrated incubator at 37°C with 5% CO₂. Cells were all maintained in T75 flasks containing 30 mL of the appropriate media.

2.2.1 Human osteoblasts

Osteoblasts-like cells were cultured from trabecular bone fragments based on the method of Robey and Termine (Figure 2.1) [212]. Bone was obtained from consenting patients undergoing hip and knee replacement surgery. Trabecular bone was cut into small fragments, washed several times with PBS then Dulbecco's modified Eagle's medium (DMEM), then incubated in DMEM containing 1% collagenase in a shaking water bath at 37°C for 30 minutes (min). Fragments were washed again to ensure removal of all bone marrow, then incubated in a flask in DMEM 10% FBS + 5 μ g/mL L-ascorbic acid 2-phosphate (A2P) until outgrowth of cells from some bone chips was noted, usually 3-4 days later. Bone fragments were changed twice weekly.

In the samples used for gene expression, bone chips were removed from the flask and 20 mL of fresh media was added 72 hours (hr) before confluence. Once confluence was attained, conditioned media was collected and frozen, and cells were counted and harvested for RNA extraction. Most of the RNA samples used in the gene expression work were collected from first generation primary cultures, however a small number were subcultured to obtain more cells.

For other experiments cells were subcultured once before use. In some cases bone chips were transferred into a new flask and further cells were released to obtain second, and subsequently third generation cells. Unless otherwise stated, cells were seeded at a density of 5 x 10^4 cells/mL in DMEM 5% FBS + 10μ g/mL A2P.



Figure 2.1: Human osteoblast outgrowth cultures from trabecular bone explants Cell growth is shown after (a) 5 days culture, and (b) 14 days culture. The arrow indicates a single osteoblast.

2.2.2 Human bone marrow cells

Bone marrow was either obtained during orthopaedic surgery or aspirated from the iliac crest of patients with Paget's disease. Bone marrow was collected in 1000 units/mL heparin. Fat and clumps were removed from the bone marrow and it was diluted approximately 1:2 in DMEM, then layered on Ficoll-Paque Plus and centrifuged at 1500 rpm with no brake for 30 min. The interface layer, enriched for mononuclear cells, was collected and washed in media,

then spun, again with no brake, for 10 min at 1900 rpm. The cells were washed again then resuspended and counted in methyl blue/acetic acid to ensure no red blood cells were included in the count. Flasks were seeded with 1.4×10^7 cells in 20 mL DMEM 10% FBS + 5 µg/mL A2P. An additional 10 mL of media was added 24 hr later. Although mesenchymal cells are more adherent to plastic than haematopoietic cells, these cultures were not subcultured, or washed vigorously ensuring haematopoietic cells were also maintained. Media were replaced twice weekly. During the final 72 hr cells were cultured in 20 mL media which was later collected and frozen, and the cells were counted and prepared for RNA extraction.

Mesenchymal cell cultures were also grown from human bone marrow. Whole bone marrow was cultured in a flask in α MEM 10% FBS, and after 24 hr the cell layer was washed thoroughly to remove non-adherent cells. Cells were grown to confluence then subcultured at least once before seeding into plates to ensure depletion of haematopoietic cells.

2.2.3 Primary rat osteoblasts

The isolation of primary rat osteoblasts was performed by other members of the bone group from 20 day old foetal rat calvariae. Calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. The calvariae were sequentially digested using collagenase and the cells from digests three and four were collected, pooled, and washed. Cells were grown in DMEM 10% FBS + 5 μ g/mL A2P for two days, and then to confluence in minimum essential medium (MEM) 10% FBS + 5 μ g/mL A2P. Cells were used on the first passage and were seeded in MEM 5% FBS + 5 μ g/mL A2P.

2.2.4 Cell lines

MC3T3-E1 Subclone 4 cells were purchased from the American Tissue Type Collection. They were maintained in ascorbic acid-free α MEM 10% FBS. UMR-106 and HEK293A cells were maintained in DMEM 5% FBS, SaOS2 cells were maintained in α MEM 10% FBS. All cells lines were passaged twice weekly.

2.2.5 Proliferation assay

2.2.5.1 Thymidine incorporation assay

Following trypsinisation, cells were seeded into 24-well plates in growth medium (rat osteoblasts, MEM + 5 μ g/mL A2P; human osteoblasts, DMEM + 10 μ g/mL A2P; SaOS2s,

 α MEM) supplemented with 5% FBS, at a density of 5 x 10⁴ cells/mL, 0.5 mL/well and grown to semi-confluence overnight. Cells were growth arrested in growth medium containing 0.1% BSA overnight. The following day fresh serum-free medium and treatments were added. Cells were incubated with [³H]-thymidine (0.5 μ Ci/well) for the last 6 hr of drug treatment, with the exception of the primary human cells where it was included for 24 hr. The experiment was terminated and thymidine incorporation was assessed using the Wallac Microbeta Trilux 1450 (PerkinElmer Life & Analytical Sciences). Most experiments were performed with six wells in each treatment group. Slight modifications were made when assays were performed using transfected or transduced cells. Transfections were performed immediately after the first addition of serum-free media using a Fugene6:DNA ratio of 6:1, and the assay then proceeded as normal without the addition of further treatments. Adenoviral transductions were also performed the day after cell seeding, however, cells were transduced in media containing 5% FBS, then after a 6 hr incubation, 0.5 mL serum-free media was added. This was replaced with completely serum- and vector-free media the following morning.

2.2.5.2 MTT assay

Cells were seeded and cultured in the same manner as the thymidine assay. Once cells were treated for the desired length of time, MTT that had been freshly dissolved in PBS was added to the wells to achieve a final concentration of 0.5 mg/mL. MTT solution was also added to control media for use as a blank. After 4 hr incubation, 10% SDS in 0.01 M HCl was added and incubated at 37°C overnight. Absorbance was measured at 595 nm on a Biotek plate reader.

2.2.6 Mineralisation assay

MC3T3-E1 Subclone 4 cells were seeded in α MEM 10% FBS at a density of 5 x 10⁴ cells/well in 6-well plates. Once cells reached confluence about three days later, media were changed to mineralisation media consisting of α MEM 10% FBS + 50 µg/mL A2P + 10 mM β-glycerophosphate (Day 0). 10 nM dexamethasone was often added to some wells as a positive control. Cells were grown for up to 21 days after addition of mineralisation media with media changes twice weekly. Cells were washed in PBS then fixed with 10% neutral buffered formalin. Mineralised areas were visualised using von Kossa staining which entailed 30 min incubation at room temperature in fresh 2.5% silver nitrate, followed by thorough washing with distilled water, then counterstaining with sodium carbonate formaldehyde for 2 min.

Human osteoblasts were subcultured, then seeded in α MEM 10% FBS + 50 µg/mL A2P at a density of 1.5 x 10⁵ cells/well in 6-well plates (Day 0). Once cells reached confluence about three days later media were changed to α MEM 5% FBS + 50 µg/mL A2P + 10 mM β -glycerophosphate, with the addition of 10 nM dexamethasone to some wells. Media were changed twice weekly, and cells were maintained for up to four weeks. Staining for mineralisation was performed using von Kossa as described above, or alizarin red S. Alizarin red staining involved fixing cells in 70% ethanol, then staining with 40 mM filtered alizarin red S for 10 min, followed by thorough washing to ensure removal of excess dye. Plate images were captured using a light box and a mounted digital SLR camera with standardised exposure settings.

Cells were collected for RNA extraction during many of the mineralisation experiments. Because of the extensive matrix that forms, cells at timepoints from approximately 10 days onwards were treated with 120 μ g/mL collagenase for 30 min before cells were harvested [213], either by trypsinisation, or by direct lysis in RLT buffer.

2.2.7 Mouse bone marrow osteoclastogenesis culture

Bone marrow was flushed from the long bones of normal Swiss male mice aged 4–6 weeks, and marrow cells were cultured for 2 hr in 90 mm Petri dishes. Non-adherent cells were then collected and grown in 48-well plates at a density of 5 x 10^5 cells/well in α MEM 10% FBS. 1,25(OH)₂D₃ (10 nM) was added to all wells except the negative controls. On day 2 and 4, the media was refreshed and treatments and 1,25(OH)₂D₃ were added. After culture for 7 days, cells were fixed and TRAP stained and TRAP-positive multinucleated cells (containing three or more nuclei) were counted in all wells. There were at least eight wells for each group.

2.2.8 Luciferase assay

Cells were seeded in 500 μ L standard media containing 5% FBS in a 48-well plate. UMR-106 cells were seeded at 10⁵ cells/mL, and SaOS2 cells were seeded at 5 x 10⁴ cells/mL. The following day cells were serum starved, then transfected using Fugene6. The 10 μ L transfection mix contained 0.6 μ L Fugene6, 100 ng vector or plasmid of interest, 40 ng pHTS-NF κ B (Biomyx), 60 ng pRL-TK (Promega) and OptiMEM (Fugene6:DNA ratio of 6:2). The pHTS-NF κ B plasmid contains a firefly luciferase gene with an NF κ B responsive promoter. The pRL-TK plasmid expresses the Renilla luciferase gene under the control of the herpes

simplex virus thymidine kinase promoter. Both plasmids were donated by Dr. Martin Philpott, Auckland Cancer Society Research Centre. Recombinant mouse TNF α was often added 3 hr before the end of the experiment. 24 hr after transfection, luminescence was measured using the Dual-Glo Luciferase Assay System. Media were removed from all wells and replaced with 75 µL fresh media and 75 µL luciferase substrate. After 10 min incubation the contents of each well was transferred to a 96-well white plate and the firefly luminescence was measured on a Wallac Victor Plate reader. 75 µL Stop & Glo substrate was added to each well, incubated for 10 min, then the Renilla luminescence was measured. Blanks and untransfected controls were included in all experiments, and results were adjusted for these, and corrected for the Renilla values. Experiments were performed in quadruplicate and repeated at least three times.

2.2.9 Cultures in three dimensional scaffolds

These experiments were performed at the Botnar Research Centre, University of Oxford under the supervision of Dr. Philippa Hulley and Dr. Zhidao Xia. Porous collagen I scaffolds that had been made at the University of Oxford using a three dimensional printer were used. Scaffolds were round and approximately 1.5 cm in diameter, and were cut into quarters before use. Scaffolds were sterilised in 70% ethanol, then washed in PBS and culture media before cells were seeded. Mesenchymal cells cultured from human bone marrow were seeded into 6-well plates and transduced with adenoviruses. After 24 hr incubation, cells were trypsinised, and cells from one well were transferred to each scaffold in a volume of 30 µL. After 2 hr incubation 1 mL α MEM 20% FBS was added to the wells. After a week media were changed to mineralisation medium (α MEM 10% FBS + 50 µg/mL A2P + 10 mM β-glycerophosphate). Media were changed twice weekly, and scaffolds were fixed in 10% phosphate-buffered formalin at the conclusion of the experiment.

Cell transduction was assessed in unfixed scaffolds using confocal microscopy to detect cells expressing green fluorescent protein. Live/Dead staining was also performed in unfixed scaffolds. Scaffolds were washed in PBS and incubated with the stain for 15 min at 37°C, then staining was visualised using confocal microscopy. Fixed scaffolds were cryosectioned, and stained for alkaline phosphatase, cell number using DAPI nuclear stain, and mineralisation using alizarin red.



2.3 Molecular biology

2.3.1 RNA extraction

RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions. Cells were lysed in the RLT buffer provided by passing through a 20g needle at least five times. All samples were treated with the RNase-free DNase kit to eliminate any genomic DNA, and the RNA was eluted in 30 μ L RNase-free water and frozen until use. 1-2 μ L of RNA was visualised on a 1% agarose gel stained with ethidium bromide to ensure that no significant degradation had occurred, and the concentration of each sample was measured using a nanodrop spectrophotometer.

2.3.2 RT-PCR

Reverse transcription and PCR were performed using an Eppendorf Mastercycler personal. Initially RNA (usually 1 μ g) was incubated at 65°C for 5 min with 4 μ g random primers and DEPC-treated water made up to a volume of 8 μ L. After incubation on ice the reaction was made up to 20 μ L containing 1x First Strand buffer, 10 mM DTT, 0.5 mM dNTPs, 200 units Superscript III reverse transcriptase and 40 units RNase OUT. Reactions were incubated at 25°C for 5 min, 50°C for 50 min then 70°C for 15 min.

Amplification of the *GAPDH* gene was performed to ensure successful reverse transcription, and to determine the approximate concentration of the cDNA. When different RNA concentrations were used in the reverse transcription reactions, semi-quantitative PCR was performed. The 50 μ L reaction mixture included 1x PCR reaction buffer, 2.5 units of Platinum Taq polymerase, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 μ M of each primer (see Table 2.1), 1 μ L of cDNA, and sterile ultrapure water, then half was transferred to another tube. Samples were heated to 94°C for 2 min, then each sample was run for 20 and 25 cycles, with each cycle consisting of 30 seconds (s) at 94°C, 30 s at 60°C (annealing) and 1 min at 72°C. A reference sample was often included in this PCR step to help determine appropriate dilutions for use in real time PCR. PCR products were run on a 1% agarose gel stained with ethidium bromide and relative concentrations were determined by visual estimation.

Standard qualitative PCR was performed in a similar manner. The primers used and their annealing temperatures are shown in Table 2.1. Reactions were performed for 35 cycles, with the exception of GAPDH which was amplified for 25 cycles, using the conditions described

above, however the annealing temperature was altered as appropriate and a final elongation step was added consisting of 72°C for 5 min at the end of the amplification.

2.3.2.1 Measles virus genome detection

In order to detect measles virus (MV) genes, either 0.5 or 1 μ g of each human RNA sample in a 20 μ L volume was sent to the National Institute of Biological Standards and Control (NIBSC), UK. All samples were supplied coded to ensure blinded analysis. Measles virus genome detection was performed by a technician using the RT-PCR-nested PCR amplification technique developed previously at NIBSC [189] under the supervision of Dr. Muhammad Afzal and Dr. Philip Minor. The nucleocapsid (*N*) protein gene and the matrix (*M*) protein gene were targeted for amplification. The sensitivity limits of *N* and *M* genespecific assays were in the range of 5.5 x 10⁻² to 2.5 x 10⁻⁴ plaque forming units of measles virus per reaction [189, 214].

The N and M gene-specific RT-PCR-nested PCR amplifications were carried out using the single-step RT-PCR amplification approach described previously [189]. In each reaction RNA template was reverse transcribed and amplified with the measles virus N gene or M gene specific primers shown in Table 2.1 using the EZrTth RNA PCR kit. The cDNA synthesis step was carried out at 60°C for 30 min on a thermal cycler (Px2) after which the reaction mixture was subjected to the following assay conditions: one cycle of 94°C for 2 min, 40 cycles of 94°C for 45 s, and 60°C for 45 s; and one cycle of 60°C for 7 min. For the nested PCR procedure, 2 µL of primary PCR product were subjected to re-amplification with template specific primer sets (MV3/MV4 for the N gene region and MV15/MV16 for the M gene shown in Table 2.1), dNTP mixture and AmpliTaq DNA polymerase (0.5 unit/reaction). All nested PCR amplifications were carried out for a single round of 30 cycles of 94°C for 1 min; 50°C for 30 s; 72°C for 1 min. The reaction products were resolved in 1% agarose gels which were stained with ethidium bromide and visualized by a U.V. lamp. The positive control RNA samples that were used in this study were derived from a measles virus tissue culture isolate and from a brain sample of a patient that had SSPE. These controls have been routinely used at NIBSC for various measles virus detection studies [189, 190, 214].

Table 2.1: PCR primers used in this thesis

Name	Forward primer	Reverse primer	Anneal- ing ^a	Size⁵
Rodent GAPDH	5'-CATCATCTCCGCCCCTTCG-3'	5'-CCTGCTTCACCACCTTCTTG-3'	60	433
Human GAPDH	5'-CATCATCTCTGCCCCCTGTG-3'	5'-CCTGCTTCACCACCTTCTTG-3'	60	436
SQSTM1 cDNA	5'-AGGAACAGATGGAGTCG-3'	5'-TGGAAGAAGGCAGAGAAAC-3'	55	463
SQSTM1 Exon 8	5'-GGCAGAGTTGAGCAGTGTG-3'	5'-CCCGTACAGAGACCTGCAAT-3'	60	445
Allelic discrimination	5'-GCTGCCTTGTACCCACATCTC-3'	5'-GCATCTGGGAGAGGGACTCA-3'	60	67
SQSTM1 for cloning	5'-GCTATGGCGTCGGTACCGGTGAAGGC-3'	5'-CCCTAACCCCTGATTCTAGAAGAAGGCAGAG-3'	55	1489
P392L mutation	5'-CCAGAGGCTGACCTGCGGCTGATTGAG-3'	5'-CTCAATCAGCCGCAGGTCAGCCTCTGG-3'	55	-
396X mutation	5'-GCTGACCCGCGGCTGATTTAGTCCCTCTC-3'	5'-GAGAGGGACTAAATCAGCCGCGGGTCAGC-3'	55	-
MV-N 1st amplification (MV1 & MV2)	5'-TTAGGGCAAGAGATGGTAAGG-3'	5'-GTTCTTCCGAGATTCCTCCCA-3'	60	432
MV-N nested primers (MV3 & MV4)	5'-AGCATCTGAACTCGGTATCAC-3'	5'-AGCCCTCGCATCACTTGCTCT-3'	50	252
MV-M 1st amplification (MV13 & MV14)	5'-GCGACAGGAAGGATGAATGC-3'	5'-GTTTGCGTTGAAGACACTCC-3'	60	286
MV-M nested primers (MV15 & MV16)	5'-TATGTACATGTTTCTGC-3'	5'-GTTGTTAGGACCTTTCTCC-3'	50	242

^a Annealing temperature used with this primer pair in °C ^b Size of PCR product (bp)
2.3.3 Sequencing

PCR products were checked for purity on an agarose gel, then purified using the PCR product purification kit. Sequencing was performed on an ABI Prism 3100 sequencer (Applied Biosystems) located in the School of Biological Sciences, University of Auckland and operated by Kristine Boxen. Sequences were compared to published sequences using BLAST and examined visually.

2.3.4 Real time RT-PCR

Real time PCR was performed using an ABI Prism 7900 (Applied Biosystems). Reactions were performed in a 10 μ L volume in 384-well plates. Multiplex reactions were carried out using 1x TaqMan Universal PCR Master Mix, a FAM-labelled TaqMan primer-probeset, and the VIC-labelled 18S rRNA primer-probeset which was included in all reactions as an internal control. Seven point validation curves were often included, and the standard curve analysis method was used if the slope was greater than 0.3, otherwise the delta delta Ct (DDCt) method was used [215]. When analysing the gene expression in the primary human samples, the average delta Ct (DCt) value of the non-pagetic samples was calculated and used as a calibrator to determine relative expression for both the groups of samples, and individual samples. Differences between the groups were calculated using a student's t test. For other experiments expression was normalised to the zero timepoint or control sample, although in some cases a standardised calibrator value was used to give some indication of absolute expression.

2.3.4.1 Real time PCR using low density arrays

Some real time PCR analysis was performed using custom Taqman Low Density Arrays. These microfluidic cards contain 384 spots of dehydrated primer-probesets which allow amplification of multiple genes using a much smaller quantity of cDNA and master mix than standard real time PCR. Two different arrays were purchased. The results from the first set of arrays, which measured expression of 24 human genes of interest including two control genes, are presented in Chapter 4, and the genes on this array are shown in Table 2.2. The second set of arrays contained primer-probesets for 48 human genes, including three control genes as shown in Table 2.3. The control genes included on the cards are shown at the bottom of these tables. All cards were analysed using the same baseline and threshold values so that, where necessary, data from separate arrays could be pooled. Data was analysed in the same manner as other real time PCR data.

Gene	Gene Name	Molecular classification
Symbol		
ALPL	Alkaline phosphatase, liver/bone/kidney	Phosphatase
BMP2	Bone morphogenetic protein 2	Signalling molecule
CCL2	Chemokine (C-C motif) ligand 2	Signalling molecule
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	Hydrolase
DLX5	Distal-less homeobox 5	Transcription factor
FGFR2	Fibroblast growth factor receptor 2	Receptor
GATA6	GATA binding protein 6	Transcription factor
IGF1	Insulin-like growth factor 1	Signalling molecule
IL11	Interleukin 11	Signalling molecule
KREMEN1	Kringle containing transmembrane protein 1	Protease
MGP	Matrix Gla protein	Signalling molecule
MMP13	Matrix metalloproteinase 13 (collagenase 3)	Protease
OMD	Osteomodulin	Extracellular matrix
PTGDS	Prostaglandin D2 synthase 21kDa (brain)	Synthase and synthetase
RGS4	Regulator of G-protein signalling 4	Select regulatory molecule
RUNX2	Runt-related transcription factor 2	Transcription factor
SATB2	SATB homeobox 2	Transcription factor
SGK1	Serum/glucocorticoid regulated kinase 1	Kinase
SQSTM1	Sequestosome 1	Molecular function unclassified
TNC	Tenascin C	Extracellular matrix
WISP2	WNT1 inducible signalling pathway protein 2	Signalling molecule
WISP3	WNT1 inducible signalling pathway protein 3	Signalling molecule
18S	Eukaryotic 18S rRNA	Molecular function unclassified
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase

 Table 2.2: Genes included in the human low density arrays containing 24 primer-probesets

 Results from these arrays are presented in Chapter 4.

Gene Svmbol	Gene Name	Molecular Classification
ALPL	Alkaline phosphatase, liver/bone/kidney	Phosphatase
BGLAP	Bone gla protein (osteocalcin)	Select calcium binding protein
BMP2	Bone morphogenetic protein 2	Signalling molecule
BMP4	Bone morphogenetic protein 4	Signalling molecule
BMP6	Bone morphogenetic protein 6	Signalling molecule
CCL2	Chemokine (C-C motif) ligand 2	Signalling molecule
CDH11	Cadherin 11, type 2, osteoblast-cadherin	Cell adhesion molecule
CDH2	Cadherin 2. type 1. neuronal-cadherin	Cell adhesion molecule
COL1A1	Collagen, type I, alpha 1	Extracellular matrix
CSF1	Colony stimulating factor 1 (macrophage)	Signalling molecule
CTNNB1	Catenin, beta 1	Cytoskeletal protein
DKK1	Dickkopf homolog 1	Signalling molecule
DKK2	Dickkopf homolog 2	Signalling molecule
DI X5	Distal-less homeobox 5	Transcription factor
EPHB4	EPH receptor B4	Receptor
EGE2	Fibroblast growth factor 2	Signalling molecule
FGFR1	Fibroblast growth factor recentor 1	Receptor
FGFR2	Fibroblast growth factor receptor 2	Receptor
FOS	FB. Losteosarcoma oncogene homolog	Transcription factor
FOSI 1	FOS-like antigen 1	Transcription factor
IBSP	Bone sialoprotein	Extracellular matrix
IGF1	Insulin-like growth factor 1	
II 1R	Interleukin 1 beta	
11.6	Interleukin 6	
		Transcription factor
	Jun B proto-oncogene	Transcription factor
KI F10	Kruppel-like factor 10	Transcription factor
LRP5	Low density lipoprotein receptor-related	Receptor
	protein 5	
MYC	v-myc oncogene homolog	Transcription factor
NFATC1	Nuclear factor of activated T-cells, calcineurin-dependent 1	Transcription factor
NFKB1	Nuclear factor kappa B 1 (p105)	Transcription factor
NFKBIA	Nuclear factor kappa B inhibitor, alpha (IkB)	Molecular function unclassified
RELA	v-rel A, nuclear factor kappa B 3, p65	Transcription factor
RUNX2	Runt-related transcription factor 2	Transcription factor
SMAD5	SMAD family member 5	Transcription factor
SMAD6	SMAD family member 6	Transcription factor
SMAD7	SMAD family member 7	Transcription factor
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	Ligase
SP7	Sp7 transcription factor (osterix)	Transcription factor
SPP1	Secreted phosphoprotein 1 (osteopontin)	Signalling molecule
TGFB1	Transforming growth factor, beta 1	Signalling molecule
TNFRSF11B	Osteoprotegerin	Receptor
TNFSF11	Receptor activator of NFkB ligand (RANKL)	Signalling molecule
VCAM1	Vascular cell adhesion molecule 1	Cell adhesion molecule
VDR	Vitamin D receptor	Transcription factor
18S	Eukaryotic 18S rRNA	Molecular function unclassified
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase
HMBS	Hydroxymethylbilane synthase	Hydrolase

Table 2.3: Genes included in the human low density arrays containing 48 primer-probesets

2.3.4.2 Allelic discrimination

Allelic discrimination experiments were performed using an Assays-by-Design primerprobeset to detect the 1215C/T (P392L) mutation in SQSTM1 cDNA. Minor Groove Binder (MGB) probes were used to distinguish wild-type (1215C) 5'-VIC-CAGCCGCGGGTCA-MGB-3', and mutant (1215T) 5'-FAM-TCAGCCGCAGGTCA-MGB-3' sequences. The primers ('Allelic discrimination') are shown in Table 2.1. Plasmids containing the wild-type and mutant SQSTM1 sequence were used to validate the assay. The plasmids alone, and in various mixtures were included as standards in all experiments, as was a no template control (NTC). The presence of SOSTM1 transcripts in all samples at high and stable levels was confirmed using real time PCR. Reactions were performed with TaqMan Genotyping Master Mix. cDNA was diluted 1:10-1:20 and plasmid standards were used at a concentration of 10 or 0.4 pg/µL. All pagetic and non-pagetic samples from patients with Paget's disease were tested in quadruplicate at least three times, and at least five control samples from patients without Paget's disease were included on each plate. Data was collected during the amplification for quality control, and endpoint data was analysed using the SDS2.3 software. The analysis required further manipulation of the data as the VIC and the FAM signals were not totally exclusive. The NTC determined the 0,0 point then all points were rotated by the angle of the line between the wild-type standard and 0,0 and the x-axis. y values of samples were then compared to the wild-type standard (which now has a y value of 0) using a student's t test.

2.3.5 Microarrays

2.3.5.1 Human osteoblast samples

All samples chosen for microarray hybridisation had RNA integrity number (RIN) values determined by a bioanalyser (Agilent Technologies) of 9.2–10. Preparation of the RNA and hybridisation and scanning of the arrays was performed at the School of Biological Sciences with the assistance of Liam Williams. RNA was reverse transcribed to cDNA using SuperScript II, and cDNA second strand was synthesized with *Escherichia coli* DNA polymerase I. Biotinylated cRNA was produced by *in vitro* transcription and fragmented to produce small RNA probes that were hybridized to the Human Genome U133A 2.0 GeneChip. After hybridization, the gene chips were automatically washed and stained with streptavidinphycoerythrin in a fluidics system and scanned with a GeneArray Scanner (Hewlett-Packard). After scanning, the Affymetrix GeneChip Operating Software (GCOS) was used to produce fluorescence intensity values for each of the perfect-match and mismatch

probes on each array. This information was exported to CEL data files, which were in turn imported into the R statistical computing environment [216] for analysis.

Analysis of the microarray data was performed by Dr. Mik Black and Sarah Song from the Department of Statistics. The Affymetrix package [217] from the Bioconductor suite of analysis tools [218] was used to apply the robust multichip analysis (RMA) algorithm [219] to the data from each array. The Limma package [220] was used to perform a linear model-based analysis of the RMA normalized data, with the goal of detecting probesets undergoing significant changes in expression level between pagetic and non-pagetic RNA. For each comparison, the false discovery rate controlling method of Benjamini and Hochberg [221] was used to produce adjusted p values for each probeset, based on a significance level of α = 0.05. Some additional analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. Pathway analysis was performed using genelists from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) [222]. Analysis of changes in gene expression in KEGG pathways was performed in Bioconductor using three methods: significance analysis of function and expression (SAFE) [223], globaltest [224], and principal coordinates and Hotelling's T² (PCOT2) [225].

2.3.5.2 SQSTM1-transfected SaOS2 cells

Twenty samples from two different experiments where SaOS2 cells were transfected with plasmids and treated with TNF α were chosen for analysis. RNA quality was confirmed using the bioanalyser and all samples had RIN values of 9.4-9.9. RNA was labelled and prepared by Liam Williams according to the manufacturer's instructions, and samples were hybridised to Human Gene 1.0 ST Arrays which contain probesets representing >28,000 genes. Chips were processed and scanned as described above. Quality control was performed using the Affymetrix Bioconductor package, and analysis was performed in Partek Genomics suite [226] and BRB-ArrayTools.

2.3.6 Cloning

The human *SQSTM1* transcript was cloned into the pcDNA3.1/His A vector. The *SQSTM1* insert was prepared from cDNA using the PCRx enhancer kit. The 50 µL reaction contained 1x PCRx amplification buffer, 2 mM MgSO₄, 15 pmol of each primer (SQSTM1 for cloning, Table 2.1), 0.5x enhancer, 0.2 mM dNTPs and 2.5 units High Fidelity Platinum Taq Polymerase. After an initial incubation at 95°C, 35 cycles of 95°C for 30 s, 55°C for 30 s and

68°C for 2 min were performed, with a final elongation step of 68°C for 10 min. These primers introduced a KpnI restriction site near the beginning of the transcript (amino acid 6 onwards were preserved), and an XbaI restriction site after the stop codon. The insert and the plasmid were sequentially digested with KpnI and XbaI with purification steps after each digest using the QIAquick Gel Extraction Kit. Ligation was performed in a 20 μ L volume containing 1x DNA ligase reaction buffer, 1 unit T4 DNA Ligase, 30 fmol of vector and 90 fmol of insert, then incubated for 5 min at room temperature. 1 μ L of ligated plasmid was used to transform competent DH5 α *E. coli* cells which were grown on LB Agar plates containing 100 μ g/mL ampicillin. Small scale plasmid preparations were produced from colonies using the High Pure Plasmid Isolation Kit, and plasmids were confirmed to be the expected size on a 0.8% agarose gel. Sequencing using the T7 (on plasmid before the insert), BGH (on plasmid after the insert) and SQSTM1 cDNA (Table 2.1) forward and reverse primers confirmed the presence of the expected sequence of the insert and the vector-insert boundaries.

The P392L (1215C/T) and 396X (1226G/T) mutations were introduced using the QuikChange Site Directed Mutagenesis Kit according to the manufacturer's instructions. PCR was performed in a 50 μ L reaction mixture containing 1x reaction buffer, 50 ng plasmid DNA, 125 ng each primer (P392L mutation and 396X mutation, Table 2.1), 1 μ L dNTPs, and 1 μ L PfuTurbo DNA polymerase. Samples were incubated at 95°C for 30 s, then 16 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 7 min. 10 units of DpnI were added to the PCR products and they were incubated at 37°C for 1 hr, then 1 μ L was used to transform the XL1-Blue cells provided. Plasmids were purified from the colonies produced and sequenced to confirm the presence of the mutation.

These plasmids and other commercial plasmids were purified initially using the High Pure Plasmid Isolation Kit, and preparations for large scale transfections were prepared using the Plasmid Mega Kit, both according to the manufacturer's instructions.

2.3.7 Adenoviral vectors

The keratin 18 adenoviral vector was constructed by Usha Bava under the supervision of Dr. Nicole Horwood at the Kennedy Institute, Imperial College, UK. The control green fluorescent protein (GFP) vector was also produced at the Kennedy Institute. A human keratin 18 (*KRT18*) IMAGE clone (number 3850724, accession number BC020982) in the pSport6

vector was purchased from Geneservice. The sequence was cut out using SalI and NotI, the ends were polished, then inserted into pENTR4.3F which was cut with SmaI. This plasmid also contains the EGFP gene which produces enhanced GFP. The *KRT18*-containing pENTR4.3 vector was then recombined with the pAd/PL DEST vector, which was digested with PacI, then transfected into HEK293A cells. An agarose overlay was performed and plaques were picked and used to infect more HEK293A cells. Expression of keratin 18 was confirmed by western blotting.

Adenoviruses were propagated in a PC2 facility at the University of Auckland. HEK293A cells were infected with the adenoviral vectors in T175 flasks, and cultured for approximately 30 hr until partial cytopathic effect was apparent. Cells and media were collected, and used for purification with the Adeno-X Maxi Purification Kit. Cell pellets were freeze-thawed three times to lyse cells. The titre of the purified virus was measured using the Adeno-X Titre Kit according to the manufacturer's instructions.

2.3.7.1 Adenoviral transduction

SaOS2 cells were transduced the day after seeding, and multiplicity of infection (MOI) was determined based on the number of cells seeded. The following day virus stocks were diluted to the appropriate concentration in α MEM 5% FBS, media was removed from the wells and 1 mL of virus-containing media was added to each well (in 6-well plates, 200 µL per well added in 24-well plates). After 6 hr incubation, additional standard growth media was added to the wells, 2 mL/well in 6-well plates, 0.5 mL/well in 24-well plates. Weak fluorescent signal was visible 24 hr later, with much stronger signal apparent by 48 hr after transduction.

Human osteoblasts and mesenchymal cells were transduced using Fugene6, based on the protocol of Fouletier-Dilling *et al.* [227]. Cells were seeded, and following overnight incubation, media was changed to fresh DMEM 5% FBS + 10 μ g/mL A2P (750 μ L/well in 6-well plates, 200 μ L/well in 24-well plates). The transfection mix for 6-well plates was made up to a volume of 500 μ L in OptiMEM. 10 μ L Fugene6 was added to the OptiMEM and incubated for 5 min, then the appropriate volume of virus for the desired MOI was added and incubated for a further 15 min, before the mix was added to the appropriate wells. 500 μ L of OptiMEM only was added to control wells. The total volume of mix per well in 24-well plates was 50 μ L, and included 2 μ L of Fugene6. The remaining protocol was identical to the procedure for the SaOS2 cells described above.

V=VI_List of research project topics and materials

2.4 Protein detection

2.4.1 ELISA

A modification of the method described by Tian et al. [74] was used to determine DKK1 protein concentrations. Microtitre plates were coated with 100 μ L of anti-DKK1 polyclonal antibody at a concentration of 1 μ g/mL in PBS. After an overnight incubation at 4°C, the reaction was blocked with 4% BSA. Conditioned media and serum samples were diluted up to 1:8 in dilution buffer (1× PBS, 0.1% Tween-20, and 1% BSA). A standard curve was created using serial 1:2 dilutions of recombinant human DKK1 with concentrations from 0.3125 to 20 ng/mL. A total of 100 μ L of standard or sample was loaded per well and incubated overnight at 4°C. The plate was then washed, and incubated with biotinylated goat anti-human DKK1 IgG diluted to a concentration of 0.2 μ g/mL in dilution buffer. This was followed by the addition of streptavidin-horseradish peroxidase, then Substrate Reagent. The reaction was stopped with 1 M sulfuric acid, and absorbance was measured at 450 nm. In addition to conditioned media, stored serum samples from patients who participated in a trial for ibandronate in Auckland were used in this assay [88], along with age- and sex-matched controls sourced from calcium supplementation studies.

The concentration of secreted OPG protein was determined with the Human Osteoprotegerin/TNFRSF11B DuoSet according to the manufacturer's instructions, used with the same Substrate Reagent pack as above.

2.4.2 Western blotting

2.4.2.1 Electrophoresis

Cell lysates were harvested from 6-well plates using HTNG lysis buffer containing protease inhibitor cocktail. Samples were mixed 4:1 with loading buffer, and boiled for 5 min to ensure denaturation. Samples were loaded into 12% Tris-HCl polyacrylamide precast minigels and run in running buffer at 200V for approximately 45 min. All gels included a prestained protein ladder and MagicMark standard.

2.4.2.2 Western blot

Proteins were transferred to a Hybond-C nitrocellulose membrane at a constant current of approximately 150mA overnight in transfer buffer. Membranes were stained with Ponceau

(0.2% in 3% trichloroacetic acid) to ensure protein was present, then blocked in TBS buffer containing 0.1% Tween 20 (TBS-T) and 5% BSA. TBS-T was also used for all washing steps. The primary antibody was diluted in the blocking buffer, and incubated for about 2 hr. The membrane was then washed and incubated with the appropriate secondary antibody diluted 1:15,000 in TBS-T containing 2.5% BSA for 1 hr. After a final thorough wash, membranes were developed using ECL reagent and the chemiluminescence imaged using a LAS3000 (Fuji). Quantification was performed using the accompanying Multigauge software.

2.4.3 Luminex

A Beadlyte 8-Plex Multi-Pathway Signalling Kit - Phosphoprotein was used to measure levels of eight phosphoproteins in SaOS2 cells. Cells were seeded in 24-well plates at a density of 5 x 10^4 cells/well in α MEM 5% FBS. The following day cells were serum starved and transduced with the SOSTM1 constructs using a Fugene6:DNA ratio of 6:1. A plasmid expressing β -galactosidase was included in some mixes as a transfection efficiency control. The following day drugs were added, and cells were harvested in the cell signalling universal lysis buffer provided with the kit with protease inhibitor cocktail added. Cells were washed in ice cold TBS, 75 µL lysis buffer was added and cells were shaken at 800 rpm for 15 min. Lysates were then spun at 4500g for 20 min at 4°C and supernatants were transferred to a fresh tube and stored at -80°C. Protein concentration was determined using the DC protein assay, and samples were diluted to a concentration of 225 μ g/mL using the cell signalling universal assay buffer provided immediately before the Luminex assay was run. βgalactosidase activity was determined in cells harvested in reporter lysis buffer to ensure that there was similar transfection efficiency with the different plasmids. ONPG substrate was incubated with the cell lysates for 1 hr at room temperature then absorbance at 420 nm was measured. The Luminex assay was run with the assistance of Sofian Tijono at the Auckland Cancer Society Research Centre according to the manufacturer's instructions. A standard curve using serial dilutions of the stimulated HeLa cell lysate provided was included.

2.5 Statistical analysis and graphs

Statistical analysis was performed as described in the figure legends and text using various software packages including GraphPad Prism and Microsoft Excel. Graphs show the mean and error bars indicate the standard error of the mean unless otherwise stated. Statistical significance is indicated as follows: * p<0.05; ** p<0.01; *** p<0.001.

CHAPTER 3: GLOBAL ANALYSIS OF GENE EXPRESSION IN PAGETIC OSTEOBLASTS

3.1 Introduction

Osteoclasts have been the focus of most of the research on Paget's disease, and it has frequently been classified as a disease of the osteoclast. However, the coupled remodelling that occurs within the pagetic lesion suggests that osteoblasts may also be abnormal, especially considering they play such an important role in regulating osteoclast development and activity. Previous studies have suggested the stromal component of pagetic bone marrow influences osteoclast precursor numbers [109]. In addition, many of the cell culture studies that show increased osteoclast formation and vitamin D hypersensitivity have been performed in bone marrow, which contains cells of the osteoblastic lineage so osteoblast abnormalities may contribute to these observations [109-112]. There are also some studies that indicate there are likely to be changes in gene expression in pagetic osteoblasts. Hankey et al. found changes in secreted protein levels in pagetic osteoblasts compared to controls on two dimensional protein gels, however only one of the proteins was identified [121]. Other studies that employed qualitative or semi-quantitative techniques have identified changes in gene or protein expression in osteoblasts, such as IL-6, IL-6 receptor and TNFα [117, 228] or in genes primarily produced by osteoblasts such as RANKL, OPG and bone matrix proteins [108, 113, 120].

We have collected RNA samples from osteoblasts and bone marrow cultured from 24 patients with Paget's disease and 32 control patients. Twelve of these samples were used for a global analysis of gene expression using microarray technology. Microarrays can be used to analyse the mRNA transcript abundance for thousands of genes, and have been utilised to identify changes in other diseases, particularly cancer. Breast cancer has been a major focus of this type of approach, and large numbers of breast cancer RNA samples have been analysed using microarrays in order to classify subtypes of disease or for class prediction in order to predict prognosis in new patients [229]. Classification analysis has also been performed in other cancers such as multiple myeloma [230], however this type of analysis was not possible in our

study given the large number of samples required. Microarrays are also useful for identifying genes and gene networks that are altered, which can lead to the identification of disease mechanisms. The microarrays performed on multiple myeloma samples identified significantly increased *DKK1* expression in patients with more advanced myeloma bone disease [74]. This culminated in the discovery that inhibition of Wnt signalling, particularly via increased DKK1 secretion, is a major cause of decreased osteoblast activity in lytic bone lesions in myeloma and metastatic bone cancer [231-235]. Development of DKK1 antibodies is now in progress and they show promise as a therapy for this deadly disease [236].

In this study we have used microarrays to investigate changes in transcript abundance in pagetic osteoblasts, and identify genes and pathways that may be important in the disease.

3.2 Methods

3.2.1 Sample details

Details of the patients with Paget's disease whose RNA samples are included in the analyses described in this thesis are shown in Table 3.1. A summary of the basic details of the pagetic and control patients is provided in Table 3.2. All samples described as 'pagetic' were derived from pagetic lesions that had been confirmed by radiology or scintigraphy. The majority of the patients had fairly mild Paget's disease, with only nine showing alkaline phosphatase levels above the reference range at the time of sample collection, and most having only one or two bones affected. At least 21 of the patients had elevated alkaline phosphatase levels at diagnosis, but only one had a measurement greater than 500 IU/litre. Most had undergone bisphosphonate treatment: 20 of the 24 patients had received treatment at some stage, and eight had received treatment during the 12 months prior to sample collection. When broken down into sample types, two of the osteoblast and three of the bone marrow RNA samples were from patients who had never received treatment, while six of each sample type were from patients who had received bisphosphonates in the past year. In addition to samples collected from pagetic lesions, some samples were collected from unaffected bone in patients with Paget's disease. In eight patients, osteoblast samples were acquired from an affected and an unaffected site, providing paired samples. Comparing gene expression in pagetic and nonpagetic paired samples had the advantage of controlling for bisphosphonate treatment and inter-patient variation.

ID	Age	Sex	ALP ^a	No. of	Pagetic	Non-	Pagetic	Non-	Family
	(years)		(IU/litre)	Bones Involved	OB	pagetic OB	BM	pagetic BM	History
1	72	Μ	320	11	+				yes
2	76	Μ	100	2	+	+	S		?
3	77	Μ	74	1			А		?
4	70	Μ	96	13			А		no
5	81	F	667	2	+		S		?
6	79	F	67	2			S		?
7	73	Μ	142	2	+	+			?
8	67	Μ	127	2			А		no
9	79	F	63	4			А		no
10	89	F	135	1	+	+			?
11	77	Μ	103	1	+				?
12	79	Μ	77	2	+		S		?
13	78	Μ	130	1	+		S		?
14	84	Μ	198	3			S		no
15	89	F	105	1	+	+		S	?
16	74	Μ	106	1	+	+			no
17	85	Μ	74	3			А		yes
18	79	Μ	115	2	+	+			no
19	82	Μ	60	1	+		S		no
20	78	Μ	318	3			А		no
21	80	F	92	2	+	+		S	?
22	63	Μ	89	2	+	+			yes
23	60	Μ	57	4			А		no
24	83	Μ	73	2		+			?

Table 3.1: Patient and sample details for the patients with Paget's disease

ALP: serum alkaline phosphatase activity at the time of tissue sampling

OB: osteoblasts

BM: bone marrow cells

A: bone marrow from iliac crest aspiration

S: bone marrow from surgery

?: not determined ^a Normal range for alkaline phosphatase 40-110 IU/litre

Table 3.2: Summar	y of	patient	details and	d samp	oles co	ollected	from	patients	with	Paget'	s disease	and	contro	ls
										<u> </u>				

· · ·	Paget's	Controls	p value
	Disease		F
Number of patients	24	32	
Sex (M/F)	18/6	11/21	p>0.05
Age – Median	78.5	68	p>0.05
– Range	60-89	30-84	-
Serum alkaline phosphatase			
– Median	101.5	-	
– Range	57-667	-	
Number of bones involved			
– Median	2	-	
– Range	1-13	-	
Samples			
 Non-pagetic osteoblasts 	9	20	
 Pagetic osteoblasts 	14	-	
 Non-pagetic bone marrow 	2	21	
 Pagetic bone marrow 	14	-	

3.2.2 Cell characteristics

The osteoblastic phenotype of cells obtained from the bone chip outgrowth cultures was confirmed using alkaline phosphatase staining (Figure 3.1), which was comparable in pagetic and non-pagetic samples. These cells were also shown to express the osteoblastic markers alkaline phosphatase, osteocalcin and type I collagen α 1 using RT-PCR (data not shown). Pagetic and non-pagetic osteoblast cultures took a similar time to reach confluence (about 15 days), and the cells showed a uniform appearance under light microscopy which was similar for pagetic and non-pagetic cultures. The bone marrow cultures were clearly a mixed cell culture, containing both stromal and haematopoietic cells, and the appearance was not obviously different between pagetic and non-pagetic samples. Pagetic cells tended to take longer to reach confluence (about 16 days compared to 13 days for non-pagetic cells). Cultures contained both alkaline phosphatase and TRAP positive cells (Figure 3.2), and in some cases mono- and multinucleated TRAP positive cells remained in the tissue culture flask after cells were trypsinised for RNA extraction.



Figure 3.1: Alkaline phosphatase staining in osteoblast cultures Cells from pagetic (a) and non-pagetic (b) cultures are shown. Most cells show some staining and have typical osteoblast morphology. The scale bar represents 200µm.



Figure 3.2: Pagetic bone marrow culture stained for alkaline phosphatase and TRAP The images demonstrate staining for alkaline phosphatase in many cells (a), and TRAP staining in some cells (b).

3.3 Microarray analysis results

3.3.1 Overview of Affymetrix microarrays

In this study we used Affymetrix Human Genome U133A 2.0 GeneChips which include over 22,000 probesets and measure transcript abundance of over 14,500 genes [237]. These Affymetrix chips are single channel arrays meaning only one RNA sample is analysed on each chip. Each spot on the array contains 25 bp probes that target the 3' end of a specific mRNA transcript, and are known as a probeset. Each probeset includes perfect-match probes which hybridise specifically to the target, and mismatch probes which are the same probe with one incorrect base in the middle of the sequence. Specific hybridisation is calculated by subtracting the intensity of the mismatch signal from the perfect-match signal. Many genes are only represented by one probeset, but some are represented by multiple probesets. Different probesets for the same gene can represent different transcript variants or perform differently, so do not always show analogous results meaning it can be advantageous to consider probesets individually in at least some of the analysis. Labelled cRNA fragments are hybridised to the array, and the chip is scanned and fluorescence levels measured to determine mRNA transcript abundance for each probeset. The data can be filtered to include only genes that are actually expressed. Present or absent calls are determined based on the relative intensities of perfect-match and mismatch probes, so if the signals are too similar or the mismatch signal is higher, a probeset is assigned an absent call. Probesets with fluorescent intensity below a certain threshold may also be excluded due to very low expression. In the analysis described later performed using BRB-ArrayTools, probesets were often excluded if they contained absent calls in over half the samples, or if over half the samples had intensity measures below 10.

3.3.2 Quality control and normalisation

Twelve samples were chosen for microarray analysis. The patient details for these samples are summarised in Table 3.3. RNA quality was determined using an Agilent bioanalyser, and only samples where enough RNA was available that had RIN values of 9.2-10 were chosen for analysis. Bioanalyser results from some of the RNA samples are shown in Figure 3.3. Eight of the samples run on the microarrays were derived from pagetic patients. Five of these samples were pagetic, and three were non-pagetic paired samples. Four non-pagetic samples from control patients were also tested. Hybridisation and scanning were performed as described in Section 2.3.5.1. Quality control, normalisation and much of the analysis was performed in the

Department of Statistics by Sarah Song under the supervision of Dr. Mik Black. The data was run through the quality control and analysis pipeline described in Section 2.3.5.1. Quality control data indicated that all arrays were suitable for analysis. Normalisation was required, and was performed using the RMA algorithm. The effects of the normalisation are illustrated in Figure 3.4.



Figure 3.3: Gel and electropherogram images of selected RNA samples examined using the bioanalyser Four of the samples (in lanes 7-10) on the gel image (a) were chosen for microarray hybridisation. One of these, sample 4P, is shown in the electropherogram (b), and has a RIN of 9.5.

Sample ID(s)	Age (years)	Sex	ALP ^a (IU/litre)	No. of bones involved	Time since bisphosphonate treatment
1P, 1NP	89	F	105	1	<1 year
2P, 2NP	74	М	106	1	>1 year
3P, 3NP	79	М	115	2	>1 year
4P	79	М	77	2	None
5P	78	М	130	1	<1 year
6	68	М	-	-	None
7	74	М	-	-	None
8	54	F	-	-	None
9	70	М	-	-	None

Table 3.3: Patient details for the 12 RNA samples run on the microarrays

Sample IDs: P - sample from pagetic lesion; NP - sample from unaffected bone in a patient with Paget's disease. ALP: serum alkaline phosphatase activity at the time of tissue sampling

Time since bisphosphonate treatment: time passed since most recent treatment, none indicates patient has never received bisphosphonate treatment.

^a Normal range for alkaline phosphatase 40-110 IU/litre

3.3.3 Global analysis of gene expression

Clustering analysis was performed in BRB-ArrayTools using centred correlation and average linkage. When performed on all the probesets that passed the expression filters, the control samples separated from the pagetic samples suggesting there are overall differences between samples from patients with and without Paget's disease (Figure 3.5(a)). When the non-pagetic paired samples were included in this analysis they grouped with their pagetic pair rather than with the control samples. This is not particularly surprising as there does tend to be a great deal of inter-patient variability in human gene expression. Likewise, when the paired samples only are analysed using all gene expression data, they group into patients (Figure 3.5).

This analysis was also performed using a subset of genes that showed more variable expression. 269 probesets passed the expression threshold in at least eight of the 12 samples, and showed more than two-fold variation from the median in at least four of the 12 samples, and these were used to perform the sample clustering analysis again (Figure 3.6). In this analysis there was separation of the pagetic samples from the non-pagetic samples with the exception of one of the paired non-pagetic samples (sample 3NP) which consistently grouped with the pagetic samples in cluster analysis using various gene sets (data not shown). Interestingly, when the paired samples only are analysed in this model they do separate into pagetic tissue, or it was an unidentified site of Paget's disease. In the analysis with the paired non-pagetic samples removed there is again clear separation of control and pagetic samples. This analysis suggests that osteoblasts from pagetic lesions have a different transcript abundance profile to control osteoblasts overall.



Figure 3.4: Box plots showing distribution of gene expression in the twelve microarrays Data is shown before (a) and after (b) normalisation using the RMA algorithm.





Figure 3.5: Dendrograms for clustering experiments using all expressed probesets Analysis was performed using centred correlation and average linkage with all 13216 probesets that were expressed above the standard threshold level in at least half the samples. Analysis is shown for samples from all nine patients with the paired non-pagetic samples excluded (a); all 12 samples (b); and in the paired samples only (c). The sample type is indicated by the colour of the label, control samples are black (6-9), pagetic samples are red (1P-5P), and non-pagetic paired samples from patients with Paget's disease are blue and have the same number as their pagetic pair (1NP-3NP).

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Figure 3.6: Dendrograms for clustering experiments using the 269 most changed probesets Analysis was performed using centred correlation and average linkage with the 269 probesets that were expressed above the standard threshold level in at least 8 of 12 samples and had over 2-fold variation from the median in at least 4 of 12 samples. Analysis is shown for samples from all nine patients with the paired nonpagetic samples excluded (a); all 12 samples (b); and in the paired samples only (c). The labels are the same as the previous figure: controls are black; pagetic samples are red; and non-pagetic paired samples are blue.

	Symbol	Name	Fold change	р
1	GCA	grancalcin, EF-hand calcium binding protein	0.673	0.016
2	FLJ23191	hypothetical protein FLJ23191	0.179	0.016
3	EPB41L4B	erythrocyte membrane protein band 4.1 like 4B	2.142	0.016
4	GULP1	GULP, engulfment adaptor PTB domain containing	2.825	0.025
5	RAI3	retinoic acid induced 3	2.500	0.025
6	SATB2	SATB family member 2	0.576	0.025
7	KRT18	keratin 18	6.761	0.042
8	RBPMS	RNA binding protein with multiple splicing	1.600	0.042
9	TNXB	tenascin XB	2.169	0.042
10	GLRB	glycine receptor, beta	0.689	0.048
11	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	0.355	0.048

Table 3.4: Genes showing statistically significant changes in expression in microarray analysis of pagetic and non-pagetic osteoblast RNA samples

Symbols are Human Genome Organization (HUGO)-approved gene symbols for the Affymetrix probesets. The numbers under 'fold change' here and in the following tables represent the pagetic/non-pagetic ratio of the means of expression in each group, and the p values are adjusted for each gene as described in Section 2.3.5.1.

Table 3.5: Top 10 up-regulated and down-regulated genes in the microarray analys	is of pagetic and non-pagetic
osteoblast RNA samples ranked according to fold change	

	Symbol	Name	Fold	p
	• • • • • •		change	P
Up-	regulated			
1	IFI27	interferon, alpha-inducible protein 27	6.86	0.08
2	KRT18	keratin 18	6.76	0.04
3	RGS4	regulator of G-protein signalling 4	6.09	0.16
4	GATA6	GATA binding protein 6	5.24	0.20
5	ZIC1	Zic family member 1 (odd-paired homolog, Drosophila)	4.76	0.05
6	MGP	matrix Gla protein	4.40	0.19
7	PARG1	PTPL1-associated RHOGAP1	4.33	0.16
8	FLG	filaggrin	4.30	0.67
9	MFAP5	microfibrillar associated protein 5	4.18	0.39
10	DKK1	dickkopf homolog 1 (Xenopus laevis)	3.98	0.41
Dov	wn-regulate	d		
1	IBSP	integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	0.16	0.05
2	FLJ23191	hypothetical protein FLJ23191	0.18	0.02
3	COL10A1	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)	0.18	0.06
4	OMD	osteomodulin	0.24	0.54
5	C2orf31	chromosome 2 open reading frame 31	0.26	0.11
6	BGLAP	bone gla protein (osteocalcin)	0.27	0.07
7	CH25H	cholesterol 25-hydroxylase	0.27	0.48
8	PPL	periplakin	0.29	0.25
9	LSP1	lymphocyte-specific protein 1	0.30	0.16
10	PDGFD	platelet derived growth factor D	0.31	0.49

3.3.4 Expression of individual genes

Differences in the transcript abundance of individual genes between the samples from pagetic sites and non-pagetic sites were also quantified as described in Section 2.3.5.1. Lists of genes

that showed the largest differences between pagetic and non-pagetic samples were created based on p value, which determines how statistically significant the differences between groups were, and based on fold change which determines which genes show the largest differences in average expression between pagetic and non-pagetic groups. Once corrections had been made for multiple statistical comparisons by controlling for the false discovery rate, eleven genes showed statistically significant changes (Table 3.4). This list is brief due to the relatively small number of samples analysed and the strict p value criteria used. Unfortunately many of these genes were not well characterised (such as FLJ23191) or did not have an obvious role in bone biology, while a number of others had very low expression levels (GCA, EPB41L4B, RAI3, and TNXB) so were not included in further studies. Genes were also sorted according to fold change between the groups of pagetic and non-pagetic samples, which can be a more reliable method of identifying differentially regulated genes [238]. The top ten upregulated and down-regulated genes are shown in Table 3.5 [239]. The data from these two tables is summarised in the heat map shown in Figure 3.7 which shows good separation of pagetic and non-pagetic samples for these genes. While many of the genes that are highly upor down-regulated do not have significant p values, there are genes that are potentially relevant to bone biology and Paget's disease, such as the osteoblast differentiation markers bone sialoprotein (IBSP) and osteocalcin (BGLAP) and other matrix proteins, matrix gla protein and osteomodulin in these lists. Therefore, the p value cut-off used in these analyses may have been too stringent. Microarray statisticians now recommend creating gene lists using multiple methods, rather than using an arbitrary p value cut-off [238, 240]. A number of the genes in these lists were chosen as candidate genes for further expression analysis in a larger group of samples, in addition to other genes that showed less highly ranked changes, but were predicted to be biologically relevant. These results are presented in Chapter 4.

A similar analysis was performed using the three paired samples included in the microarrays to produce a list of the most highly changed genes in the pagetic lesion compared to control tissue from the same patient. A list of genes that showed 2-fold change or greater in each pair was compiled, then ranked according to the highest fold changes overall. The top 30 genes in this list are shown in Table 3.6 and a heat map representing most of the top 50 probes is shown in Figure 3.8. Many of the differentially expressed genes in the paired samples were also changed overall in the comparison of expression in pagetic and non-pagetic samples, so the overall analysis was generally used when choosing genes for further investigation.



Figure 3.7: Heat map showing expression levels of genes with the most significant changes or highest fold changes between pagetic and non-pagetic gene expression overall

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Each spot represents the data from one probeset for each sample. Samples are indicated across the top, and genes are down the side. Green spots indicate relative lower expression while red spots indicate relative higher expression. The samples numbers and colours are the same as in previous figures. The genes in the top half are up-regulated in Paget's disease while the bottom ones are down-regulated.

			Fold Change			
			Patient	Patient	Patient	
	Symbol	Name	1	2	3	Overall
1	CHI3L1	chitinase 3-like 1	5.60	6.09	14.97	8.89
2	ZIC1	Zic family member 1	6.81	7.32	8.33	7.49
3	COL10A1	collagen type X alpha 1	0.10	0.24	0.13	0.16
4	THBD	thrombomodulin	2.09	7.09	7.63	5.60
5	FLJ23191	hypothetical protein FL 123191	0.23	0.20	0.15	0.19
6	MMP1	matrix metalloproteinase 1 (interstitial	3.02	9.06	2 69	4 92
•		collagenase)	0.02	0.00	2.00	
7	MGP	matrix Gla protein	5.20	6.10	2.80	4.70
8	KRTAP1-1	keratin associated protein 1-1	0.27	0.20	0.18	0.22
9	CD24	CD24 antigen	0.16	0.29	0.24	0.23
10	SNAP25	synaptosomal-associated protein, 25kDa	0.20	0.35	0.19	0.25
11	KRT18	keratin 18	4.20	4.87	2.75	3.94
12	HAPLN1	hyaluronan and proteoglycan link protein 1	3.26	5.42	2.91	3.86
13	PTGIS	prostaglandin I2 (prostacyclin) synthase	5.36	2.35	3.48	3.73
14	DMD	dystrophin	4.46	3.35	3.04	3.62
15	IBSP	integrin-binding sialoprotein (bone sialoprotein)	0.41	0.14	0.29	0.28
16	C14orf78	chromosome 14 open reading frame 78	2.04	4.05	4.71	3.60
17	CDKN1C	cvclin-dependent kinase inhibitor 1C	3.62	2.04	4.84	3.50
		(p57, Kip2)				
18	FBLN2	fibulin 2	2.45	2.45	5.54	3.48
19	CCND2	cvclin D2	3.39	2.39	4.61	3.46
20	BGLAP	bone gla protein (osteocalcin)	0.32	0.24	0.31	0.29
21	KRT7	keratin 7	0.31	0.33	0.30	0.31
22	HLA-DRA	major histocompatibility complex, class	0.49	0.21	0.25	0.31
		II, DR alpha				
23	SERPINB2	serine (or cysteine) proteinase inhibitor,	0.33	0.37	0.26	0.32
24	SI C16A6	clade B (ovalbumin), member 2	0.20	0.47	0.20	0.25
24	SLCTOAD	acid transporters) member 6	0.29	0.47	0.29	0.35
25	EFEMP1	EGF-containing fibulin-like extracellular	2.69	3.48	2.01	2.73
		matrix protein 1			-	-
26	GULP1	GULP, engulfment adaptor PTB domain containing 1	2.53	2.54	2.97	2.68
27	SCRN1	secernin 1	3.32	2.13	2.45	2.63
28	HAS1	hvaluronan synthase 1	0.27	0.45	0.44	0.39
29	MAN1C1	mannosidase, alpha, class 1C, member	2.18	2.06	3.42	2.55
		1		2.00	0.12	2.00
30	ENPP1	ectonucleotide	0.47	0.49	0.22	0.39
		pyrophosphatase/phosphodiesterase 2				
29 30	MAN1C1 ENPP1	mannosidase, alpha, class 1C, member 1 ectonucleotide pyrophosphatase/phosphodiesterase 2	2.18 0.47	2.06 0.49	3.42 0.22	2.55 0.39

Table 3.6: Top differentially regulated genes in the three paired samples



Figure 3.8: Heat map showing most highly changed genes in the paired samples The sample numbers and colours are the same as previous figures and the image is generated and presented in the same way as Figure 3.7.

3.3.5 Pathway analysis

Analysis of gene expression changes within the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways was performed using three different methods, SAFE, PCOT2 and globaltest, as described in Section 2.3.5.1. Globaltest showed the largest number of significantly changed pathways and SAFE showed the fewest. The lists generated using PCOT2 and globaltest had many pathways in common, consistent with reports in the literature

[241]. The pathways that showed more changes in gene expression than would be expected by chance using two or all three methodologies are shown in Table 3.7. Gene Ontology (GO) analysis was also performed using BRB ArrayTools. This analysis identifies gene groups with statistically significant changes overall. The groupings are based on biological processes, cellular components and molecular functions. Out of the 1475 GO categories examined, 82 showed statistically significant changes between pagetic and non-pagetic samples (p<0.005). The top 20 GO categories and three others that may have particular relevance to Paget's disease are shown in Table 3.8. The LS p value is calculated based on the mean of the p values of the genes within the group, and the KS p value indicates whether the p values of individual genes are uniformly distributed. Many of the pathways and categories in these lists do not have an obvious role in Paget's disease. However, it was interesting to see that the extracellular matrix-receptor interaction pathway was significantly changed using all three methods in the KEGG pathway analysis. Four of the most significantly changed GO categories were also associated with extracellular matrix, namely the most significantly altered pathway 'collagen', 'extracellular matrix structural constituent', 'structural constituent of bone' and 'cell-matrix adhesion'. These lists all include a number of genes found in bone matrix. It was also interesting to note that there are potential changes in the Wnt and TGFB signalling KEGG pathways which have important roles in osteoblast biology. Two GO categories associated with G-protein signalling, and 'second-messenger-mediated signalling' which can be stimulated by G protein-coupled receptor activation are in the list of most significantly regulated categories. This suggests there may also be changes in G-protein signalling, another pathway that has important functions in osteoblasts.

Given that *DKK1* was highly up-regulated we were interested in changes in the Wnt signalling pathway, however the KEGG pathway includes genes involved in signal transduction, but very few of the transcriptional targets of the pathway, which are likely to have altered levels of expression if the level of signalling has changed. A combined list excluding *DKK1*, but including other genes in the Wnt signalling KEGG pathway (hsa04310) and target genes from the list provided on the Wnt home page [242] was constructed and the pathway analysis was performed again. Both the global test and SAFE methods showed p values of <0.01 with this list of genes, further indicating that alterations in Wnt signalling may be important in Paget's disease. Unfortunately it is difficult to gauge from this analysis whether the pathway is more or less active than in controls, so while up-regulation of the inhibitor of Wnt signalling *DKK1* suggests the pathway may be less active in the pagetic

samples, *DKK1* is also a target gene of the pathway [243, 244]. Many of the target genes of the pathway were unchanged, but of those that were altered there is also disagreement. Some targets such as *WISP2* and *WISP3* tended to have decreased transcript abundance in pagetic samples, but others like *IL-6* and alkaline phosphatase showed increased abundance.

KEGG ID	KEGG Cate- gory ^a	KEGG Name	PCOT2 p value	SAFE p value	Global test p value
Statisti	cally sig	nificant using all three methods			
4512	3.3	Extracellular matrix-receptor interaction	0.002	0.049	0.000
760	1.9	Nicotinate and nicotinamide metabolism	0.015	0.029	0.001
770	1.9	Pantothenate and CoA biosynthesis	0.006	0.024	0.001
350	1.5	Tyrosine metabolism	0.029	0.041	0.006
Statisti	cally sig	nificant using two methods			
31	1.1	Inositol metabolism		0.008	0.037
730	1.9	Thiamine metabolism		0.024	0.012
521	1.10	Streptomycin biosynthesis		0.026	0.044
4350	3.2	TGF-beta signalling pathway		0.028	0.044
626	1.11	Nitrobenzene degradation		0.030	0.033
940	1.10	Stilbene, coumarine and lignin biosynthesis	0.009	0.005	
740	1.9	Riboflavin metabolism	0.000		0.001
220	1.5	Urea cycle and metabolism of amino groups	0.003		0.001
4610	4.5	Complement and coagulation cascades	0.005		0.001
4020	3.2	Calcium signalling pathway	0.008		0.001
500	1.1	Starch and sucrose metabolism	0.010		0.001
340	1.5	Histidine metabolism	0.026		0.004
4080	3.3	Neuroactive ligand-receptor interaction	0.028		0.008
4810	4.1	Regulation of actin cytoskeleton	0.029		0.002
360	1.5	Phenylalanine metabolism	0.032		0.006
4510	4.3	Focal adhesion	0.033		0.003
4310	3.2	Wnt signalling pathway	0.042		0.017
10	1.1	Glycolysis / Gluconeogenesis	0.043		0.017
40	1.1	Pentose and glucuronate interconversions	0.046		0.006
252	1.5	Alanine and aspartate metabolism	0.048		0.012

Table 3.7: Pathway analysis showing KEGG pathways that had statistically significant changes using more than one method of analysis

^a KEGG Category definitions are as follows:

1. Metabolism

- 1.1 Carbohydrate metabolism
- 1.5 Amino acid metabolism
- 1.9 Metabolism of cofactors and vitamins
- 1.10 Biosynthesis of secondary metabolites
- 1.11 Xenobiotics biodegradation and metabolism
- 3. Environmental information processing
 - 3.2 Signal transduction
 - 3.3 Signal molecules and interaction
- 4. Cellular processes
 - 4.1 Cell motility
 - 4.3 Cell communication
 - 4.5 Immune system

	GO category	GO description	Number of genes	LS permutation p value	KS permutation p value
1	0005581	collagen	42	1x10 ⁻⁵	1x10 ⁻⁵
2	0005604	basement membrane	45	1x10⁻⁵	1x10 ⁻⁵
3	0001653	peptide receptor activity	14	1x10⁻⁵	0.00333
4	0005201	extracellular matrix structural constituent	80	1x10 ⁻⁵	1x10 ⁻⁵
5	0008528	peptide receptor activity, G-protein coupled	14	1x10 ⁻⁵	0.00333
6	0030674	protein binding, bridging	45	1x10⁻⁵	0.00495
7	0006909	phagocytosis	7	1x10⁻⁵	0.08776
8	0006959	humoral immune response	51	1x10⁻⁵	0.00421
9	0008277	regulation of G-protein coupled receptor protein signalling pathway	12	1x10 ⁻⁵	0.00065
10	0009611	response to wounding	92	1x10⁻⁵	1x10 ⁻⁵
11	0009790	embryonic development	12	1x10 ⁻⁵	0.00013
12	0050817	coagulation	47	1x10⁻⁵	1x10 ⁻⁵
13	0006817	phosphate transport	47	9x10⁻⁵	0.00067
14	0019932	second-messenger-mediated signalling	38	1x10 ⁻⁴	4x10 ⁻⁴
15	0005901	caveola	10	1x10 ⁻⁴	0.00464
16	0042060	wound healing	45	0.00013	1x10 ⁻⁵
17	0005605	basal lamina	22	0.00013	0.00016
18	0008083	growth factor activity	70	0.00015	1x10 ⁻⁵
19	0007517	muscle development	71	0.00015	1x10 ⁻⁴
20	0045012	MHC class II receptor activity	12	0.00016	1x10⁻⁵
31	0005125	cytokine activity	66	0.00065	6x10 ⁻⁴
43	0008147	structural constituent of bone	5	0.00153	0.09079
44	0007160	cell-matrix adhesion	42	0.00185	0.01079

Table 3.8: Gene Ontology analysis showing the most significantly changed categories between pagetic and non-pagetic samples

3.4 Discussion

The microarray analysis was an effective way of identifying changes in mRNA transcript abundance in the pagetic osteoblasts. The microarrays produced a large amount of data, and a number of exciting results that warranted further investigation. Firstly, the fact that the control samples clearly separated from the pagetic samples, both in the analysis using all genes that passed the basic expression filters, and in the list of genes with more variable expression, suggests that osteoblasts derived from pagetic lesions have a different gene expression phenotype to those from patients without the disease. This is particularly interesting considering that most of the patients had been treated and did not tend to have highly active disease when the samples were taken. The cells had also been cultured in vitro which ensured they were not influenced by the pagetic bone microenvironment, although primary samples were used so that the cells did not lose their phenotype due to serial passaging. The bone chips did remain in the culture, creating a possible source of factors influencing the cellular phenotype, although presumably without the action of osteoclasts they would not release matrix-bound factors. Additionally, bone chips were removed during the final 72 hr of the cultures. v List of research project topics and materials

The lists of highly differentially regulated genes include a number of interesting candidate genes that had not previously been considered in the context of Paget's disease. Many of the highly differentially expressed, or interesting candidate genes have been examined in further detail using real time PCR which will be discussed in Chapter 4.

The pathway analysis also produced interesting results, particularly the possible changes in Wnt signalling, which has recently been shown to play a critical role in osteoblast commitment and differentiation [245]. Unfortunately it is difficult to interpret alterations in signalling pathways based on changes in mRNA transcript abundance alone, so further investigation is required to determine if particular cell signalling pathways are genuinely changed, and whether they are up- or down-regulated. The changes in extracellular matrixrelated gene lists were also interesting given the function of osteoblast cells, and the fact that disordered matrix with abnormal distribution of non-collagenous proteins is found in Paget's disease [108]. It is also clear from the lists of highly differentially regulated genes that the expression of a number of bone matrix proteins is altered. Most of the other KEGG pathways (16 of the 24) that were significantly altered were metabolic pathways with no obvious role in Paget's disease. Changes in TGFB and calcium signalling, however, could certainly be relevant, as could changes in focal adhesion and the actin cytoskeleton. It should be noted that KEGG pathways are very general lists compiled from information from many cell types, so are not at all specific to bone biology. While some of the lists, particularly the signal transduction-related lists are certainly likely to be relevant to bone biology, there are no lists that represent bone-specific processes such as RANKL-OPG signalling. However, the Gene Ontology categories contain a much more extensive range of genelists. This analysis also showed a number of results that did not appear to be relevant to Paget's disease, such as categories related to the immune system and wound healing. Changes in the categories related to extracellular matrix appear relevant, as do changes in different signalling pathways, including cytokine activity, which is thought to be involved in increased osteoclastogenesis in Paget's disease.

The microarray study provided evidence that there were overall differences in gene expression between pagetic and control osteoblasts, and provided a number of candidate genes for further analysis. Previously our group had examined gene expression based on candidate genes that were differentially expressed in other studies. Given that abnormal osteoclast formation and function is such a striking feature of Paget's disease, most of these genes had roles in stimulating osteoclastogenesis, such as RANKL and interleukin 6 [113, 117, 228]. The microarray analysis identified changes in genes involved in osteoblast function that had not been considered previously using the candidate gene approach. It also identified differentially regulated genes that did not previously have any known role in osteoblast function. This provided an excellent starting point for examining gene expression further in a larger subset of samples using real time PCR.

CHAPTER 4: DIFFERENTIAL GENE EXPRESSION IN PAGETIC CELLS

4.1 Introduction

Microarrays provide a huge amount of gene expression data, which like all biological results need to be replicated and validated. The results from the microarrays used in this study can be affected by probe design and sensitivity, efficiency of cRNA labelling and hybridisation conditions, and the bias towards measuring gene expression using only the 3' ends of genes [246]. Additionally, these microarrays are not particularly sensitive when measuring low gene expression levels. For this reason real time RT-PCR is commonly used to validate microarray expression data. Real time PCR measures the accumulation of fluorescent signal produced throughout the PCR amplification. This makes it an accurate and quantitative way to determine levels of gene expression using much smaller amounts of RNA than alternate techniques. Real time PCR validates most results obtained from microarrays, and usually indicates larger fold changes than microarray data [246-248]. In this study, it allowed us to validate the microarray results using a much larger set of samples that included bone marrow cultures as well as primary osteoblasts.

4.2 Methods

We have examined gene expression in pagetic and non-pagetic RNA samples from both osteoblast and bone marrow cultures using TaqMan gene expression assay-based real time RT-PCR. Gene expression was examined using real time PCR in 14 pagetic and 28 control (including 8 non-pagetic paired) osteoblast samples, and 14 pagetic and 21 non-pagetic bone marrow samples. The details of the patients and samples are shown in Tables 3.1 and 3.2. Many of the genes were chosen as a result of the microarray analysis, but some were also chosen prior to the microarray analysis because they play important roles in bone biology, and have been previously implicated in Paget's disease. Of the genes chosen as a result of the microarray analysis, many were chosen because of their potential role in bone biology and Paget's disease rather than because they had the largest changes or most significant p values. The microarray results, where available, for the genes examined by real time PCR are shown in Table 4.1.

Gene svmbol	Gene name	Fold change	Overall fold	p value	p value rank
-,		5.15.15	change		
			rank		
ALPL	Alkaline phosphatase, liver/bone/kidney	1.619	237	0.661	609
BGLAP	Osteocalcin	0.272	17	0.070	14
BMP2	Bone morphogenetic protein 2	0.595	202	0.652	584
CCL2	Monocyte chemotactic factor 1	2.483	52	0.462	271
CHI3L1	Chitinase 3 like 1	2.414	62	0.895	2270
DKK1	Dickkopf 1	3.978	14	0.475	285
DLX5	Distal-less homeobox 5	0.454	79	0.228	86
FGFR2	Fibroblast growth factor receptor 2	0.408	58	0.440	253
GATA6	GATA binding protein 6	5.244	7	0.226	83
IBSP	Bone sialoprotein	0.156	3	0.056	12
IFI27	Interferon alpha-inducible protein 27	6.864	1	0.081	16
IGF1	Insulin-like growth factor 1	1.717	191	0.823	1479
IL11	Interleukin 11	0.802	937	0.887	1784
IL1B	Interleukin 1β	1.219	1113	0.393	127
IL6	Interleukin 6	2.473	56	0.644	560
KRT18	Keratin 18	6.761	2	0.042	7
MAFB	v-maf musculoaponeurotic fibrosarcoma				
	oncogene homolog B	0.355	36	0.048	10
MGP	Matrix gla protein	4.397	9	0.215	75
MMP13	Matrix metalloproteinase 13	0.403	53	0.784	950
OMD	Osteomodulin	0.238	12	0.610	502
PTGDS	Prostaglandin D2 synthase 21kDa				
	(brain)	0.326	25	0.120	23
RGS4	Regulator of G-protein signalling 4	6.028	4	0.201	47
RUNX2	Runt-related transcription factor 2	0.817	1090	0.520	434
SATB2	SATB family member 2	0.576	181	0.025	4
SGK1	Serum/glucocorticoid regulated kinase 1	2.941	33	0.111	25
SQSTM1	Sequestosome 1	1.263	882	0.801	1018
TNC	Tenascin C	0.480	93	0.349	119
TNFRSF11B	Osteoprotegerin	0.934	7692	0.995	10358
TNFSF11	RANK ligand	0.841	4974	0.928	4401
WISP2	WNT1 inducible signalling pathway				
	protein 2	0.540	140	0.940	5721
WISP3	WNT1 inducible signalling pathway				
	protein 3	0.353	35	0.458	263

Table 4.1: Microarray results for genes that have been examined using real time PCR

Rankings show the position of fold change and p values within all genes (about 14,000) examined on the microarrays. Absolute fold changes were calculated so that both up- and down-regulated genes could be ranked. Two of the genes that were included in the real time PCR analysis, *KREMEN1*, and *SP7*/osterix are not on this list as they were not represented on the microarrays.

Many of the real time PCR results presented in this chapter were generated using the low density arrays described in Section 2.3.4.1. The genes included on these arrays are shown in Table 2.2. The remaining results were generated using standard multiplexed real time PCR. Alkaline phosphatase expression was evaluated using both methodologies, and results were similar. The real time PCR results presented in this chapter all show relative expression and individual gene expression values have been corrected for differences in starting material using expression of the housekeeping gene *18S* rRNA. For the comparisons between non-pagetic and pagetic expression, the samples have been grouped, and the non-pagetic samples have been normalised to a value of 1. In eight of the patients with Paget's disease osteoblasts

were also cultured from unaffected areas of the skeleton. These samples provide a good control for inter-patient variability, and for the effects of bisphosphonate treatment. The results of the paired samples, where significant, are also presented.

4.3 Results – Differential gene expression in pagetic cultures

4.3.1 Local regulators of bone metabolism

4.3.1.1 RANKL/OPG

Expression of a number of local bone regulators was examined because of their important role in bone metabolism, and previous reports of involvement in Paget's disease. Levels of RANKL and OPG, and their ratio play a major role in determining the level of osteoclastogenesis occurring. Real time PCR data showed that *RANKL* and *OPG* levels were not significantly changed in pagetic osteoblasts, although *RANKL* levels tended to be decreased and hence, the *RANKL/OPG* ratio also tended to be decreased [239]. In the bone marrow *RANKL* levels also tended to be decreased while *OPG* levels were significantly increased by approximately 3-fold. *RANKL/OPG* was also lowered, although this was not statistically significant (Figure 4.1(a)). *RANK* expression in the bone marrow (Figure 4.1(d)), and *M-CSF* expression in both cell types were unchanged (data not shown). OPG protein levels were quantified by ELISA in conditioned media collected at the time of RNA extraction. These results mirrored the gene expression results with significantly increased OPG in the pagetic bone marrow conditioned media, and a good correlation between RNA and protein levels (Figure 4.1). As expected, OPG protein secretion was higher in the osteoblast cultures which are more differentiated cells that express higher levels of OPG.

4.3.1.2 Interleukins

Proinflammatory cytokines such as IL-1 β and IL-6 are produced in the bone microenvironment and affect bone turnover, primarily by promoting osteoclast differentiation and activity. *IL-6* expression was increased by more than 2-fold in the pagetic samples in both cell types, although this was only significant in the osteoblasts, while *IL-1\beta* was similarly upregulated in the osteoblasts (Figure 4.2). *IL-1\beta* expression was also increased in all but one of the pagetic paired samples indicating levels are increased in the pagetic lesions compared to unaffected bone in patients. Changes in IL-6 expression are often accompanied by changes in IL-11, which is a related cytokine with similar biological activity. In this case, however, *IL-111* levels were unchanged.

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Figure 4.1: Relative expression levels of *RANKL*, OPG and *RANK* in non-pagetic and pagetic cells (a) mRNA levels of *RANKL*, *OPG* and the ratio between *RANKL* and *OPG* expression levels. White bars show average non-pagetic expression (normalised to 1), black bars show pagetic expression. (b) Concentration of secreted OPG protein measured by ELISA in conditioned media collected after the last 72 hr of cell culture before RNA extraction. (c) Correlation between OPG mRNA and secreted protein levels. The correlation was calculated using Deming (model II) linear regression. (d) mRNA levels of *RANK* in bone marrow, *RANK* expression was not detectable in osteoblasts. Graphs for real time PCR results are from a representative experiment, ELISA data is pooled from at least three replicates. Statistical significance was determined using a Student's t test. Statistical significance is indicated here, and in all other graphs in this thesis as follows: * p<0.05; ** p<0.01; *** p<0.01. Error bars on all graphs in this chapter indicate the standard error of the mean.



Figure 4.2: Relative expression levels of *IL-6*, *IL-1* β and *IL-11* mRNA in non-pagetic and pagetic cells Normalised relative expression is shown in non-pagetic (white bars) and pagetic (black bars) samples. (c) Expression of *IL-1* β in paired samples from 8 patients with Paget's disease. White bars are non-pagetic samples and black bars are pagetic samples. The result was not significant when expression levels were normalised to the non-pagetic samples, but a paired t-test of the non-normalised data presented in the graph showed a statistically significant increase in the pagetic pairs (p = 0.0338). The gene expression results from all samples and the paired samples in the next six figures are also presented in this manner.



Figure 4.3: Relative expression of BMP2 and IGF1 mRNA in non-pagetic and pagetic cells

4.3.1.3 BMP2 and IGF1

The microarray results indicated that there may be a modest decrease in *BMP2* expression, and an increase in *IGF1* expression. The real time PCR results indicated that *BMP2* expression was significantly reduced by about 3-fold in pagetic osteoblasts. The decrease in

bone marrow did not reach significance (Figure 4.3). *IGF1* expression in the osteoblasts was low (Figure 4.10(b)), and neither cell type showed statistically significant changes in expression (Figure 4.3).

4.3.2 Osteoblast matrix proteins

A number of non-collagenous matrix proteins were among the genes showing the highest fold changes in the microarray experiments. Markers of mature osteoblasts, bone sialoprotein and osteocalcin, were both significantly down-regulated (8- and 5-fold respectively) in the pagetic osteoblasts (Figure 4.4). Both genes were also significantly down-regulated in the paired samples, with lower levels of expression in the pagetic site in all eight patients. Both these genes were expressed at much lower levels in the bone marrow, particularly osteocalcin, which was expressed at levels 70-fold lower than the osteoblast samples (Figure 4.10(a)). Osteocalcin levels in the pagetic bone marrow samples were unchanged, and bone sialoprotein levels were decreased (Figure 4.4). We attempted to measure osteocalcin protein levels in conditioned media samples using a clinical Roche kit (performed by staff at LabPlus under the supervision of Dr. James Davidson). Unfortunately this assay showed high cross-reactivity with the FBS in the conditioned media, so results were inconclusive (data not shown).

Expression of alkaline phosphatase, an early marker of the osteoblastic phenotype, was also measured, and found to be significantly up-regulated by approximately 2.5-fold in the pagetic osteoblasts (Figure 4.4). In the bone marrow alkaline phosphatase expression was over 10 times higher than in the osteoblasts (Figure 4.10(a)), and expression tended to be decreased in the pagetic samples. Together these results indicate that the normal bone marrow culture contains osteoblastic cells with a less mature phenotype (higher alkaline phosphatase, lower osteocalcin and bone sialoprotein) than the normal osteoblast cultures. They also suggest that the pagetic osteoblasts have a less mature phenotype than the controls despite being cultured in comparable conditions for a similar period of time. The expression levels of alkaline phosphatase in osteoblasts and bone marrow did not correlate with serum alkaline phosphatase activity at either the time of sample collection, or at diagnosis (data not shown).

Changes in three other matrix proteins were examined. Matrix gla protein was significantly up-regulated 2.5-fold in pagetic osteoblasts. Levels were also significantly increased in the paired osteoblast samples, but expression was unchanged in bone marrow (Figure 4.4).



Figure 4.4: Relative expression of osteoblast matrix protein mRNA in non-pagetic and pagetic cells Relative expression in paired samples is shown for osteocalcin (c), bone sialoprotein (d), matrix gla protein (e) and tenascin C (f). p values here and in the following figures are calculated from normalised results.
Tenascin C is a large hexameric glycoprotein that was expressed at very high levels in both the osteoblasts and the bone marrow (Figure 4.10). In both cell types tenascin C expression was significantly down-regulated to approximately 50% of the non-pagetic level and levels were also significantly decreased in the osteoblastic paired samples (Figure 4.4). Osteomodulin, a protein that shows similar expression and distribution to bone sialoprotein [249], had variable expression levels in the non-pagetic samples, and while levels tended to be decreased in both the pagetic osteoblast and bone marrow samples this did not reach significance (Figure 4.4).



Figure 4.5: Relative expression of osteoblast transcription factor mRNA in non-pagetic and pagetic cells Osterix expression was undetectable in most of the bone marrow samples. Expression levels in paired osteoblast samples of *DLX5* (c) and *SATB2* (d) are also shown.

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4.3.3 Osteoblast transcription factors

The expression of four transcription factors that are important in osteoblast differentiation was examined. *RUNX2* and *DLX5* were both significantly down-regulated in osteoblasts and bone marrow. *SATB2* was down-regulated in osteoblasts, but the change in bone marrow was not significant. *DLX5* and *SATB2* were both significantly decreased in the paired osteoblast samples (Figure 4.5). Surprisingly, osterix expression was very low in both the osteoblast samples, although generally at very low levels, and in 11 (out of 14) of the pagetic samples. Levels in pagetic samples tended to be lower, but due to the variability in the control samples this was not statistically significant (Figure 4.5). In the bone marrow, expression was only detected in 12 (out of 21) of the control samples and two of the 14 pagetic samples (data not shown).

4.3.4 Wnt signalling molecules

The microarray results indicated that there may be changes in Wnt signalling. DKK1 expression was up-regulated 3-fold in both osteoblasts and bone marrow (Figure 4.6(a)). The concentration of secreted DKK1 in conditioned media was measured using ELISA, and showed similarly increased levels in media from pagetic osteoblasts and bone marrow (Figure 4.6(b)). There was a good correlation between mRNA and protein levels measured (Figure 4.6(c)). DKK1 levels were also measured in serum from patients with Paget's disease and age-matched controls using ELISA. Levels in patients with Paget's disease before treatment (n = 9), after ibandronate treatment (n = 24), and controls (n = 25) all showed very similar levels of serum DKK1, around 10 ng/mL, and there was no evidence of changes in response to treatment (data not shown). These levels are similar to measurements in control patient serum in other studies [250-252]. DKK1 inhibits Wnt signalling by binding to the Wnt coreceptor LRP5/6 and Kremen [253, 254]. KREMEN1 was not represented on the microarray, but real time PCR indicated that expression levels were unchanged in osteoblasts, but were significantly down-regulated in bone marrow (Figure 4.6). Expression levels of two Wnt signalling target genes which appeared to be down-regulated in the microarrays, WNT1 inducible signalling pathway protein (WISP) 2 and WISP3 were tested using real time PCR. WISP2 mRNA levels were unchanged in both cell types, but WISP3 was significantly downregulated in the osteoblasts (Figure 4.6).



Figure 4.6: Expression of genes involved in the Wnt signalling pathway in non-pagetic and pagetic cells (a) Relative expression of *DKK1* in osteoblasts and bone marrow. (b) DKK1 protein levels secreted into conditioned media as determined by ELISA. (c) Correlation between levels of DKK1 mRNA and secreted protein calculated using Deming (model II) linear regression. (d) Expression of the Wnt signalling pathway gene *KREMEN1* and the Wnt signalling target genes *WISP2* and *WISP3* are shown in osteoblasts and (e) bone marrow.

4.3.5 Other genes with known roles in bone biology

FGF signalling is important in bone development. One of the four FGF receptors, *FGFR2* was significantly down-regulated by at least 2-fold in both pagetic osteoblasts and bone marrow (Figure 4.7). There are multiple splice variants of *FGFR2*, and two isoforms with different ligand specificities, the epithelial isoform IIIb and the mesenchymal isoform IIIc. The mesenchymal isoform is activated by a number of ligands including FGFs 1, 2, 4, 6, 8, and 9 [255, 256]. The real time PCR and microarray probesets do not distinguish between these two isoforms, although presumably the mesenchymal isoform is expressed. The microarray data indicates that *FGFR1* is also expressed in osteoblasts, but there was no indication of changes in the pagetic samples.

The chemokine monocyte chemotactic factor 1 (*CCL2*) was significantly up-regulated in the pagetic osteoblasts and paired osteoblast samples, but was not significantly different in bone marrow (Figure 4.7). CCL2 acts as a chemoattractant for monocytes and macrophages, and can stimulate osteoclastogenesis [257, 258]. Prostaglandin D2 (PGD₂) synthase catalyses the final step in PGD₂ production. Osteoblasts express the brain isoform of PGD₂ synthase (*PTGDS*), but not the haematopoietic one [259]. *PTGDS* was significantly down-regulated in the pagetic osteoblasts and in the paired samples, but expression was unchanged in the bone marrow (Figure 4.7). *MMP13*, also known as collagenase 3, appeared to be down-regulated in the microarrays, however real time PCR did not show any significant changes in expression of this gene (Figure 4.7).

4.3.6 Genes with unexplored roles in osteoblast biology

We examined expression levels of a number of genes that showed large changes in the microarray results and had easily detectable expression, but no known function in osteoblast biology. The type I intermediate filament keratin 18 was up-regulated approximately 3-fold in both pagetic osteoblasts and bone marrow, and was also significantly increased in the paired osteoblast samples (Figure 4.8). Interferon-alpha inducible protein 27 (*IFI27*) was up-regulated over 4-fold in the pagetic osteoblasts, and tended to be down-regulated in pagetic bone marrow (Figure 4.8). *IFI27* expression in the non-pagetic bone marrow was about 7 times higher than in the non-pagetic osteoblasts (Figure 4.10(a)).

MAFB is a transcription factor that is mainly expressed in cells of the monocyte lineage [260], and it is commonly up-regulated as a result of chromosomal translocations in multiple

myeloma [261]. *MAFB* expression was significantly down-regulated in the pagetic osteoblasts, and paired pagetic samples, but was unchanged in the bone marrow (Figure 4.8). Surprisingly, although it is supposed to be specific to haematopoietic cells, expression levels between the osteoblast and bone marrow cultures were very similar (Figure 4.10).



Figure 4.7: Relative expression of *CCL2*, *FGFR2*, *PTGDS* and *MMP13* mRNA in non-pagetic and pagetic cells Expression of *CCL2* (c) and *PTGDS* (d) is also shown in paired osteoblast samples.



Figure 4.8: Relative mRNA expression of genes with unknown functions in bone biology in non-pagetic and pagetic cells

Relative expression in paired osteoblast samples is shown for keratin 18 (c), MAFB (d), GATA6 (e) and SGK1 (f).

Another transcription factor, *GATA6*, which is expressed in various tissues of mesodermal and endodermal origin, and interacts with other transcription factors to mediate tissue-specific gene expression [262] was significantly up-regulated about 4-fold in pagetic osteoblasts. Levels also tended to be increased in pagetic bone marrow, but since expression levels were quite variable this was not consistently statistically significant. *GATA6* was also significantly up-regulated in the pagetic paired samples (Figure 4.8).

Expression of the modulator of signal transduction, regulator of G-protein signalling (RGS) 4 was significantly up-regulated by 4-5-fold in both the osteoblasts and the bone marrow (Figure 4.8). Serum and glucocorticoid induced kinase 1 (*SGK1*) is a ubiquitously expressed inducible kinase that was significantly up-regulated 2-fold in the pagetic osteoblasts and 1.5-fold in the pagetic bone marrow, and was also significantly increased in the pagetic paired osteoblast samples (Figure 4.8). Chitinase 3-like 1 (*CHI3L1*) expression was also examined as it was the gene with the largest fold change in the paired samples on the microarray. It stimulates cell proliferation and is thought to play a role in tissue remodelling [263]. *CHI3L1* expression levels were very variable, and there were no significant differences in expression in the osteoblasts or bone marrow (Figure 4.8).

4.3.7 Overall changes in gene expression in pagetic and non-pagetic samples

As described above, there were a number of significant changes in gene expression in the pagetic samples compared to the non-pagetic controls. A summary of these changes is shown in Figure 4.9. A summary of the differences in gene expression between non-pagetic osteoblast and non-pagetic bone marrow samples is also shown in Figure 4.10(a). Some of these changes have been mentioned in the text previously, but overall many of the genes have similar levels of expression in these two different cell populations. However, there are large differences in a number of bone markers consistent with the bone marrow cultures containing less mature osteoblasts. The bone marrow cultures also express elevated levels of *IGF1*, *CCL2*, *IF127* and *SGK1* in comparison to the osteoblasts. Figure 4.10(b) shows a comparison of expression levels in the different genes in the non-pagetic osteoblasts. This figure is comparing data obtained using both standard real time PCR and results from low density arrays, and different primer-probesets tend to show slightly different amplification efficiencies, but it gives some indication of overall relative expression levels. The genes that were most highly expressed were *OPG*, bone sialoprotein and tenascin C, while *IL-1β* and osterix were expressed at very low levels.



Figure 4.9: Summary of changes in gene expression in pagetic cells Relative expression of genes in pagetic osteoblasts (a), and pagetic bone marrow (b). Expression of pagetic samples is shown in comparison to gene expression in non-pagetic samples which was normalised to 1.

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Figure 4.10: Differences in gene expression between cell types and relative expression levels of different genes (a) Relative levels of gene expression between non-pagetic osteoblasts and bone marrow cells. Expression in the osteoblasts samples was normalised to 1. No results are shown for *RANK* and osterix as expression was not detectable in both cell types, and *IL-6* and *IL-1* β results are not shown as experiments were performed separately so were not directly comparable, although *IL-1* β expression appears to be higher in the bone marrow. (b) Relative expression levels of the different genes in non-pagetic osteoblasts. Values are calculated using 2^{-DCt}x10⁶.

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Figure 4.11: Correlation of expression of different pairs of genes in osteoblasts and bone marrow The eight pairs of genes that either had correlation coefficients of over 0.7 in both osteoblasts and bone marrow (a-e), or a correlation coefficient over 0.8 in one cell type and slightly poorer but still highly significant correlation in the other cell type are shown. Combined DCt data from both cell types are plotted and r and p values from the combined Pearson correlation analysis are shown. The genes in (f) showed very strong correlation in osteoblasts (r = 0.854) while (g) and (h) showed very strong correlations in bone marrow (r = 0.836 and 0.807 respectively).



Figure 4.12: Correlation of expression of different pairs of genes in osteoblasts only Pairs of genes that showed strong correlation in the osteoblasts, but poor correlation (*MAFB* and *RGS4* (d); *FGFR2* and *MMP13* (e); *IL-1* β and *SGK1* (f)) or no correlation (bone sialoprotein and osteocalcin (a); bone sialoprotein and *MAFB* (b); *MAFB* and *FGFR2* (c)) in the bone marrow cultures. DCt values are plotted and r and p values from the Pearson correlation analysis are shown. Bone sialoprotein and osteocalcin (a), and *IL-1* β and *SGK1* (f) also show very high correlation when osteoblast and bone marrow data was combined (r = 0.745 and 0.811 respectively).

4.3.8 Effects of patient age and sex on gene expression

As shown in Table 3.2, the pagetic patients tended to be older than the controls, and there were more males in the group with Paget's disease. Although these differences in demographics were not statistically significant, we were concerned that there could be age- or sex- related changes in gene expression that could be affecting the results. The effect of age on gene expression was investigated by performing linear regression on the DCt values for

each gene and testing if the slope was significantly different from zero. The DCt values were used for correlation analyses as they are effectively log transformed values which means that equal weighting is given to samples with high and low expression. Analysis on the pagetic and control samples was performed separately. None of the genes consistently showed plots where the slope was different from zero indicating no age-related changes in expression of the genes examined, and therefore no need to adjust the data for this variable.

The influence of sex on expression levels of the various genes analysed was also tested. Samples were split into four groups, pagetic male and female and non-pagetic male and female, and 1-way ANOVA with Tukey's post test was performed. There was no consistently significant difference between the pagetic male and female groups, or the non-pagetic male and female groups for any of the genes. This suggests that the expression of these genes in osteoblastic and bone marrow cells is not significantly influenced by the sex of the patient.

4.3.9 Correlation of gene expression for different genes

In order to determine which genes showed correlation with each other, the DCt values for most of the genes that had been included in the real time PCR analysis were compared to DCt values of other genes from the same patient sample. Pearson correlation coefficients and p values were calculated. Both pagetic and non-pagetic samples were included in this analysis, and a large number of combinations were statistically significant, so only combinations with a p value of <0.0001 and a high r value (generally >0.7 or <-0.7) were considered likely to be biologically relevant. In the osteoblasts there were 25 combinations that fitted these criteria, and in the bone marrow there were 21. When data from the two cell type were combined, there were 13 such combinations. Graphs depicting some of these analyses are shown in Figures 4.11 and 4.12. The majority are positive correlations, however RGS4 expression increases as MAFB decreases in the osteoblasts (Figure 4.12(d)). The transcription factors DLX5 and RUNX2 showed strong correlation with each other and with FGFR2 and tenascin C in both the osteoblasts and bone marrow (Figure 4.11). Bone sialoprotein and osteocalcin expression was strongly correlated in the osteoblasts and the combined analysis, but not in the bone marrow. Relationships between the expression levels of these genes and other osteoblast markers such as alkaline phosphatase and matrix gla protein were much weaker in the osteoblasts, and completely absent in the bone marrow. However, osteocalcin and alkaline phosphatase did show a strong negative correlation in the combined analysis (r = -0.730, data not shown), and bone sialoprotein and osteomodulin correlated positively (r = 0.712). It is difficult to determine the biological relevance of many of these correlations, particularly between genes with undefined functions in bone such as *MAFB*, *RGS4*, *GATA6* and keratin 18 which show a number of significant correlations with other genes.

4.4 Results – Gene expression in differentiating osteoblasts

In order to help elucidate the role of the differentially regulated genes in Paget's disease we examined their expression in differentiating osteoblasts. This was of particular interest given that the changes in the bone marker genes alkaline phosphatase, osteocalcin and bone sialoprotein suggested that the pagetic osteoblasts may be less differentiated than control cells. These studies were performed in MC3T3-E1 Subclone 4 cells and primary human osteoblasts. The MC3T3-E1s formed mineralised structures within three weeks. The cells were grown either in standard growth media, mineralisation media, or mineralisation media supplemented with dexamethasone for up to three weeks and RNA was harvested at regular timepoints. As shown in Figure 4.13 mineralisation began to occur by day 16 and was extensive by day 21, with more mineralisation apparent in the dexamethasone-treated wells. No mineralisation occurred in standard growth media.

Mineralisation in the human osteoblasts was less reliable. Mineralisation experiments were performed using cells derived from six different patients. However, after a period of at least three weeks only four showed any evidence of mineralisation, so only these were included in further analyses. Plates from one of the patients stained with von Kossa and alizarin red are shown in Figure 4.14. Cells were cultured with and without dexamethasone. In all but one patient, mineralisation occurred more readily with dexamethasone, however in this particular patient there was only mineralisation in the absence of dexamethasone. Sometimes cells spontaneously peeled off the wells, possibly because of overcrowding due to continuing proliferation [43]. In some patients this loss of the cell layer was completely prevented by dexamethasone treatment, but in others cells in both types of media showed this behaviour.



10mm

Figure 4.13: Mineralisation in MC3T3-E1 subclone 4 cells over time

Mineralised areas are stained black with von Kossa stain. Representative wells are shown from cells grown in control growth media, mineralisation media, and mineralisation media supplemented with 10 nM dexamethasone for the times indicated.



Figure 4.14: Mineralisation in human osteoblasts

Representative wells showing mineralisation in cells from one patient are shown stained with von Kossa or alizarin red. Cells were grown in either standard mineralisation media or in mineralisation media supplemented with 10 nM dexamethasone for the times indicated.

4.4.1 Osteoblast matrix proteins

In order to confirm that the cells were differentiating as expected, expression of bone markers was examined. MC3T3-E1 subclone 4 cells do not express alkaline phosphatase [264], however there were dramatic increases in both osteocalcin and bone sialoprotein expression over time in the mineralisation media with and without dexamethasone (Figure 4.15). As expected, levels of bone sialoprotein expression started to increase earlier (day 2) than

osteocalcin (day 7). Interestingly dexamethasone had different effects on the expression levels of these two genes. Expression of both osteocalcin and bone sialoprotein increased over time in the control cells, although to a much lesser degree than in the treated cells. This suggests these cells were also differentiating albeit at a much slower rate. This is not surprising given that the lack of space in the wells is likely to limit proliferation.

The human osteoblasts often showed different patterns of expression between the different patients. Alkaline phosphatase was strongly expressed and tended to show at least transiently increased expression during the culture period (Figure 4.15(a)). Osteocalcin was expressed at very low levels in most of the samples, and while there did tend to be increased expression as the cells began to mineralise, these increases were of a much smaller magnitude than in the MC3T3-E1s (Figure 4.15(c)). Bone sialoprotein expression increased over time in all the human osteoblast samples, and in some of the patients the pattern was similar to the MC3T3-E1s grown in mineralisation media (Figure 4.15(e)). Overall, these results suggest that there is differentiation in the MC3T3-E1 cells, however the results in the human osteoblasts vary among individuals and may not be as reliable.

Matrix gla protein expression is generally suppressed during osteoblast differentiation, as it is an inhibitor of mineralisation. This was observed in the MC3T3-E1s, although expression in the control cells also decreased over time. The dexamethasone-treated cells tended to have higher levels of matrix gla protein expression than the other groups in the first week of culture (Figure 4.16(a)). In contrast, matrix gla protein expression in the human osteoblasts tended to increase over the timecourse examined, with expression levels in three of the four patients increasing at least 100-fold from baseline after two weeks in culture (Figure 4.16(b)). In most cases expression began to decrease in the final two weeks of culture, but these decreases were modest compared to the large increases in expression that occurred earlier.

Expression of tenascin C in the MC3T3-E1s did not change over time, with very high expression persisting throughout differentiation (Figure 4.16(c) and data not shown). The decreased expression over time shown in the figure was not a consistent finding. Three of the four human osteoblast experiments showed modest increases in expression towards the end of the timecourse (Figure 4.16(d)). Osteomodulin levels were not examined in the MC3T3-E1s, however in the human osteoblasts there were very large increases in expression in three of the experiments (Figure 4.16(e)). These corresponded to the three samples that were treated with

dexamethasone. The cells from the final patient that showed mineralisation only in the absence of dexamethasone showed modest increases in osteomodulin expression during differentiation (up to about a 10-fold increase from baseline).



Figure 4.15: Expression of osteoblast marker genes in differentiating MC3T3-E1 and human osteoblast cells MC3T3-E1 cells were cultured in either control growth media (blue), mineralisation media (green) or mineralisation media supplemented with 10 nM dexamethasone (red) for the times shown. Human osteoblasts were cultured from four different patients in mineralisation media. The different colours represent samples derived from different patients. All except the purple one were treated with 10 nM dexamethasone. Relative expression is calculated from real time PCR data in relation to the Day 0 timepoint. Data from one representative experiment is shown.



Figure 4.16: Expression of matrix gla protein, tenascin C and osteomodulin in differentiating MC3T3-E1 and human osteoblast cells

Results are presented in the same manner as the previous figure. The MC3T3-E1 cells were cultured in control (blue), mineralisation (green) or dexamethasone-containing (red) media.

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Figure 4.17: Expression of local regulators of bone metabolism *OPG*, *IL-6* and *BMP2* in differentiating MC3T3-E1 and human osteoblast cells

Results are presented in the same manner as the previous figures. The MC3T3-E1 cells were cultured in control (blue), mineralisation (green) or dexamethasone-containing (red) media.

4.4.2 Local regulators of bone turnover

Previous reports suggest that levels of RANKL tend to decrease as osteoblastic cells differentiate while levels of OPG increase [265, 266]. *RANKL* expression in both the cell types was very low or undetectable (data not shown). *OPG* was expressed at much higher levels with fairly stable expression in both cell types throughout the differentiation timecourse, although there was a transient increase in *OPG* levels in the dexamethasone-treated MC3T3-E1 cells (Figure 4.17). *IL-6* expression in the human osteoblasts was consistently suppressed

as differentiation proceeded (Figure 4.17(c)). *IL-1\beta* is not expressed in MC3T3-E1s, and expression was not examined in differentiating human osteoblasts. *BMP2* expression tended to increase over time in the differentiating human osteoblasts, although the magnitude of this increase varied in the different patients (Figure 4.17(d)). *Bmp2* and *IL-6* expression were not examined in the MC3T3-E1 cells.

4.4.3 Osteoblast transcription factors

Expression levels of *RUNX2* and *DLX5* were examined in both cell types. Despite playing roles in osteoblast differentiation, and the negative effect of RUNX2 on terminal osteoblast differentiation [42], there was very little change in the expression of these two genes over time in either of the cell types (Figure 4.18). Osterix and *SATB2* were not examined.



Figure 4.18: Expression of osteoblast transcription factors *RUNX2* and *DLX5* in differentiating MC3T3-E1 and human osteoblast cells

Results are presented in the same manner as the previous figures. The MC3T3-E1 cells were cultured in control (blue), mineralisation (green) or dexamethasone-containing (red) media.

4.4.4 Wnt signalling molecules

DKK1 expression was not reliably detectable in MC3T3-E1 cells, and *KREMEN1* and *WISP3* levels were not examined in these cells. However, all these genes were examined in the

differentiating human osteoblasts. *DKK1* has been reported to be up-regulated as osteoblasts differentiate [31, 33], however, in the human osteoblasts levels tended to decrease early in the culture with at least 3-fold decreases from baseline observed after the first week. In most cases the levels returned to baseline or above at the later stages (Figure 4.19(a)). *KREMEN1* and *WISP3* both showed modest increases in expression over the timecourse (Figure 4.19).



Figure 4.19: Expression of genes involved in Wnt signalling during human osteoblast differentiation Expression of *DKK1* (a), *KREMEN1* (b) and *WISP3* (c) over time in cells from four different patients is shown.

4.4.5 Other genes with known roles in bone biology

The chemokine *CCL2* showed minimal changes during MC3T3-E1 differentiation, and the transient increase shown in the dexamethasone-treated cells was not a consistent finding (Figure 4.20(a)). Levels increased in two of the human osteoblast experiments, but changed little in the other two (Figure 4.20(b)). *FGFR2* levels decreased during MC3T3-E1 differentiation, although decreases in the control cells were similar to the cells that underwent mineralisation (Figure 4.20(c)). In the human osteoblasts, if anything, there tended to be modest increases in expression (Figure 4.20(d)).





Figure 4.20: Expression of other genes involved in bone metabolism *CCL2*, *FGFR2*, *PTGDS* and *MMP13* in differentiating MC3T3-E1 and human osteoblast cells

Results are presented in the same manner as the previous figures. The MC3T3-E1 cells were cultured in control (blue), mineralisation (green) or dexamethasone-containing (red) media.

PTGDS expression was not detectable in the MC3T3-E1s, and in the human osteoblasts expression tended to increase over the course of the experiment. The increased expression was particularly dramatic in one patient (Figure 4.20(e)). *MMP13* expression was not examined in the MC3T3-E1s since it did not change significantly in the pagetic samples. However, it showed more consistent results than many of the genes in the differentiating human osteoblasts with all four experiments showing a maximum increase in expression of at least 100-fold from baseline (Figure 4.20(f)). This is consistent with reports in the literature showing that *MMP13* increases during osteoblast differentiation and is a target gene of RUNX2 [267, 268].

4.4.6 Genes with unknown roles in bone biology

We were particularly interested in characterising the expression patterns of the genes with previously unidentified roles in osteoblast biology in the hope of shedding some light on their potential functions. *GATA6* expression was very low in the MC3T3-E1s and there were minimal changes in expression in both cell types (Figure 4.21). *IF127* showed a small transient increase in expression early on in the MC3T3-E1 timecourse and then levels decreased considerably. If anything, the decrease in expression was smaller in the cells cultured in mineralisation media than those in control media or treated with dexamethasone (Figure 4.21(c)). In the human osteoblasts three out of four experiments showed very little change over time (Figure 4.21(d)). At some timepoints equivalent samples with and without dexamethasone treatment were compared (data not shown). These results suggested that *IF127* expression was suppressed by dexamethasone treatment, in agreement with the MC3T3-E1 results.

RGS4 was also expressed at low levels in the MC3T3-E1s, and showed decreased expression as differentiation proceeded, although this occurred in the control cells as well as the mineralising ones (Figure 4.21(e)). No consistent pattern of expression was apparent in the differentiating human osteoblasts (Figure 4.21(f)). *SGK1* levels did not change much over time in either of the cell types, however in the MC3T3-E1s dexamethasone treatment consistently resulted in levels about twice as high as the untreated cells (Figure 4.21(g,h)). Unfortunately a comparison between human osteoblast cells with and without dexamethasone treatment was not performed for this gene.



Figure 4.21: Expression of *GATA6*, *IFI27*, *RGS4* and *SGK1* in differentiating MC3T3-E1 and human osteoblast cells

Results are presented in the same manner as the previous figures. The MC3T3-E1 cells were cultured in control (blue), mineralisation (green) or dexamethasone-containing (red) media.

Keratin 18 and *MAFB* were both undetectable in the MC3T3-E1 cells. Keratin 18 expression tended to decrease during differentiation in the human osteoblasts, while *MAFB* expression was consistently increased by 5-8-fold after 3-4 weeks in differentiating conditions (Figure 4.22).



Figure 4.22: Expression of KRT18 (a) and MAFB (b) in differentiating human osteoblast cells

4.4.7 Summary of gene expression in differentiating osteoblasts

Two culture systems were used to examine expression of the genes of interest during differentiation. MC3T3-E1s show reliable differentiation and reproducible results, however, being a mouse cell line means not all results are indicative of what really occurs during the differentiation of human cells. The absence of alkaline phosphatase, an enzyme that plays an important role in mineralisation, is one example of a non-physiological feature of this cell line. Other genes like *DKK1*, *RANKL* and *IL-1* β which are normally expressed in osteoblastic cells, are also absent or expressed at very low levels in these MC3T3-E1 cells. Differentiation in human osteoblast cells was also examined as these were the cells used for the original investigations into gene expression. Unfortunately the culture conditions used to stimulate human osteoblast differentiation did not consistently result in mineralisation, and the results of both the mineralisation and gene expression appear to be donor-specific. A summary of the changes in gene expression during osteoblast differentiation shown in this study, and results reported by others, are shown in Table 4.2.

	Expression in patient samples	Expressi differentiatin <i>vitro</i>	on in g cells <i>in</i> o		
Gene	OB / BM	MC3T3-E1	HOBs	Literature	References
Alkaline phosphatase	$\downarrow\downarrow$	NE	1	↑	[269]
Osteocalcin	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow	$\uparrow\uparrow$	[269]
Bone sialoprotein	$\uparrow\uparrow$	$\uparrow\uparrow$	$\uparrow \uparrow$	$\uparrow\uparrow$	[269]
Matrix gla protein	\rightarrow	\downarrow	$\uparrow\uparrow$	\downarrow	[33, 270]
Tenascin C	\rightarrow	\rightarrow	\rightarrow	?	
Osteomodulin	1	ND	$\uparrow\uparrow$	$\uparrow\uparrow$	[271, 272]
OPG	1	\rightarrow	1	1	[265, 266]
RANKL	\rightarrow	NE	NE	\downarrow	[265, 266]
IL-6	ND	ND	$\downarrow\downarrow$	↓↓ or ↑↓	[273, 274]
BMP2	↑	ND	1	1	[33]
RUNX2	↑	\rightarrow	\rightarrow	1	[269]
DLX5	\rightarrow	\rightarrow	\rightarrow	$\uparrow \downarrow$	[43]
DKK1	\rightarrow	NE	$\downarrow\uparrow$	$\uparrow\uparrow$	[33]
KREMEN1	\rightarrow	ND	1	?	
WISP3	\rightarrow	ND	1	\rightarrow	[275]
CCL2	\downarrow	\rightarrow	\rightarrow	1	[274]
FGFR2	\rightarrow	\downarrow	1	1	[268]
PTGDS	\rightarrow	NE	1	?	
MMP13	↑	ND	$\uparrow\uparrow$	$\uparrow\uparrow$	[268]
GATA6	\rightarrow	\rightarrow	\rightarrow	?	
IFI27	$\downarrow\downarrow$	$\uparrow \downarrow$	\rightarrow	?	
RGS4	\rightarrow	\downarrow	\rightarrow	\downarrow	[267]
SGK1	\downarrow	\rightarrow	\rightarrow	?	
Keratin 18	\rightarrow	NE	\downarrow	?	
MAFB	\rightarrow	NE	↑	?	

Table 4.2: Changes in expression of genes of interest during osteoblast differentiation

OB / BM: ratio of expression between the human osteoblast and bone marrow cultures (Figure 4.10(a)) MC3T3-E1s and HOBs: changes during differentiation in MC3T3-E1 subclone 4 and human osteoblast cells ↑: increased expression

 \rightarrow : unchanged expression

↓: decreased expression

- NE: not expressed
- ND: not determined

?: no literature identified/unknown

Double arrows in the same direction indicate large changes in expression, while multiple arrows in different directions indicate sequential changes in expression.

4.5 Discussion

These results have identified a number of changes in gene expression in pagetic osteoblasts and bone marrow cultures that could contribute to the development of pagetic lesions. A summary of the effects that some of these genes have on bone cells is shown in Figure 4.23. Many of these effects are discussed further below.



Figure 4.23: Effects of genes with altered transcript abundance in pagetic osteoblasts on bone cell differentiation The changes in expression in the pagetic osteoblasts are indicated by the colours of the boxes (changes shown are for the osteoblasts with the exception of OPG which only changed in the bone marrow). Stimulators of the differentiation processes indicated are in the left panel and inhibitors are in the right panel.

4.5.1 Agreement between real time PCR and microarray results

A comparison of the fold-changes in expression between the groups of pagetic and nonpagetic samples within the 12 samples included in the microarray analysis is shown in Table 4.3. This data is for the 29 genes where appropriate data was available from both assay formats. 19 of the genes had a higher fold change in the real time PCR data than in the microarrays, which is a common finding when comparing these two formats [246-248]. In many cases the change in expression was only modestly higher in the real time PCR data, although for four of the genes the change was over 50% higher in the real time PCR data. Six genes showed very similar changes with the two techniques, and four showed lower fold changes using real time PCR analysis.

Table 4.3: Fold changes in gene expression between	non-pagetic and pagetic samples in the 12 samples that were
analysed on the microarrays determined using microa	array analysis and real time PCR

	Fold change			
Gene	Microarray	Real time		
RANKL	0.841	0.730		
OPG	0.934	1.244		
IL-6	2.473	3.000		
IL-11	0.802	1.256		
BMP2	0.595	0.530		
IGF1	1.717	2.495		
Alkaline phosphatase	1.619	4.035		
Osteocalcin	0.272	0.126		
Bone sialoprotein	0.156	0.126		
Matrix gla protein	4.397	5.344		
Osteomodulin	0.238	0.177		
Tenascin C	0.480	0.544		
DLX5	0.454	0.545		
RUNX2	0.817	0.814		
SATB2	0.576	0.662		
DKK1	3.978	4.616		
WISP2	0.540	0.863		
WISP3	0.353	0.373		
CCL2	2.483	3.014		
FGFR2	0.408	0.393		
MMP13	0.403	0.271		
PTGDS	0.326	0.268		
CHI3L1	2.414	4.175		
GATA6	5.244	6.926		
IFI27	6.864	13.660		
KRT18	6.761	6.734		
MAFB	0.355	0.262		
RGS4	6.028	7.860		
SGK1	2.941	4.268		

The 12 microarrays provided an excellent source of candidate genes for the whole group of samples. Choosing candidate genes based on a combination of p value, fold change, and potential relevance was an effective approach. The real time PCR results suggested that p values alone were not necessarily the best tool for choosing candidate genes, although only three of the 11 genes with significant p values in the microarray analysis were included in the real time PCR analysis. For example, the results from SATB2 (which was significantly downregulated in the microarray analysis) in the real time PCR were not as statistically significant as other genes like IF127, CCL2 and SGK1 that had higher, non-significant p values in the microarray analysis. Additionally, some genes with large p values in the microarray analysis (>0.6) such as alkaline phosphatase and *IL-6* still showed significant results in the real time PCR analysis. However, other genes with large p values and large fold changes like CHI3L1, MMP13 and osteomodulin also showed changes in the real time PCR analysis, but these were not statistically significant due to the variability in expression. All the genes tested from within the top 10 differentially regulated genes on the microarrays according to fold change had reasonably low p values (all in the top 100), and they all showed statistically significant changes in the real time PCR analysis. The results from RUNX2, which did not appear to be differentially regulated in the microarrays, but was significantly down-regulated in the full set of samples used for real time PCR, indicate that the microarray analysis may have missed potentially interesting and significant changes in gene expression. In the case of RUNX2 the small number of samples run on the microarray affected the result as the differences between the groups within the subset of samples on the microarray was much smaller than the difference overall, as shown by the agreement in fold change between the two techniques in Table 4.3. The change in expression in this gene is fairly small, but given the important role it plays in bone biology it is still likely to be biologically relevant.

4.5.2 RANKL/OPG

Changes in RANKL and OPG levels are a common mechanism for altered osteoclastogenesis. The changes identified in this study, however, do not support a role for an increased *RANKL/OPG* ratio in the development of the pagetic lesion, as the ratio tended to be decreased rather than increased, and OPG was significantly up-regulated at both the mRNA and protein level in the bone marrow cultures. This finding is different from Menaa *et al.*, who found increased *RANKL* expression in pagetic bone marrow cultures [113]. The differences between these two studies may be due to the different culture systems used, and it should be noted that the present study utilised a more quantitative method for determining

levels of gene expression. Sun *et al.* also reported increased *RANKL*, and decreased *OPG* expression in pagetic osteoblasts from a small number of patients, although again, this study used standard RT-PCR [115]. In agreement with our results, there are reports of increased OPG levels in the serum of patients with Paget's disease [118, 120]. Our findings suggest that other factors are driving the development of the pagetic lesions and the RANKL/OPG ratio is decreasing in response to this, rather than being a driver of the altered bone turnover.

4.5.3 Interleukins

A second well-characterised mechanism by which osteoblasts influence osteoclasts is through the production of cytokines such as IL-6 and IL-1. IL-1 is a direct up-regulator of osteoclastogenesis, and can also stimulate IL-6 release from stromal or osteoblast cells [276, 277], while IL-6 stimulates osteoclastogenesis and activity by altering the levels of RANKL and OPG produced by osteoblastic cells [278]. Thus, the over-expression of these two cytokines in pagetic osteoblasts provides a synergistic mechanism for the local stimulation of osteoclastogenesis. These findings are consistent with a number of earlier results that have reported increased IL-6 concentrations in bone marrow plasma from patients with Paget's disease, and increased expression in osteoblastic cells [111, 116, 117]. There is also evidence that an IL-6 neutralising antibody can block the increased osteoclastogenesis stimulated by conditioned media from pagetic bone marrow [113] or pagetic serum [119]. However, neutralising antibodies had no significant effect on osteoclastogenesis in cultures of peripheral blood mononuclear cells [114], suggesting the presence of pagetic stromal and osteoblast cells may be a major source of increased IL-6 in Paget's disease. There is also a report of increased IL-1 activity produced by both pagetic bone marrow and peripheral blood cells consistent with our results [279]. One of the advantages of the current study is that it has identified these changes using a quantitative method in a population of osteoblastic cells, whereas other studies have used mixed cell populations. Our results indicate that osteoblasts are contributing to the increases in these cytokines that have previously been reported in Paget's disease. While IL-6 clearly supports osteoclast generation in a high bone turnover situation, it also stimulates osteoblast proliferation and differentiation [280]. Therefore it may also play a role in the osteoblast over-activity in the pagetic lesion.

4.5.4 CCL2

CCL2, or monocyte chemoattractant protein 1, is one of approximately 50 chemokines in humans. It transmits signals mainly via its high affinity receptor CCR2 [281]. CCL2 can also

bind to non-signalling glycosaminoglycan receptors on vascular epithelial cells to create a chemokine gradient that promotes monocyte and macrophage migration to sites of inflammation [257]. The up-regulation of CCL2 seen in the pagetic osteoblasts is likely to have a pro-osteoclastogenic effect. The main function of CCL2 appears to be recruitment of monocytes and macrophages, which can serve as osteoclast precursors. However, it also promotes osteoclastogenesis and can stimulate formation of multinucleated, but non-resorbing osteoclasts in the absence of RANKL [257, 258, 282]. Expression in osteoblasts is stimulated by inflammatory mediators such as IL-1 [257], but in this study there was no correlation between CCL2 and IL-1 β expression in either of the cell types, and only a weak correlation between CCL2 and IL-6 expression in the osteoblasts. CCL2 does not have a direct effect on osteoblasts as they do not express appropriate receptors, although treatment of jaw lesions in rats with CCL2 stimulated increased osteoblast numbers around the lesion [283]. Other reports suggest that CCL2 is expressed at low levels in unstimulated osteoblasts [257], however in both human osteoblasts and MC3T3-E1s expression was easily detectable in all samples, and was generally higher than levels of *IL-6* and *IL-1\beta*. All these cells were grown in media containing FBS which may be sufficient to stimulate CCL2 expression. CCL2 expression did not appear to change greatly during differentiation in the cells used in this study, although others have reported increased CCL2 secretion in differentiating human mesenchymal stem cells [274].

4.5.5 BMP2 and osteoblast transcription factors

Another important local regulator of bone turnover, BMP2, was down-regulated in the pagetic osteoblasts and was decreased, although not significantly so, in the bone marrow. *BMP2* appears to be up-regulated during osteoblast differentiation so reduced expression may be a result of the pagetic osteoblast being less differentiated than the non-pagetic controls, as suggested by the changes in the markers of osteoblast differentiation, alkaline phosphatase, osteocalcin and bone sialoprotein. Alternatively, BMP2 is a potent stimulator of osteoblast differentiation, so reduced levels of this factor may be a cause of the changes in the differentiation markers mentioned above as well as the reduced levels of osteoblastic transcription factors *RUNX2* and *DLX5* which are both transcriptional targets of BMP2 [284]. BMP2 expression is also up-regulated by RUNX2, and BMP-activated Smads interact with RUNX2 to stimulate osteoblast-specific gene expression and differentiation [285, 286]. Osterix expression is also stimulated by BMP2 and DLX5 [287], and while there was no significant change in the expression of this transcription factor, it was expressed at lower both transcription factor.

levels in the pagetic cells. The KEGG TGF β signalling pathway, which includes BMP signalling molecules, was significantly altered in the microarray analysis, suggesting that changes in this pathway may be important in Paget's disease. Loss of BMP signalling in osteoblasts of developing mice resulted in enhanced bone mass, but the bone formed was disorganised or woven. This suggests that disrupted BMP signalling could contribute to the abnormal bone formation observed in Paget's disease [288].

SATB2 expression was also reduced in the pagetic osteoblasts. This protein functions as both a regulator of transcription, and as a factor that modulates the activity of other transcription factors. *SATB2* haploinsufficiency or mutations cause syndromes associated with craniofacial abnormalities and osteoporosis [289]. Since this gene has not been studied as thoroughly as many of the other factors involved in osteoblast activity, it is unclear how it is regulated. However, it does up-regulate bone sialoprotein expression directly, and can function synergistically with RUNX2 to up-regulate osteocalcin expression [290]. The down-regulation of BMP2, and many of the transcription factors with important roles in osteoblast differentiation and activity suggests there are fundamental differences in the osteoblastic cells derived from pagetic lesions compared to those from normal tissue. These changes are likely to contribute to the disordered bone formation observed in the pagetic lesion.

4.5.6 Osteoblast matrix proteins

The changes observed in markers of osteoblastic differentiation, particularly increased alkaline phosphatase, and decreased osteocalcin and bone sialoprotein, may indicate that the pagetic osteoblasts are less differentiated than their non-pagetic counterparts as previously mentioned. Matrix gla protein expression has been reported to decrease during osteoblast differentiation *in vitro* [270], which we have shown in MC3T3-E1s, so the increased *MGP* expression in this study could also indicate that the pagetic osteoblasts are less differentiated. Impaired differentiation may mean there is a larger population of immature cells that produce factors that stimulate osteoclastogenesis. Decreased levels of *BMP2* and *RUNX2* are also likely to contribute to the decreased expression of their target genes osteocalcin and bone sialoprotein. Expression levels of osteocalcin and bone sialoprotein are strongly correlated in the osteoblasts, and they are also both significantly correlated with *RUNX2*, *DLX5*, and *BMP2*, although the correlation coefficients for these latter combinations are all below 0.7.

The changes in these marker genes are also interesting in relation to clinical observations of serum bone markers. While serum alkaline phosphatase is a very good clinical marker for Paget's disease activity, osteocalcin, which is also in use clinically as a marker of osteoblast activity, is not a good marker in Paget's disease, with serum levels frequently in the normal range in patients with active disease [88, 89]. Although bone sialoprotein is not used routinely in diagnosis, there is a report of increased serum bone sialoprotein levels in Paget's disease, however levels were not elevated as significantly as alkaline phosphatase [291].

The role and regulation of tenascin C, another matrix protein that is significantly downregulated in the pagetic cells, is less clear. Its expression does not appear to change during osteoblast differentiation but it does show strong correlation with both *RUNX2* and *DLX5* expression in both the osteoblasts and the bone marrow cultures. Tenascin C is known to have stimulatory effects on osteoblast differentiation and affect cell morphology, but it is not found in mature bone matrix other than at reversal lines [292, 293]. There were no obvious differences in cell morphology between pagetic and control cells using light microscopy.

4.5.7 Wnt signalling molecules

The up-regulation of DKK1 expression was of interest as Wnt signalling is such an important regulator of osteoblast activity, and alterations in DKK1 levels play a role in bone cancers and metastases and bone destruction in rheumatoid arthritis [294]. DKK1 is thought to inhibit Wnt signalling by combining with Kremens to internalise the Wnt co-receptor LRP5/6 [254]. Global analysis of the microarray data also suggested there could be changes in the Wnt signalling pathway, and WISP3, a target of Wnt signalling was significantly down-regulated in the osteoblasts, and decreased in the bone marrow. This suggests that Wnt signalling may be inhibited in the pagetic cells. WISPs are members of the CCN family of extracellular proteins that modulate signalling, particularly associated with the extracellular matrix [295]. Mutations in WISP3 cause progressive pseudorheumatoid dysplasia, an autosomal recessive disorder associated with cartilage loss and destructive bone changes from a young age [296]. Chondrocytes are thought to be the main cell type affected, possibly due to accumulation of reactive oxygen species [297]. WISP3 has also been shown to modulate BMP and Wnt signalling, and act as a chemoattractant for mesenchymal stem cells [298, 299]. FGFR2 which was down-regulated in both cultures may also be a target of Wnt signalling [300]. However, the bone marrow also had decreased expression of the DKK1 co-receptor KREMEN1, which may affect the DKK1 activity in these cells. Additionally, alkaline phosphatase and IL-6,

which were both up-regulated in the pagetic osteoblasts, are also targets of Wnt signalling, although the transcription of these factors is also affected by various other signalling cascades [301, 302]. The TCF/LEF transcription factors activated by Wnt signalling can also antagonise RUNX2 activity which could lead to decreased expression of bone sialoprotein and osteocalcin [245]. To complicate matters further, *DKK1* is also a transcriptional target of Wnt signalling [243, 244]. Therefore, it is very difficult to determine if there is increased or decreased Wnt signalling in the pagetic cells using a snapshot of gene expression data alone, and more work is needed to determine the role of Wnt signalling in Paget's disease.

4.5.8 FGF signalling

The decreased expression of *FGFR2* suggests there may also be an alteration in FGF signalling. The overall effect of FGF signalling appears to be promoting osteoprogenitor replication and differentiation resulting in increased numbers of functional osteoblasts, and FGFR1 and FGFR2 are both important for mediating these effects [303]. Activating mutations in *FGFR2* in humans cause syndromes characterised by craniofacial defects such as Apert and Crouzon syndromes [303]. Alterations in *FGFR2* expression have been reported in breast and prostate cancer [304, 305]. The FGF pathway did not show significant changes in the microarray results, but is not specifically included in the KEGG lists other than as one of many stimulators of MAPK signalling. Again, further investigation is required before any role in the disease can be surmised.

4.5.9 Prostaglandin D synthase

The decrease in *PTGDS* expression in the osteoblastic cells is a little surprising given what is known about the regulation of this gene. PTGDS is the only prostaglandin D synthase expressed by osteoblasts and is critical for the production of PGD₂ [306]. While the actions of this prostaglandin are not as well characterised as PGE₂ which plays important roles in the stimulation of osteoclastogenesis and bone formation *in vivo*, it does also appear to influence bone cell activity. PGD₂ production only occurs when cells are stimulated and it is induced by various factors, in particular IL-1 and PTH [259]. The very low to undetectable expressed in osteoblasts and osteoclasts, and PGD₂ can stimulate osteoblast differentiation and chemotaxis, while, unlike PGE₂, the effects of this prostaglandin on osteoclastogenesis appear to be inhibitory [259, 307]. PGD₂ can also induce IL-6 expression in osteoblasts [259]. There is also preliminary evidence that levels of a stable PGD₂ metabolite are increased in the urine

of patients with Paget's disease [308]. While we have not measured PGD_2 levels in the conditioned media, the reduction in *PTGDS* expression suggests that PGD_2 production would be decreased in pagetic cells, despite increased expression of cytokines. Presumably reduced PGD_2 levels would have a positive effect on osteoclastogenesis.

4.5.10 Genes with unexplored roles in osteoblast biology

Given that many of the other genes that show changes in expression have not been previously studied in relation to osteoblast biology it is difficult to establish what their role in Paget's disease may be. Keratin 18 is discussed further in Chapter 5. IFI27, which was up-regulated in the osteoblasts, is an interferon α and β responsive gene, and although there is no evidence that interferon expression was increased in these cells, other authors have shown increased expression of interferons α , β and γ in monocytes and lymphocytes derived from patients with Paget's disease [309]. However, expression of IFI27 in pagetic bone marrow, which is more likely to contain monocytes and lymphocytes, tended to be decreased. Expression was also much higher in the bone marrow than in the osteoblasts, and decreased in the MC3T3-E1s during differentiation, although this occurred in all conditions tested, and expression seemed to be further suppressed by dexamethasone. Therefore the increased expression in the human osteoblasts may be a result of the apparently less differentiated phenotype of the pagetic cells. IFI27 expression has also been reported to be up-regulated in diseased tissues in various microarray studies, including breast, ovarian and other cancers, focal segmental glomerulosclerosis, hepatitis C and psoriasis [310-313]. It is localised to the nuclear membrane and appears to inhibit proliferation and have antiviral activity [314, 315].

Decreased *MAFB* and increased *RGS4* levels may also be related to the differentiation stage of the cells. *MAFB* expression increased during osteoblast differentiation while *RGS4* showed decreased expression in the MC3T3-E1s as differentiation progressed. Others have also reported decreased *RGS4* as MC3T3-E1 cells lose their proliferative potential and differentiate [267]. The expression of *MAFB* in these cultures is actually a little surprising as it is a transcription factor that is important in the monocyte lineage and an inhibitor of osteoclast differentiation that is down-regulated by RANKL [260, 316]. A recent publication has suggested that osteal macrophages are generally present in primary osteoblast cultures and promote osteoblast differentiation [317], so it is possible that changes in *MAFB* expression in the human osteoblast cultures may be due to changes in the numbers or activity of these contaminating cells.

GATA6, which showed increased expression in pagetic osteoblasts, is a transcription factor that is expressed in a variety of tissues of mesodermal and endodermal origin. GATA4-6 have roles in cell-type specific gene expression in a number of endocrine organs [318]. Expression levels of *GATA4* and *GATA6* are interrelated, although based on the microarray data, only *GATA6* is expressed in osteoblasts [262]. There were no clear changes in expression during osteoblast differentiation, and levels of this gene were very low in the MC3T3-E1 cells. It would certainly be interesting to identify osteoblast-specific targets of this transcription factor, particularly given that loss of GATA6 in lung cells was associated with increased Wnt signalling [300].

RGS4, which was up-regulated in pagetic samples in both cell types, is a modulator of Gprotein signalling. Many factors including PTH, prostaglandins and calcium initiate cell signalling cascades through the actions of G protein-coupled receptors which are associated with heterotrimeric G-proteins. Regulators of G-protein signalling regulate the activity of heterotrimeric G-proteins. RGSs are key desensitisers of heterotrimeric G-protein signalling and scaffolds to overcome diffusion limitations and facilitate rapid, receptor-specific signal onset and termination [319]. They markedly alter cellular responses to hormone and neurotransmitter signals. RGS4 is one of the family of 'small' RGS proteins and it is ubiquitously expressed [320]. Its functions, including any in bone biology, have not been well studied, but it is required for correct regulation of $G\beta\gamma$ -gated potassium ion channels [321]. It will be interesting to determine if RGS4 expression affects the response of osteoblastic cells to various stimulators of G protein-coupled receptors, or if it inhibits differentiation, as it does in chondrocytes [322]. The Gene Ontology results in the microarray analysis implied that changes in G protein-coupled receptor signalling may be important in Paget's disease. A recent report showed that Rgs4 expression is repressed by RUNX2, and while the related RGS2 could alter proliferation in osteoblastic cells, suppression of RGS4 expression had no effect [267]. The increased expression of RGS4 in these samples is therefore consistent with the decreased RUNX2 expression, although the correlation between these two genes was not particularly strong in either the osteoblasts or bone marrow.

The increased expression of *SGK1* is perhaps not surprising given that this gene is responsive to various cytokines and growth factors including glucocorticoids, $1,25(OH)_2D_3$, TGF β , IL-6, FGFs, and PDGF [323]. Expression was strongly correlated with *IL-1\beta* in the primary pagetic
and non-pagetic cultures. It is activated by a number of different signalling pathways, and is an immediate early gene with appearance and disappearance of expression within 20 minutes [323]. However, the results in the MC3T3-E1 cells treated with dexamethasone suggest that extended treatment with a factor that stimulates its transcription can cause long term modestly increased expression, similar to what was detected in the pagetic cultures. The functions of *SGK1* include regulation of various ion channels, carriers and pumps, and the Na⁺-K⁺-ATPase. It interacts with transcription factors including NF κ B and can confer cell survival and affect cell proliferation [323]. It is not clear how the increased expression of this gene may affect the behaviour of osteoblastic cells, but its increased expression is probably another indicator that these cells are producing elevated levels of factors that could be stimulating osteoclastogenesis.

4.5.11 Differences between osteoblasts and bone marrow

For some genes, similar changes in gene expression were detected in both the osteoblast and bone marrow cultures, but there were also differences in the results between these two culture systems. Both contain osteoblastic cells, although the bone marrow is a mixed population which complicates the interpretation of results from these samples. Given that many of the genes were chosen based on microarray results from osteoblast samples it is not surprising that more of the results were significant in the osteoblasts. Sample numbers in both groups were similar, so should not have influenced the results. It is particularly surprising that many of the cytokines and other stimulators of osteoclastogenesis are only significantly increased in the osteoblasts, as increased osteoclastogenesis occurs in pagetic bone marrow *in vitro* when appropriate culture conditions are used [110, 111, 113]. There may well be important changes in gene expression in the bone marrow cultures that are not present in the osteoblasts in genes that have not been tested.

4.5.12 Paired osteoblast samples

The paired samples provided excellent controls for inter-patient variation and bisphosphonate treatment. Many of the genes that showed statistically significant changes in the osteoblasts also showed similar changes in the paired samples, and the results in the paired samples always showed the same trend as the overall changes. In addition, when samples were split into three groups, with the paired non-pagetic samples analysed separately, the mean of the non-pagetic paired samples was never significantly different from the controls, although in some cases it did fall in between the pagetic and the control group. These results provide

reassurance that the changes in gene expression observed are likely to be a consequence of the disease process occurring within the pagetic lesion. The fact that these changes persist in patients that have been treated and have low disease activity suggests that there is still an underlying abnormality in the cells, and the changes detected are not an epiphenomenon of high bone turnover which could itself affect the osteoblast phenotype.

4.5.13 Conclusion

Overall the results suggest that the pagetic osteoblasts are different from controls, both from patients without the disease, and from unaffected bone from patients with the disease. The changes suggest that the osteoblasts are producing more osteoclastogenic factors to stimulate the increased osteoclast numbers and activity seen in Paget's disease. In addition, a large number of genes involved in osteoblast differentiation and activity have altered expression, in particular the transcription factors *RUNX2*, *DLX5* and *SATB2*, *BMP2* and the bone markers alkaline phosphatase, osteocalcin and bone sialoprotein. These changes may contribute to the abnormal bone formation by pagetic osteoblasts which may result from a primary defect in osteoblasts rather than as a consequence of high bone turnover.

CHAPTER 5: EFFECTS OF DICKKOPF 1 AND KERATIN 18 ON BONE CELLS

5.1 Introduction

In order to understand how the changes in gene expression that we have identified in pagetic lesions contribute to the local accelerated bone turnover in Paget's disease it is important to understand the function of these genes in bone. Many of the genes showing altered expression have well-studied roles in bone cell biology, however others have not been investigated at all. This chapter will describe briefly the effects of DKK1 in *in vitro* bone cell cultures. The effects of DKK1 in osteoblast cultures were of interest due to its involvement in other bone diseases, and the importance of Wnt signalling in osteoblast biology. However, given that there are already numerous reports about the effects of DKK1 on bone cells, it was not investigated extensively. We have also examined some of the effects of over-expression of keratin 18 in osteoblastic cells. Keratin 18 was chosen because it was one of the most significantly up-regulated genes identified in both the pagetic osteoblasts and bone marrow, and it has not previously been studied in relation to bone biology.

5.1.1 Dickkopf 1

In recent years there have been multiple publications exploring the effects of Wnt signalling and DKK1 on various aspects of bone biology. *In vivo* studies show that DKK1 reduces bone mass, and is involved in the development of lytic bone lesions in multiple myeloma and metastatic cancers, indicating that DKK1 is a negative regulator of bone formation [74, 233, 324-326]. However, *in vitro* evidence suggests that inhibition of Wnt signalling is necessary for terminal differentiation of osteoblasts, and DKK1 contributes to inhibition of Wnt signalling in maturing osteoblasts [31, 32]. DKK1 is also a stimulator of cell division in mesenchymal stem cells, and can stimulate adipogenesis [327, 328].

5.1.2 Keratin 18

Keratin 18 is a type I intermediate filament protein, which assembles with a type II filament, most commonly keratin 8, in the cytoplasm to form functional intermediate filaments. The make-up of these cytoskeletal structures is shown in Figure 5.1. While keratin filaments often play a structural role and protect cells from physical stress, the keratin 18/keratin 8 pair is

expressed mainly in simple epithelia of internal organs, such as the liver, which experience little mechanical stress. Keratin 18/keratin 8 filaments, however, are thought to protect cells from metabolic stress and injury, regulate the cell cycle and prevent apoptosis [329-331]. The role of keratin 18 in osteoblast biology has not been previously determined. In order to investigate the effects of increased keratin 18 expression we engineered an adenoviral vector to over-express keratin 18.



Figure 5.1: Hierarchical structure of an intermediate filament Monomers form a coiled-coil dimer. In the case of keratin filaments, type I and II monomers form a heterodimer. These assemble in an antiparallel, staggered fashion to form tetramers. Tetramers assemble into protofilaments, and eight protofilaments combine to form a mature intermediate filament. From Darnell *et al.* 1990 [332].

5.2 Results – Dickkopf 1

Since DKK1 is a secreted protein, the effect of adding recombinant mouse Dkk1 to cultures was examined. Dkk1 inhibited primary rat osteoblast proliferation, and this was statistically significant at a concentration of 100 ng/mL (Figure 5.2(a)). Previous reports have demonstrated inhibition of mineralisation at concentrations of 10 ng/mL and above [325]. When added to a mouse bone marrow assay Dkk1-treated cells tended to show slightly increased numbers of osteoclasts, although this was only statistically significant at the 50

ng/mL concentration in some experiments (Figure 5.2(b)). These results are congruous with the known positive effect of Wnt signalling on osteoblast proliferation and differentiation, and modulation of *RANKL* and *OPG* expression to inhibit osteoclastogenesis [29, 30, 245]. In fact, given that the higher concentrations of Dkk1 inhibit Wnt signalling, which should be stimulated by the FBS in the bone marrow cultures, it is surprising that the effects on osteoclastogenesis are so mild. It is possible, however, that the 1,25(OH)₂D₃ treatment may be achieving such large changes in RANKL and OPG levels that the effects of inhibition of Wnt signalling are negligible. Previous studies have shown inhibition of osteoclastogenesis with stimulation of Wnt signalling in similar mixed cell culture systems [30].



Figure 5.2: Effect of Dkk1 on osteoblast proliferation and osteoclastogenesis The effects of Dkk1 on thymidine incorporation in primary rat osteoblasts (a), and in mouse bone marrow osteoclastogenesis (b) are shown. Thymidine incorporation data is pooled from three independent experiments. Osteoclastogenesis was also repeated three times, but one representative experiment is shown. Treatments that were significantly different from the control using one-way ANOVA with Dunnett's post test are marked.

5.3 Results – Keratin 18

Adenoviral vectors can be used to transduce a range of cell types and tissues. Expression lasts longer than plasmid transfections, generally for a number of weeks in dividing cells (until levels are diluted by cells division), and for months in non-dividing cells. The keratin 18-expressing adenoviral vector was produced as described in Section 2.3.7. Like most adenoviral vectors it is based on the Type 5 serotype adenovirus, with the E1 and E3 genes removed from the genome ensuring that the recombinant virus is replication-deficient in normal cells, and there is minimal expression of adenoviral genes. These vectors do not require active cell division or integration into the genome for gene expression to occur [333].

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Adenoviruses enter cells by binding to the Coxsackie-adenovirus receptor (CAR). A schematic of adenovirus vector structure and entry into the cell is shown in Figure 5.3.



Figure 5.3: Structure and gene transduction pathway of an adenovirus vector (a) The double-stranded virus genome is packaged within an icosahedral protein capsid. The geometrical faces of the capsid are comprised of hexon proteins, while penton bases associate with fibre proteins to form penton capsomer complexes at each of the 12 vertices. (b) The adenoviral vector binds to CAR and is internalised. The viral DNA is released into the nucleus where it is transcribed by the host cell. Reproduced with permission from Kawabata *et al.* 2006 [334].

5.3.1 Optimising the transduction conditions

Viral titres are expressed in plaque-forming units (pfu), or infection-forming units (ifu) per mL, and transductions are performed by adding the virus at a certain MOI, which indicates the number of pfu per cell. Most protocols use MOI ranging from about 1-100 in order to achieve good transduction efficiency. Both the keratin 18-expressing adenovirus and the control adenovirus express GFP allowing straightforward qualitative assessment of the transduction efficiency using a fluorescent microscope. Preliminary experiments in MC3T3-E1 cells showed very poor transduction efficiency unless very high MOIs (over 250) were used.

While many publications report the use of adenoviral vectors in osteoblastic cells using basic transduction protocols (for example [335-337]), it has also been reported that fibroblastic and osteoblastic cells express low levels of CAR so are difficult to transduce [227]. We therefore used a modification of the protocol described by Fouletier-Dilling *et al.* [227] which used the transfection reagent GeneJammer to improve transduction efficiency in MC3T3-E1 cells. While this protocol did not greatly improve the results in MC3T3-E1 cells, we also tested a

number of other cell types with varying success. Primary human osteoblasts were successfully transduced, and the transduction worked much better in the presence of GeneJammer, as shown in Figure 5.4. SaOS2s were also successfully transduced (Figure 5.5(c,d)), and they did not require the presence of GeneJammer. Rat osteoblasts (Figure 5.5(a,b)) and UMR-106 cells (data not shown) could also be transduced, although not as well as the human cells, and MC3T3-E1 (Figure 5.5(e,f)) and ST2 cells (data not shown) showed very poor transduction. These observations suggested that the species of origin of the cells may affect their ability to take up adenoviruses, with human cells performing the best, rat cells less well, and mouse cells very poorly, although this may be coincidental. Subsequent tests indicated that Fugene6, which has very similar properties to GeneJammer, worked equally well at improving transduction. Since Fugene6 was already used routinely for other transfection protocols in our lab, later experiments were performed with this reagent.



Figure 5.4: Primary human osteoblasts transduced with adenoviral vectors with and without GeneJammer transfection reagent

Primary human osteoblasts were transduced with MOI 100 of keratin 18 adenovirus (which also expresses GFP). 48 hr later images were taken. Fluorescent images (a, c) indicate transduced cells in green, while the phase contrast images (b, d) show all the cells present in the same field of view. The results indicate much lower transduction efficiency in the absence of GeneJammer transfection reagent (a), compared to the presence of GeneJammer (c). Scale bar represents $100\mu m$.

Chapter 5



Figure 5.5: Transduction of various osteoblastic cell types with keratin 18 adenovirus Fluorescent images (a, c, e) indicate transduced cells in green, while the phase contrast images (b, d, f) show all the cells present in the same field of view. Primary rat osteoblasts (a, b), show some GFP-positive cells when transduced at MOI 100 in the presence of GeneJammer. SaOS2s (c, d) show good transduction efficiency, even in the absence of GeneJammer at MOI 100, and MC3T3s (e, f) show very poor transduction efficiency at MOI 200 in the presence of Fugene6 or GeneJammer (not shown). All images were taken 48 hr after transduction, scale bar represents 100µm.

Preliminary experiments also indicated that long term expression was possible using the adenoviral vectors, with GFP expression still detectable 20 days after transduction (data not shown). Transduction appeared to affect cell morphology, and microscopic analysis indicated there were fewer cells in wells transduced using high MOIs, probably due to increased cell death. For this reason most experiments were performed over the 72 hr following transduction, and MOIs were chosen at a range that did not appear to have negative effects on the cells. Western blots were routinely performed on lysates from transduced cells, and keratin 18

protein was detectable. A representative blot is shown in Figure 5.6. The keratin 18 band from the human osteoblast samples appears to have a slightly higher molecular weight than in the SaOS2s. This may be due to variations in posttranslational processing but was not investigated. Real time PCR also indicated much higher levels of keratin 18 gene expression in cells transduced with the keratin 18 adenovirus compared with controls. The extent of this increase depended on the MOI and the experiment, but was generally between 500-1000 times higher than the control. No keratin 18 expression was detectable in samples from control cDNA reactions lacking reverse transcriptase, indicating that the RNA samples are not contaminated with viral vector DNA.



Figure 5.6: Western blot showing keratin 18 expression in transduced cells

Expression of keratin 18 was not detectable at the protein level in control cells (Lanes 1 and 5). Lanes 1-4 contain lysates from human osteoblast cells (HOB). Cells were transduced at MOI 30 (lane 2), 60 (lane 3) and 100 (lane 4). Lanes 5-7 contain lysates from SaOS2 cells. Cells were transduced at MOI 30 (lane 6) or MOI 100 (lane 7). All lysates were collected 48 hr after transduction. Lane 8 contains lysate from HEK293A cells used to grow up the adenovirus stock as a positive control, while lane 9 contains MagicMark. Bands were detected at about 45 kDa as expected. The graph below shows intensity of the bands corrected to total ERK expression, which was detected after stripping and reprobing the blot.

5.3.2 Effects of keratin 18 over-expression on cell proliferation

The effects of keratin 18 over-expression on osteoblast proliferation were evaluated in both human osteoblasts and SaOS2 cells. The effect of transduction on human cells is summarised in Figure 5.7 using combined data from between three and six experiments performed using

cells derived from different patients. Transduction of the cells did not appear to have any reproducible effect on proliferation. The effects of keratin 18 on human osteoblast proliferation were also inconsistent. The inconsistency in these results may be due to the use of primary human cells, with cells from different donors showing different responses. The proliferative response of these cells to potent factors like PDGF is reproducible, although the magnitude of the response can differ, but responses to other factors can be more variable. Additionally, the baseline radioactive counts which are indicative of the baseline proliferation rate can vary considerably between individuals, which may also affect the response of the cells.



Figure 5.7: Human osteoblast proliferation in cells transduced with keratin 18

Proliferation was measured by 24 hr thymidine incorporation either between (a) 30 and 54 hr after transduction, or (b) between 54 and 78 hr after transduction in serum-free conditions. Significance is determined by 1-way ANOVA with Dunnett's post test.

Control: non-transduced cells;

GFP: cells transduced with the control GFP-expressing adenovirus;

KRT18: cells transduced with the keratin 18-expressing adenovirus;

PDGF: 10 ng/mL PDGF added as a drug immediately before the earlier thymidine addition

The effect of the adenoviruses on proliferation in SaOS2 cells was also evaluated. Unlike the primary human osteoblasts, in SaOS2 cells transduction itself produced a very large increase in thymidine incorporation (Figure 5.8(a)), much greater than with bovine lactoferrin or TGF β , both of which are potent stimulators of osteoblast proliferation. MTT assays were also performed to rule out the possibility that this effect was a result of GFP expression interfering with the assay. The MTT assay measures cell number and activity rather than DNA replication, and tends to be less sensitive than thymidine incorporation. Although not statistically significant overall, the MTT results also suggest increased proliferation in the

transduced SaOS2 cells (Figure 5.8(b,c)). This was a very surprising result, as we would have expected that over-expression of proteins, if anything, was more likely to have a negative effect on cell proliferation. Based on the thymidine incorporation assay, transduction with keratin 18-expressing adenovirus reduced proliferation by about 25% compared with the appropriate GFP-transduced controls (Figure 5.8(a)). The results were similar for both MOIs tested. The MTT assay did not show a significant change in proliferation with the keratin 18 over-expression compared to GFP alone (Figure 5.8(b,c)). In summary, over-expression of keratin 18 in human osteoblast cells does not appear to have any reproducible effects on cell proliferation, but the proliferation in SaOS2 cells may be inhibited by over-expression of keratin 18 in comparison to the vector control.



Figure 5.8: SaOS2 proliferation in cells transduced with keratin 18

Proliferation was measured using 6 hr thymidine incorporation (a), or MTT assay 48 hr (b) or 72 hr (c) after transduction. Bovine lactoferrin (bLF) at 50 μ g/mL was included as a positive control, and was added as a drug 30 hr after the transductions of the other cells. Significance is determined by 1-way ANOVA with Dunnett's post test. Student's t tests were also performed to compare *KRT18*-transduced cells to the appropriate GFP control and results of these are indicated if significant.

5.3.3 Effects of keratin 18 over-expression on osteoblast gene expression

In order to determine whether keratin 18 over-expression affected gene expression, real time PCR was performed on RNA samples extracted from human osteoblasts and SaOS2 cells at various timepoints up to 72 hr after transduction. Gene expression in many of the samples was examined using TaqMan low density array microfluidic cards. The cards used in these experiments contained primers for 45 genes involved in various aspects of bone formation, stimulation of osteoclastogenesis, and cell signalling, and three control genes (genes listed in Table 2.3). Candidate genes identified in either the human osteoblast or in the SaOS2 samples were verified using standard real time PCR. The nine genes chosen were *BMP6*, *CCL2*, *DKK1*, *FGF2*, *IL-1* β , *IL-6*, *M-CSF*, *OPG* and osteopontin, a number of which had shown changes in expression in the pagetic osteoblasts as indicated by the microarray and real time PCR results. The remaining 36 genes examined on the low density arrays either showed minimal changes, or did not show consistent results in the human osteoblast cells.

Gene expression in human osteoblasts was examined in cells from six different patients 48 hr and 72 hr after transduction at two different MOIs. The gene that was most consistently upregulated in cells transduced with keratin 18 was *BMP6* (Figure 5.9(a)). Basal *BMP6* expression tended to be low, but keratin 18 over-expression caused increases in *BMP6* expression of up to 38-fold in five of the six patients, and there was often a dose-dependent effect observed with increasing MOI. *DKK1* also tended to be up-regulated, with increases in four of the six patients (Figure 5.9(b)). However, transduction with the GFP vector often resulted in lower levels of *DKK1* expression than the untransduced control, so in some cases expression in the keratin 18-transduced cells was higher than the GFP control, but similar to the untransduced control. *M-CSF* expression also tended to be up-regulated in the keratin 18-transduced cells, with significant increases compared to the GFP-transduced cells in four out of six patients (Figure 5.9(c)). Conversely, in one patient levels tended to decrease, although this was not statistically significant.



Figure 5.9: Relative expression of *BMP6*, *DKK1* and *M-CSF* in human osteoblasts transduced with keratin 18 Each graph shows real time PCR results from one representative experiment. Significance was determined with 2-way ANOVA and keratin 18 samples that showed a significant difference from the appropriate GFP-transduced control are marked. Relative expression was corrected to a standard calibrator value, so expression values give some indication of absolute expression, although since amplification efficiency varies between probes, they are not directly comparable between different genes.



Figure 5.10: Relative expression of *CCL2*, *IL-1\beta* and *IL-6* in human osteoblasts transduced with keratin 18 These representative experiments are presented in the same manner as the previous figure.

Expression of the chemokine, *CCL2* also tended to be increased in the human osteoblasts, although transduction with the control GFP adenovirus lowered basal expression of this gene in all experiments, meaning that overall the highest expression tended to be in the control cells (Figure 5.10(a)). *IL-1\beta* was expressed at much lower levels, but showed significant increases in three of the patients, a trend towards increased expression in one further patient, but very little change in the remaining two patients (Figure 5.10(b)). While there was a significant increase in *IL-6* expression in one of the patients, there was very little change in the others suggesting this gene was not up-regulated by the presence of keratin 18 (Figure 5.10(c)). The remaining three genes did not show any reproducible changes (data not shown).

Expression of the same nine genes was examined in SaOS2 cells transduced with the adenoviral vectors. IL-1 β expression was not detectable at all in the SaOS2s, and BMP6 and CCL2 were barely detectable, so results from these genes are not shown. As expected, the results from replicate SaOS2 experiments were much more consistent than in the primary human cells. FGF2 expression was up-regulated at the higher MOI tested with keratin 18 over-expression, as was osteopontin (Figure 5.11). Osteopontin expression in the SaOS2 cells was much lower than in the human osteoblasts. IL-6 was also significantly up-regulated by up to 20-fold in the keratin 18 over-expressing cells, and showed dose-dependent increases in expression, particularly 48hr and 72hr after transduction (Figure 5.11). The other genes tested, DKK1, M-CSF and OPG, did not show any significant changes in expression (data not shown). A number of other genes from the low density arrays tended to be up-regulated in the keratin 18 over-expressing cells. These included the transcription factors MYC, JUN and FOSL1; SMAD7, an inhibitory Smad that attenuates TGFB signalling; and LRP5, a Wnt coreceptor. However the expression of these genes was only examined in a subset of the samples from transduced SaOS2 cells. MYC and JUN showed no clear trends in the human osteoblast samples run on the low density arrays, however FOSL1, SMAD7 and LRP5 showed upward trends in at least half of the experiments. Therefore, there are likely to be other genes that are up-regulated in response to keratin 18 over-expression, possibly within the genes examined, and others not included in the low density arrays. Overall there did not seem to be changes in bone markers, or osteoblastic genes, although this may be due to the limited timecourse performed. However, some of the increases in cytokines and other secreted proteins observed are similar to the changes in gene expression detected in pagetic osteoblasts.





Figure 5.11: Relative expression of *FGF2*, *IL-6* and osteopontin in SaOS2 cells transduced with keratin 18 Results show one representative real time PCR experiment, and are presented and analysed in the same manner as the gene expression results from the human osteoblasts in the previous figures.

5.3.4 Other effects of keratin 18 in osteoblasts

One of the most striking features of the cells transduced with keratin 18 was the presence of unusual structures associated with some cells as shown in Figure 5.12. These were not seen in the GFP-transduced cells. They were still present after trypsinisation and replating of transduced cells (data not shown). These structures are clearly visible using light microscopy, but it would be interesting to perform some more advanced imaging on these structures to characterise them.



Figure 5.12: Unusual cellular structures in keratin 18-transduced cells Cells transduced with keratin 18 (a) show unusual structures in the phase contrast image (left panel) associated with some of the transduced cells (green cells in the right panel), two examples are marked with red and pink arrows. These structures were not present in equivalent cells transduced with the control GFP vector (b). An example of a transduced cell is shown in this image with a yellow arrow. Scale bars represent 50µm.

There has previously been a report of IL-6 mediated induction of keratin 18 expression in intestinal epithelial cells [338]. We were interested to see if IL-6 treatment of human osteoblasts would result in increased *KRT18* expression. In a preliminary experiment, *KRT18* expression was unchanged in cells treated with 100 ng/mL IL-6 for up to 24 hr, in the presence or absence of IL-6 soluble receptor which is often required for IL-6 effects on

osteoblasts. This concentration of IL-6 was used in the previous report, however this is a high concentration of IL-6 which can have negative effects on human osteoblast proliferation (data not shown). This, in addition to the variable results in experiments using human osteoblast cells, produced inconclusive results.

Keratin 18- and GFP-transduced cells were also grown on three dimensional collagen scaffolds in order to observe their effect on cells in a more 'bone-like' environment. This was a preliminary experiment that we plan to repeat. Mesenchymal cells derived from human bone marrow were transduced, then trypsinised and seeded onto collagen scaffolds. Cells were then cultured for a week in growth media, and then up to three weeks in mineralisation media. Confocal microscopy confirmed that GFP-positive cells were still present after four weeks of culture (Figure 5.13), and live/dead staining showed there was still good cell survival at this point (Figure 5.14).



Figure 5.13: GFP-positive cells after four weeks growth on collagen scaffolds Confocal microscopy showing two different fields of GFP-positive mesenchymal cells that have been cultured on a collagen scaffold for four weeks after transduction with the keratin 18 adenovirus.

Scaffolds were fixed at weekly intervals, and cryosections were stained. These were examined qualitatively. DAPI staining indicated that cells were present in all the sections examined regardless of the treatment, however, surprisingly, alkaline phosphatase staining was only observed in the final timepoint (Figure 5.15). Alizarin red staining indicated that there was no mineralisation before the addition of mineralisation media (data not shown), however, staining was present at later timepoints (Figure 5.15). The scaffolds with the transduced cells showed alizarin red staining as early as one week after the addition of mineralisation media,

while the control cells did not show extensive staining until the three week timepoint. This possible increase in mineralisation is not specific to the keratin 18-expressing cells, as it is also seen in the GFP-transduced cells, suggesting the GFP expression, or adenoviral transduction may cause increased alizarin red staining. While preliminary, this experiment indicates that mesenchymal cells transduced with adenoviruses can be grown successfully in three dimensional scaffolds for long periods, with good cell survival, and that mineralisation is probably occurring under these conditions.







Figure 5.14: Live/dead staining of cells cultured on collagen scaffolds for four weeks

Mesenchymal cells were cultured on collagen scaffolds then live/dead stained. Confocal microscopy shows live cells in green while dead cells are red. The images show fields from scaffolds that were cultured with untransduced controls (a), GFP-transduced (b), or keratin 18-transduced (c) cells.



Figure 5.15: Sections of collagen scaffolds stained for alkaline phosphatase, mineralisation, and cell number after four weeks in culture

Mesenchymal cells cultured on collagen scaffolds at week 3 (4 weeks after seeding, 3 weeks after addition of mineralisation media). Scaffolds were fixed then cryosectioned and stained. Shown are sections from scaffolds cultured with control (a-c), GFP-transduced (d-f), and keratin 18-transduced (g-i) cells. The stains shown are for alkaline phosphatase (pink) (a, d, g), alizarin red which stains mineral red (b, e, h), and blue DAPI nuclear stain (c, f, i). Scale bars represent $200\mu m$.

5.4 Discussion

One of the interesting findings of the present study is the increased expression of the Wnt signalling antagonist, DKK1, in both osteoblast and bone marrow cultures. DKK1 overproduction has been implicated in the development of focal bone lesions in multiple myeloma, which are radiologically similar to lytic pagetic lesions [74, 233]. Tian *et al.* have suggested that the release of DKK1 from malignant plasma cells in multiple myeloma results in an inhibition of osteoblast proliferation, accentuating the imbalance between bone formation and bone resorption and facilitating local bone loss [74]. The *in vitro* findings in the current study showing decreased osteoblast proliferation and increased osteoclastogenesis in cells treated with Dkk1 support this hypothesis. *DKK1* over-expression has also been observed in prostate and breast cancer cell lines that produce lytic bone lesions when inoculated into animals [252, 339]. Over-expression of *DKK1* has also been implicated in the development of lytic lesions in patients with breast cancer metastases [231], and expression levels influence whether lytic or osteoblastic lesions develop in prostate cancer metastases

[339, 340]. Interestingly, DKK1 production by osteoblastic cells can also inhibit breast cancer cell growth [341]. DKK1 has also been implicated in bone destruction in rheumatoid arthritis, and development of paediatric osteosarcoma [250, 251]. Unlike these diseases, where serum DKK1 was increased in addition to local increases in expression and protein production [250-252, 342], we were unable to identify increased DKK1 levels in serum from patients with Paget's disease.

In the context of Paget's disease, overproduction of DKK1 by the osteoblast itself could have analogous effects on bone formation to DKK1 overproduction by cancer cells. Gunn et al. have shown that DKK1 from myeloma cells increases IL-6 expression from undifferentiated mesenchymal stem cells, which promotes further myeloma cell proliferation [343]. In the context of Paget's disease, stimulation of IL-6 production as a result of over-expression of DKK1 would contribute to the development of the lytic pagetic lesion by further accelerating local bone turnover. Subsequent studies have indicated that the role DKK1 (and DKK2, which was not expressed in pagetic or non-pagetic osteoblasts in these studies) plays in osteoblast development is more complex. van der Horst et al. showed that inhibition of Wnt signalling is necessary for late-stage osteoblast differentiation and mineralisation and that this inhibition is achieved by the up-regulation of the expression of DKK1 and DKK2 [32]. Similar results were found in studies of the role of Dkk2 in terminal osteoblast differentiation in mice [344]. Thus, DKK1 over-expression could both promote the development of the lytic pagetic lesion (similar to its role in myeloma) but ultimately also contribute to the sclerotic stage of Paget's disease, to which most lytic lesions progress. In vivo, this transition takes place over a period of months to years, and often there is co-existence of both, with sclerosis in the older part of a lesion, whereas the advancing edge shows a lytic process. Possibly, DKK1 inhibits the proliferation of osteoblasts in the lytic pagetic lesion and facilitates local mineralisation as osteoblast differentiation takes place, and this transition is a time-dependent process within a given cohort of pagetic cells.

The bone-specific role of keratin 18, which was similarly up-regulated in both pagetic osteoblasts and bone marrow, was also investigated. Using adenoviral vectors in the presence of transfection reagents enabled over-expression of keratin 18 in primary human cells, which are very difficult to transfect with plasmids. Transgene expression was still detectable four weeks after transduction, although because adenoviruses do not incorporate into the genome like lentiviral and retroviral vectors, expression is diluted as cells divide. The transduction and

expression of GFP did not seem to be harmful to the cells, and in the SaOS2s cell proliferation increased in the transduced cells. The adenoviral vectors limited the use of certain cell lines, but were useful for experiments involving primary osteoblasts.

The expression of keratin 18 in the human osteoblast and bone marrow cultures demonstrated by the microarray and real time PCR results is unexpected, as keratins are not usually expressed in mesenchymal cells. However, expression of simple epithelial keratins including keratin 18 has previously been reported in mesenchymal cells, particularly foetal cells, and certain smooth muscle cells where they are coexpressed with other intermediate filaments like vimentin and desmin [329]. The background expression of keratin 18 in the human osteoblasts was consistently detectable at low levels, and although expression was not directly compared to epithelial cells, keratins are reported to be abundantly expressed in these cells [345]. It is possible that the expression levels of keratin 18 in the osteoblast cultures are low because it is not expressed in all the cells in the culture. Keratin 18 expression was not detectable in the MC3T3-E1 cells, but expression levels in the SaOS2 cells were similar to in the primary human osteoblasts. It would be interesting to identify how many of the cells in the primary human osteoblast cultures are expressing keratin 18 using immunohistochemical or cell sorting techniques, and to characterise how this changes in the pagetic cultures.

The simple epithelial keratins have proved to be useful cancer markers, and their fragments are present in the traditional cancer marker tissue polypeptide antigen [330]. Tumours release these keratin fragments into the blood, but dying epithelial cells do not appear to. Keratin 18 is a particularly good marker as an antibody is available that specifically identifies caspase-cleaved fragments. This potentially allows measurement of relative amounts of apoptosis and necrosis of tumour cells [346]. Over-expression of keratin 18 or 8 in mice produced no overt phenotype, and keratin 18 knockout mice are fertile with normal life expectancy [345, 347, 348]. The loss of keratin 18 is possibly compensated by keratin 19, although these mice do develop Mallory bodies containing keratin 8 in their hepatocytes [345]. These accumulations of abnormally folded keratins and SQSTM1 are a feature of liver conditions such as alcoholic hepatitis [349].

Keratin 18 over-expression may have a negative effect on osteoblast proliferation, particularly in SaOS2 cells, although the effects in human osteoblasts were inconsistent. There were also some changes in gene expression, including up-regulation of *IL-6*, *FGF2* and osteopontin in

SaOS2 cells, and increased *BMP6*, *IL-1β*, and possibly *DKK1*, *M-CSF* and *CCL2* in human osteoblasts. There were no consistent changes in osteoblast marker genes, although the timeframe of the experiments was brief, and differentiating media was not used (although the media used for the human osteoblasts did contain A2P, albeit at a concentration five times lower than what is generally used in osteoblast differentiation cultures). Changes in *OPG* were minimal or inconsistent, and *RANKL* expression in these cells is very low, so significant changes were not observed. Given that a number of the up-regulated genes play a role in stimulating osteoclastogenesis it would be interesting to investigate the effects of keratin 18 over-expression on this aspect of osteoblast biology. It would also be interesting to see if there is an effect of keratin 18 over-expression on apoptosis, given that keratin filaments can have anti-apoptotic function.

Formation of keratin filaments requires equimolar amounts of type I and type II keratins. When keratin 18 was over-expressed, there were no significant increases in its usual binding partner, keratin 8, and there was no indication of increased expression of keratin 8 or other type I keratins in the microarrays (data not shown). Single keratins without a matching pair are rapidly degraded [329]. Therefore large increases in keratin 18 protein levels are likely to be transient. Increased protein levels were detectable on western blots, although the viral lysates from HEK293A cells used as a positive control, which probably contained much smaller protein concentrations due to the different lysis method used, consistently showed much stronger signal. This may mean changes observed are a result of cell stress due to excessive protein degradation, and the unusual structures observed associated with the cells over-expressing keratin 18 in culture may be some form of protein aggregate. Keratin 18 can certainly be a component of protein aggregates, such as Mallory bodies, although the formation of these structures appears to be driven by excess keratin 8 rather than keratin 18 [345, 349]. Notably, an alternative hypothesis for the identity of the 'viral-like' inclusions found in cells in Paget's disease is that they are some form of protein aggregate.

The results presented here suggest that in combination with other factors, increased DKK1 expression could contribute to the abnormal bone turnover observed in Paget's disease but increased keratin 18 expression is unlikely to be a primary driver of the changes. While some of the changes in gene expression in cells over-expressing keratin 18 mimic the changes we found in pagetic osteoblasts, this required increases in expression orders of magnitude higher than what was observed in the pagetic osteoblasts. An alternative explanation for increases in

keratin 18 expression could be that expression is increased in response to increased IL-6 levels [338], although we found no evidence that this occurred in human osteoblasts and there was only a weak correlation between IL-6 and keratin 18 expression levels measured by real time PCR in the human osteoblasts (r = 0.427, p = 0053), and no correlation in the bone marrow. There was a much stronger correlation between *KRT18* and *DKK1* (r > 0.6, p < 0.0001) in both cell types. More research is required to identify a role for keratin 18 in osteoblast biology and Paget's disease.

CHAPTER 6: EFFECT OF WILD-TYPE AND MUTANT SQSTM1 IN OSTEOBLASTS

6.1 Introduction

Mutations in the UBA domain of *SQSTM1* were first identified in patients with Paget's disease in 2002 [127], and since then around 20 different mutations, located either in or near the UBA domain have been reported. These mutations predispose patients to Paget's disease in an autosomal dominant fashion, although they show incomplete penetrance [139]. Approximately one third of families with the disease have mutations in *SQSTM1*, and mutations are also found in around 6% of sporadic cases [350]. SQSTM1 is a ubiquitously expressed adaptor protein, which can play a role in a diverse array of cellular processes (see Section 1.13.1.1) [143]. *Sqstm1* knockout mice are grossly normal, and young mice do not have an overt bone phenotype, although they do show reduced bone resorption in response to PTHrP, and lower levels of RANKL-induced osteoclastogenesis *in vitro* [149]. They also develop mature-onset diabetes and insulin resistance [146], and aged mice have increased BMD [148]. When this study was started there was very little known about the mechanisms by which mutations in *SQSTM1* predisposed patients to developing Paget's disease. The mutations were shown to inhibit the ubiquitin-binding ability of the protein *in vitro*, but the consequences of this change on protein function were not understood [131, 132].

SQSTM1 acts as an adaptor in the NF κ B signalling pathway. In bone biology, NF κ B signalling is considered to be a particularly important signalling pathway in the osteoclast lineage, as NF κ B signalling stimulated by RANKL is critical for osteoclastogenesis. Recent studies exploring the consequences of *SQSTM1* mutations *in vitro* and *in vivo* have therefore focussed on their effects on NF κ B signalling and differentiation in cells of the osteoclast lineage [133, 156, 157]. However, the NF κ B cascade is a ubiquitous signalling pathway, and also functions in osteoblastic cells. TNF α , signalling through NF κ B, stimulates osteoblast proliferation, inhibits differentiation, and antagonises Smad signalling stimulated by TGF β or BMPs [351, 352]. TNF α knockout mice have higher peak bone mass than controls due to increased osteoblast numbers and bone formation while osteoclastogenesis is normal [351].

V=vt=List of research project topics and materials

The mutations in *SQSTM1* in patients with Paget's disease are germline mutations present in all cells including osteoblasts. Our data suggest that osteoblasts play an important role in Paget's disease so it is possible that the effects of these mutations in osteoblasts contribute to the development of the disease. To investigate this hypothesis, we have constructed plasmids that express wild-type *SQSTM1* and two common mutants found in patients with Paget's disease, and examined the differential effects when these proteins are over-expressed in osteoblastic cell lines. These cells still expressed high background levels of endogenous wild-type *SQSTM1*, but given that the mutations in Paget's disease are dominant and heterozygous, their effects should still be manifested in this system.

6.2 Methods

6.2.1 Vector construction

The pcDNA3.1/His vector from Invitrogen was chosen for *SQSTM1* expression. This vector contains a bacterial origin of replication, and a gene conferring ampicillin resistance allowing large-scale production in bacteria. Gene expression in mammalian cells is controlled by the cytomegalovirus promoter which drives high constitutive expression, and neomycin resistance allows selection of stably transfected clones. This vector encompasses an N-terminal tag containing a polyhistidine region, an Xpress tag, and an enterokinase cleavage site which allows removal of the tag. An N-terminal tag was chosen because one of the mutations we planned to introduce was a truncation mutation, which would introduce a stop codon that would exclude any C-terminal tags from the recombinant protein. Other studies have used N-terminal tagged *SQSTM1* constructs without apparent impairment of protein function [133, 156].

The wild-type human *SQSTM1* sequence for insertion into the plasmid was amplified from SaOS2 cell cDNA as described in Section 2.3.6. This produced a fragment of 1449 bp with a KpnI restriction site 10 bp downstream of the start codon, and an XbaI site following the stop codon. The beginning and end of the human *SQSTM1* sequence depicting the introduction of the restriction sites in relation to the start and stop codons is shown in Figure 6.1. The tags and *SQSTM1* sequence were cloned in frame to ensure the correct amino acid sequence was produced. After the insert and the plasmid were digested with both enzymes, ligation was performed. The resulting plasmid was the predicted size (approximately 7000 bp), and digestion with HindIII produced bands of the expected sizes as shown in Figure 6.2. Sequencing showed the expected sequence of the insert, and the insert-vector boundaries.

There were two single nucleotide polymorphisms detected in the *SQSTM1* gene that were not reported in the NCBI databases, namely 945G/A, which is within the protein coding sequence, but does not change the amino acid sequence, and 1381T/C, which is downstream of the stop codon. Since these are both silent changes we did not consider them problematic. The resulting protein contains the N-terminal tag from the plasmid (32 amino acids), but is missing the first five amino acids of the normal SQSTM1 protein sequence. The lysine residue at position 7 which is important in the activity of the PB1 domain is maintained [353].

SQSTM1 for cloning F GCT ATGGCGTCGG T ACCGGTGAA 1 cgaccgggac ggcccgttt ccgccagctc gccgctcgc atggcgtcgc tcacc gtgaa GGC 1321 tctggacacc atccagtatt caaagcatcc cccgccgtt tgaccactt tgcccacctc 1381 ttctgcgtgc ccctcttctg tctcatagtt gtgttaagct tgcgtagaa tgcaggtctc SQSTM1 for cloning R CTCTG CCTTCTTCTA GAATCAGGGG TTAGGG 1441 tgtacgggcc agtttctctg ccttcttcca ggatcagggg ttagggtgca agaagccatt

Figure 6.1: Parts of the *SQSTM1* cDNA sequence (genbank accession number NM_003900) showing the introduction of restriction sites for cloning

The start (ATG) and stop (TGA) codons are labelled in red, and the primers used to introduce restriction sites (SQSTM1 for cloning forward (F) and reverse (R)) are shown. The restriction site sequences of KpnI in the forward primer and XbaI in the reverse primer are shown in blue.

L 1 2 3 4 5 6 7 8 9 10 11 12 13



Figure 6.2: Gel showing the plasmid after insertion of the SQSTM1 sequence.

Lanes L and 7 contain 1 kilobase and 100 bp ladders respectively. Lanes 1-6 show undigested plasmid preparations. Lane 1 contains the vector without an insert. Lanes 2-6 show plasmids purified from bacterial colonies generated from the ligation reaction. All but one (lane 5) contain the insert. This was confirmed by a HindIII digest of the same plasmid preparations (lanes 8-13). Both the plasmid and insert have one HindIII site, so the plasmids containing inserts form two fragments when digested, one about 5500 bp and another about 1400 bp as shown in lanes 9-11 and 13.

6.2.2 Site-directed mutagenesis

Mutations were introduced using site-directed mutagenesis. Two mutations were chosen for this study. The first mutant was proline 392 to leucine (P392L) which is the most common *SQSTM1* mutation [350]. The second mutation introduces a stop codon at position 396 (396X),

which is one of a number of possible truncation mutations that may predispose patients to more severe disease [136], and is likely to have a more drastic effect on protein function. The P392L mutation results from a 1215C/T change at the nucleotide level, while 396X is usually encoded by 1224insT. However, for the site directed mutagenesis we used a primer that changed bp 1226 from G to T which also results in the formation of a stop codon at position 396. Sequence analysis confirmed that the mutations were introduced at the appropriate locations, and there were no changes to the surrounding sequences.

6.2.3 Transfection and protein expression

Preliminary testing of transfection efficiency in various osteoblastic cells indicated that the rat osteosarcoma cell line UMR-106, and the human osteosarcoma cell line SaOS2 could be readily transfected, so these cell lines were used to determine the effects of SOSTM1 mutants in osteoblastic cells. Real time PCR indicated that SOSTM1 expression was increased approximately 50 to 80-fold from controls in SaOS2 cells 24 hr after transfection. SOSTM1 transcripts were also detected in no reverse transcriptase controls, suggesting there was plasmid contamination in the RNA preparations, so these controls were always included in real time PCR analysis of SQSTM1 expression in transfected cells in order to correct the results appropriately. Attempts to demonstrate protein expression using western blotting were unsuccessful. The Xpress antibody showed very poor results with the first batch purchased, and production of this antibody subsequently ceased. Results with various His tag antibodies were also very poor, with high background signal, but no specific bands of the expected size. SQSTM1 antibodies were not suitable as many used C-terminal regions of the protein as the epitope, meaning the mutants may not have been as easily detected. Since we were unable to confirm SQSTM1 protein expression due to technical problems, the production of protein from the plasmids was confirmed using a functional assay as detailed in the results section below.

6.3 Results

6.3.1 Basal SQSTM1 expression in osteoblasts

We examined the expression of *SQSTM1* in pagetic osteoblasts and bone marrow as well as differentiating MC3T3-E1 cells and human osteoblasts using the same approach as the results presented in Chapter 4. *SQSTM1* was consistently expressed at high levels, and the expression in human osteoblasts was similar to levels of tenascin C and bone sialoprotein shown in

Figure 4.10(b). There was no significant difference between the non-pagetic and pagetic cells in either the osteoblasts or the bone marrow (Figure 6.3). Expression in the bone marrow tended to be about 30% lower than in the osteoblasts, although this was not consistently statistically significant. Expression was maintained at very similar levels throughout differentiation of the MC3T3-E1 cells, and dexamethasone did not have any effect on the results. There was generally some increase in expression during human osteoblast differentiation (Figure 6.3). The results suggest that osteoblasts express high levels of *SQSTM1*, and expression is not affected greatly in the pagetic lesion or during osteoblast differentiation.



Figure 6.3: Expression of SQSTM1 in osteoblastic cells

Relative expression of SQSTM1 is shown in pagetic osteoblasts (a) and bone marrow (b), and in differentiating MC3T3-E1 cells (c) and primary human osteoblasts (d). MC3T3-E1 cells were cultured in either control (blue), mineralisation (green) or dexamethasone-containing (red) media. The different coloured lines in (d) represent results from cells derived from four different patients.

6.3.2 Over-expression of SQSTM1 in transfected cells

Increased expression of *SQSTM1* mRNA in transfected cells was shown using real time PCR (Figure 6.4(a)). We used a functional assay to demonstrate the production of active SQSTM1 in the transfected cells. Previous studies using a luciferase reporter assay have indicated that wild-type SQSTM1 over-expression in HEK293 cells results in reduced NF κ B activity, while over-expression of SQSTM1 with pagetic mutations causes little or no inhibition of NF κ B [133]. We were able to replicate these results in HEK293 cells, suggesting functional SQSTM1 protein was being produced from the plasmids, and the mutations were having the expected effect (Figure 6.4(b)).



Figure 6.4: Functional SQSTM1 is over-expressed by the plasmids

(a) Expression of *SQSTM1* in SaOS2 cells 24 and 48 hr after transfection. Expression was measured using real time PCR and corrected for plasmid contamination in the RNA by subtracting the *SQSTM1* signal detected in appropriate no reverse transcriptase controls.

(b) NF κ B activity in HEK293 cells 24 and 48 hr after transfection. NF κ B activity was measured using a luciferase reporter assay, with firefly luciferase produced under the control of an NF κ B responsive promoter. Luciferase values were corrected for β -galactosidase activity produced by a co-transfected plasmid.

6.3.3 Effect of wild-type and mutant SQSTM1 expression on proliferation

Many factors and pathways can alter osteoblast proliferation, so we investigated the mitogenic effects of over-expression of wild-type and mutant SQSTM1. Proliferation experiments were performed in SaOS2 cells. UMR-106 cells had a very high rate of cell division that was not affected by factors known to stimulate osteoblast proliferation, so these cells were not utilised for proliferation experiments. There were no changes observed in proliferation in SaOS2 24 hr after transfection, however, there was a small but significant decrease in proliferation (approximately 10%) in the cells transfected with the 396X mutant after 48 hr (Figure 6.5). The transfection protocol did not appear to affect baseline cell proliferation (data not shown).



Figure 6.5: Proliferation in SaOS2 cells transfected with wild-type or mutant SQSTM1 Proliferation in SaOS2 cells 24 hr (a) and 48 hr (b) after transfection determined by 6 hr thymidine incorporation. Data is pooled from three experiments, and significance is determined by repeated measures ANOVA with Dunnett's post test using vector as the control.

6.3.4 Effect of SQSTM1 over-expression on NFkB signalling in osteoblasts

Since over-expression of wild-type and mutant SQSTM1 has been shown to have differential effects on NF κ B activity in both HEK293 cells and osteoclast-like cells, we examined the effects of over-expression of these constructs on NF κ B signalling in osteoblastic cells using a luciferase reporter assay. Results in MC3T3-E1s and rat osteoblasts were inconsistent due to poor transfection efficiency. UMR-106 and SaOS2 cells showed good transfection efficiency and reproducible results. Basal NF κ B activity in both UMR-106 and SaOS2 cells was suppressed by over-expression of wild-type SQSTM1. Higher levels of NF κ B activity were

maintained when either of the mutants was over-expressed (Figure 6.6). In the UMR-106 cells activity in the mutants was not significantly different from the vector, or from each other, while in the SaOS2 cells the activity in the mutant-transfected cells was still significantly lower than the vector. In both cell types the truncation mutation tended to have higher activity than P392L, although this was only statistically significant in some experiments. In SaOS2 cells NF κ B activity remained suppressed by wild-type SQSTM1 up to 72 hr after transfection (Figure 6.6(c) shows relative luminescence, Renilla luciferase activity levels increased up to 30 hr, then remained stable suggesting transcription from the plasmids was still occurring), while both mutants showed higher activity, although not as high as the vector control.



Figure 6.6: NFkB activity in cells transfected with wild-type and mutant SQSTM1

NFκB activity was determined using a luciferase reporter assay in UMR-106 (a) and SaOS2 cells (b). Luminescence produced from an NFκB-responsive firefly luciferase reporter plasmid was corrected for Renilla luciferase activity from a co-transfected plasmid. Measurements were performed 24 hr after transfection. Data is pooled from at least three experiments, and normalised to the vector control. Repeated measures ANOVA analysis was performed, and significance is indicated based on comparison to wild-type SQSTM1 using Dunnett's post test. NFκB activity at various timepoints after transfection in SaOS2 cells is also shown (c). Values from this experiment are not normalised. Significance is shown based on 1-way ANOVA analysis for each timepoint with Dunnett's post test using wild-type SQSTM1 as the control value.

Chapter 6



Figure 6.7: Effects of TNFa on osteoblast-like cells



We also examined the effects of the wild-type and mutant SQSTM1 on NF κ B signalling when stimulated by TNF α . TNF α dose-dependently stimulated NF κ B activity in SaOS2 cells (Figure 6.7(a)), and stimulated NF κ B activity in all other osteoblastic cells that were tested (data not shown), although generally not as potently as in the SaOS2s. TNF α also stimulated proliferation in osteoblastic cells, although the effect on proliferation in SaOS2 cells was not significant (Figure 6.7). TNF α (1 ng/mL) stimulated a 10-fold increase in NF κ B activity in UMR-106 cells, and the effects of SQSTM1 over-expression were similar to the untreated cells (Figure 6.8(a)). The P392L mutant did not show significantly higher NF κ B activity than the wild-type construct in the ANOVA analysis, however they were significantly different with a Student's t test (p = 0.0144). A t test also indicated that there was a significant difference between the two mutants (p = 0.0195). SQSTM1 consistently reduced NF κ B activity in the SaOS2 cells treated with TNF α (Figure 6.8). Two concentrations of TNF α were

tested, and although the higher concentration caused a much greater increase in NF κ B activity, the extent to which SQSTM1 inhibited the activity was very similar. Wild-type SQSTM1 did not inhibit NF κ B signalling in comparison to the vector control as strongly as in the untreated cells. The mutants still showed significantly higher activity than the wild-type. In the 396X-transfected cells treated with 0.25 ng/mL this was only statistically significant using a t test (p = 0.0290). Overall these results suggest that, similar to results reported in osteoclasts, stronger inhibition of NF κ B activity results from over-expression of wild-type SQSTM1 than the mutant forms of the protein in osteoblastic cells. They also suggest that the different mutants may have slightly different effects, with the less severe P392L mutant showing activity more similar to the wild-type, than the 396X truncation mutant.



Figure 6.8: NF κ B activity in transfected osteoblast-like cells treated with TNF α

NF κ B activity in UMR-106 cells (a) treated with 1 ng/mL TNF α for 3 hr, or SaOS2 cells treated with 0.25 ng/mL (b) or 1 ng/mL (c) TNF α for 3 hr was determined 24 hr after transfection. Values are normalised to the untreated vector control, so indicate the fold activation stimulated by TNF α treatment. UMR-106 data is pooled from three experiments and analysed using repeated measures ANOVA, while the SaOS2 graphs show single representative experiments analysed by 1-way ANOVA. Significance is based on Dunnett's post test using wild-type SQSTM1 as the control.

6.3.5 Effects of SQSTM1 over-expression on cell signalling pathways

Over-expression of wild-type and mutant SQSTM1 had differential effects on NFkB activity in osteoblastic cells. In order to determine what effects SQSTM1 wild-type and mutants have on other signalling pathways, we used a Luminex assay that measures levels of phosphorylated proteins. The assay chosen was an 8-plex assay containing beads conjugated to antibodies for phosphorylated forms of ERK/MAPK, p38 MAPK, jun N-terminal kinase (JNK), STAT3, STAT5A/B, IkB-a, p70 S6 kinase and cAMP response element binding (CREB). These are markers of activation of many of the major cell signalling pathways, including the MAPK pathways (ERK, p38 and JNK) which are involved in transduction of extracellular signals to influence cellular processes including proliferation, transcription, apoptosis and differentiation. Activation of p38 MAPK, for example, is required for osteoblast differentiation to occur [354]. STAT3 and STAT5A/B are involved in cytokine signalling via the Janus kinase (JAK)/STAT pathway, and are phosphorylated in response to stimuli including IL-6. IκB-α phosphorylation is required for NFκB activation, and CREB phosphorylation is stimulated by cAMP which acts as a second messenger for numerous molecules including PTH. Phosphorylation of p70 S6 kinase occurs via the PI3K/Akt pathway, and p70 S6 kinase functions to up-regulated ribosomal biosynthesis allowing increased protein translation to occur.

Cell lysates from transfected SaOS2 cells were harvested after treatment with TNF α (1 ng/mL), TGF β (10 pM), or 1,25(OH)₂D₃ (10 nM) for 10, 30 or 120 min. Drug treatments were included in order to measure the effects of SQSTM1 constructs under basal conditions as well as when stimulated by extracellular factors. TNF α was used because of the previous demonstration that NF κ B signalling was altered. TGF β and 1,25(OH)₂D₃ both stimulate signalling pathways involved in osteoblast function, and since NF κ B signalling can directly oppose the effects of Smad signalling in osteoblasts [351], we hypothesised that the response to TGF β may also be altered. The experiment was only performed once, with each sample run in duplicate. In many of the assays the majority of the readings from the SaOS2 cell lysates were below the lower detection limit of the kit. Phospho-STAT5A/B was not detectable in any of the SaOS2 lysates, while phospho-ERK, p38 and I κ B- α were only detected at levels above background in some samples.













Figure 6.9: Phospho-JNK levels detected using Luminex in transfected SaOS2 cells Cells were transfected with different constructs, then 24 hr later treated with TNF α (a), TGF β (b) or 1,25(OH)₂D₃ (c) for the times shown. Median fluorescent intensity readings that indicate relative abundance are shown.
Phospho-CREB was detected at the highest levels, but none of the treatments greatly affected the levels of phosphorylation of this protein, and there were no differential effects with the SQSTM1 constructs (data not shown). A decreased signal in the SQSTM1-transfected cells treated with TGF β for 2 hr was noted, but since results at the other timepoints do not show a similar trend, it is probably coincidental. JNK phosphorylation did appear to increase transiently with TNF α treatment, but there was no consistent difference between the different constructs (Figure 6.9). ERK phosphorylation also appeared to be responsive to TNF α and possibly TGF β , although this was also a transient increase (data not shown). Phosphorylated I κ B was barely detectable, but did increase in response to TNF α . However, contrary to the luciferase assay results, levels did not appear to be affected by over-expression of SQSTM1 in any consistent manner (data not shown).

6.3.6 Effects of SQSTM1 over-expression on gene expression

We also expected the changes in NF κ B signalling to lead to downstream changes in gene expression. The effects of wild-type and mutant SQSTM1 over-expression on osteoblast gene expression were examined using SaOS2 cells. Transfected cells were treated with TNF α or TGF β and gene expression was examined using low density arrays (genes listed in Table 2.3). These results failed to show any consistently significant differences between the vector, wild-type and mutant constructs. There were differences in gene expression between constructs in some cases, but these either tended to be very small changes, or were not repeated at different timepoints, or with different treatments. A number of genes did show responses to TNF α . I κ B in particular was strongly up-regulated (Figure 6.10(a)), as were *NFKB1* (also known as p50), a component of the NF κ B transcription factor, the transcription factor *FOSL1*, and *M-CSF* (data not shown). *IL-6* and *CCL2* expression also increased from very low basal expression levels in response to TNF α (data not shown). Fewer of the genes tested appeared to be responsive to TGF β treatment. *DKK1* expression appeared to be suppressed by both TNF α and TGF β treatment, while osterix expression increased in TGF β -treated cells, and decreased in TNF α -treated cells (Figure 6.10).



Figure 6.10: Gene expression in SaOS2 cells transfected with SQSTM1 and mutants Cells were transfected, then 24 hr later treated with either vehicle control, 1 ng/mL TNF α , or 10 pM TGF β for the times shown. Gene expression levels were determined by real time PCR using low density arrays and results for selected genes are shown. Results are corrected for *GAPDH* expression, and adjusted by a standard calibrator value, so there is some indication of relative expression levels between genes.

Since we may have missed relevant changes in gene expression by examining only selected genes on the low density arrays, samples that had been transfected with the SQSTM1 constructs, and were either untreated, or treated for 2 or 6 hr with TNF α were examined on Affymetrix HuGene 1.0 ST arrays. These are new generation microarrays that examine the expression of >28,000 transcripts using probes from the whole mRNA sequence rather than just the 3' end like traditional Affymetrix GeneChips [355, 356]. Quality control indicated that the arrays were suitable for analysis. Analysis of this data is still in progress, but preliminary investigation failed to identify genes with known roles in osteoblastic cells that showed consistent differences between the wild-type and mutant SQSTM1 constructs.

6.4 Discussion

SQSTM1 was expressed in osteoblastic cells at high levels, which were not changed due to Paget's disease or at different stages of osteoblast differentiation. Nagy *et al.* also failed to find changes in SQSTM1 gene expression in pagetic monocytes and lymphocytes [309]. Conversely, Collet *et al.* found increased levels of SQSTM1 mRNA and protein in immortalised B cell lines derived from patients with Paget's disease, and expression levels in Paget's patients were not affected by the presence of mutations in the *SQSTM1* gene [138]. SQSTM1 expression is rapidly up-regulated after induction of adipocyte differentiation, and during RANKL-induced osteoclastogenesis [146, 149].

Our results failed to demonstrate many differences between osteoblastic cells over-expressing wild-type and mutant SQSTM1. While there were changes in NF κ B signalling, these did not appear to translate to changes in proliferation, gene expression or other signal transduction pathways. Unlike other reports, NF κ B activity in the SaOS2 cells was still suppressed by the mutant constructs, just to a lesser degree than with the wild-type. There also tended to be more signal inhibition by the P392L mutant than 396X. This is consistent with the fact that P392L is a less severe mutation that may retain some ubiquitin binding ability [131]. So far, we have not examined differentiation or support of osteoclastogenesis by osteoblastic cells over-expressing SQSTM1, primarily because enhanced gene expression in transiently transfected cells is too short-term, lasting up to four days whereas these assays take 1-3 weeks. These experiments will require the development of either viral vectors or stably transfected cells.

The minimal changes in osteoblast proliferation in the cells transfected with wild-type and mutant SQSTM1 are consistent with another report that showed unchanged proliferation in stromal cells expressing mutant SQSTM1 [158]. Other investigators have shown modest differences in proliferation between cells of the osteoclast lineage expressing wild-type and mutant SQSTM1. Over-expression of wild-type SQSTM1 in RAW 264.7 cells which can differentiate into osteoclasts reduced proliferation on day 5 while mutants showed similar results to the control [133]. Osteoclast precursors from mice expressing mutant SQSTM1 showed increased proliferation compared to controls [157, 158].

There have recently been other reports regarding the effects of *SQSTM1* mutations on protein function. Cells of the haematopoietic lineage expressing mutant SQSTM1 have increased NF κ B signalling compared to cells with the wild-type protein [133, 149, 157]. Conversely, one report showed that wild-type SQSTM1 was necessary for TRAF6-induced NF κ B activation in HEK293 cells, and the UBA domain was required for this effect [357]. Osteoclast precursors expressing either the P392L mutant, or a mutant lacking the complete UBA domain show increased osteoclastogenesis and resorption [133, 156-158]. Consistent with the NF κ B reporter assays, osteoclastogenesis was inhibited in RAW 264.7 cells over-expressing wild-type SQSTM1, and the osteoclast-like cells formed did not resorb bone.

There is also evidence that mutant SQSTM1 increases ERK and p38 MAPK signalling [156-158]. SQSTM1 has been shown to associate with p38, and mediate cytokine-stimulated p38 signalling [353]. ERK also binds to SQSTM1, but this results in inhibition of ERK signalling [146]. These interactions require different domains of the SQSTM1 protein. ERK interaction requires the N-terminal PB1 domain [146], while p38 interacts with amino acids 173-182 and 335-344 [353], which fall outside the domains described in Figure 1.17, although none of these regions are affected by the mutations found in Paget's disease. The results from this study failed to demonstrate changes in either of these signalling pathways in SaOS2 cells transfected with wild-type and mutant SQSTM1, but phospho-ERK and p38 were barely detectable in the assay used. Loss of the UBA domain also alters the subcellular localisation of the SQSTM1 protein. Wild-type SQSTM1 forms cytoplasmic aggregates, which increase in size when proteasome function is impaired [151, 156]. SQSTM1 is found in protein aggregates in multiple diseases including alcoholic hepatitis and neurodegenerative diseases [145]. SQSTM1 that has lost most or all of the UBA domain does not form aggregates, and is

distributed diffusely throughout the cytoplasm, but when the protein contains point mutations P392L or P387L the aggregates formed are larger than observed with the wild-type [139, 156].

In the last couple of years there have been a number of reports of transgenic mouse models expressing mutant SOSTM1. Mice expressing human P392L SOSTM1 under the control of the TRAP promoter showed increased osteoclast numbers in vivo and progressive bone loss, but did not develop pagetic-like lesions. There was no coupled increase in osteoblast numbers or activity, and osteoclasts did not contain nuclear inclusions [157]. Mice with a 'knock-in' of the P394L mutation, which is the murine equivalent of P392L, showed no evidence of histological bone abnormalities in either homozygous or heterozygous animals aged up to 18 months [158]. Increased osteoclastogenesis was apparent in bone marrow cultures from these mice, and stromal cells supported increased osteoclastogenesis in normal osteoclast precursors. Stromal cells produced more RANKL in response to 1,25(OH)₂D₃ than control cells and showed increased p38 MAPK activation [158]. There are reports of two other mutant SQSTM1 mouse models, although these have only been presented in abstract form at this stage. Mice with a 409X mutation develop focal lytic lesions in their lower limbs by the age of 12-15 months, and lesions contain increased osteoclast and osteoblast numbers. Cells from the mutant mice show increased osteoclastogenesis in vitro, while osteoblast growth is unchanged [160]. P392L knock-in mice also develop lytic lesions by 12 months of age which are more severe in homozygous animals [159]. Knockout of Sąstm1 in mice does not cause a Paget's disease-like syndrome, but does result in obesity, increased bone mass, and neurodegeneration in aged mice [146, 148, 153]. These mice also showed reduced IL-6 expression in response to PTHrP compared to wild-type mice [149]. These results are consistent with increased IL-6 expression in Paget's disease, presuming the mutations have an activating effect. While the phenotypes of these mouse models vary, none appear to entirely recapitulate a pagetic phenotype.

We hoped that examining the effects of over-expression of wild-type and mutant *SQSTM1* in osteoblastic cells would provide evidence for mechanisms by which these mutations predispose patients to developing Paget's disease. Over-expression of similar constructs in osteoclast precursor cells provided some insight into the direct effects of SQSTM1 and mutants on osteoclastogenesis [133, 156]. However, the results presented here suggest that, apart from altering NF κ B activity, mutations in the *SQSTM1* gene have minimal effects on cell proliferation, gene expression and cell signalling in osteoblasts. A recent publication

using a mouse model suggests that the mutations have some effect on osteoblast function, in particular their ability to support osteoclastogenesis, which was not tested in the current study [158]. This mouse model may provide a better system for identifying changes caused by mutant SQSTM1 in osteoblastic cells than transient over-expression of the genes in an osteoblastic cell line. The observation that reduced RANKL-induced osteoclastogenesis occurs in cells from Sąstm1 knockout mice suggests that SQSTM1 has a positive effect on NFkB signalling [149], so it is surprising that over-expression of the protein inhibits NFkB activation. It is possible that over-expression of wild-type SQSTM1 results in sequestration of its binding partners in aggregates, which are then targeted for proteasomal degradation or autophagy. The results of studies of wild-type and mutant SQSTM1, and the autosomal dominant inheritance pattern of Paget's disease, suggest that loss of ubiquitin binding function results in either a gain of function for SQSTM1, or increased ability to perform certain functions that do not require ubiquitin binding. While recent publications have shed some light on the functional effects of the SOSTM1 mutations found in Paget's disease, it is still unclear how these changes trigger focal bone disease. In fact, it is perplexing that mutations in a protein that is expressed ubiquitously at high levels, and affects such a variety of important cellular processes, should result in development of a focal bone disease with incomplete penetrance.

CHAPTER 7: DETECTION OF MEASLES VIRUS RNA IN PAGETIC CELLS

7.1 Introduction

While genetics undoubtedly play an important role in the development of Paget's disease, the epidemiology of the disease suggests that environmental factors may be important as well. Factors such as the marked differences in prevalence over fairly small geographic areas, and the declining prevalence and severity of the disease in recent years suggest altered exposure to an environmental agent [80, 83]. One such possibility is long-term infection with a virus. A number of studies suggest that viruses are associated with Paget's disease and could be one of the non-genetic factors contributing to the development of the condition. A number of publications report measles virus mRNA or protein in samples from Paget's patients [162-168]. Other groups, however, have repeatedly failed to detect viral RNA or antigens [175-179]. There is also evidence that infection of bone marrow with measles virus or measles virus nucleocapsid protein can cause the development of osteoclasts with a pagetic phenotype [181, 182, 184]. However, live measles virus has never been isolated from pagetic cells or tissue.

7.2 Methods

Seventy five RNA samples from the cultured pagetic and non-pagetic bone samples were sent on dry ice to the NIBSC in Hertfordshire, UK. This was the majority of the primary human RNA samples, and included 13 pagetic osteoblast and 13 pagetic bone marrow samples (from 22 different patients), eight non-pagetic osteoblast and two non-pagetic bone marrow samples from patients with Paget's disease, and 18 control osteoblast and 21 control bone marrow RNA samples (from 31 patients). RT-PCR nested PCR analysis for detection of the measles virus nucleocapsid (*N*) and matrix (*M*) genes was performed at the NIBSC using the method described previously [189] and in Section 2.3.2.1.

7.3 Further characterisation of bone marrow cells

Given that many reports of measles virus expression in patients with Paget's disease have involved either whole bone marrow, or bone marrow or blood that has been enriched for various haematopoietic cell populations, we performed real time PCR for a number of osteoclast lineage genes to ensure that cells of the haematopoietic lineage were present in all samples. As previously mentioned, *RANK* was expressed in the bone marrow, as were *TRAP* and the monocyte marker *CD14* (Figure 7.1). The calcitonin receptor which is expressed at certain stages of osteoclastogenesis was detectable in all but one of the pagetic samples, and 18 of the 23 non-pagetic samples that were sent for measles virus RNA detection (data not shown). There were no significant differences in the expression of these genes between pagetic and non-pagetic samples. The expression levels of *RANK*, *CD14*, *TRAP*, calcitonin receptor, and the monocyte transcription factor *MAFB* in individual samples all showed very strong correlation with each other. These results confirmed that cells of the osteoclast lineage were present in these cultures. The expression of alkaline phosphatase, osteocalcin and bone sialoprotein in these samples, as described in Chapter 4, indicates that there are also osteoblastic cells present in both culture systems.



Figure 7.1: Relative expression of osteoclast precursor marker genes in bone marrow cell cultures Real-time PCR was used to determine the levels of expression of, (a), *RANK*, (b), *TRAP*, and (c), *CD14*. The expression levels in the different samples were normalized to the expression of *18S* RNA and are presented relative to the sample with the highest expression level, which was normalized to 10.

7.4 Measles virus detection

RNA samples extracted from primary cultures of osteoblasts and bone marrow cells were amplified using the N gene and M gene specific primers for the measles virus genome sequences. The results of RT-PCR-nested PCR amplifications were negative for all 75 samples tested (data not shown). Measles virus specific cDNA fragments corresponding to the N and M gene regions were detected in the positive controls of a measles virus culture isolate and an SSPE brain sample. The RT-PCR-nested PCR assay for detection of measles virus has previously been shown to detect viral RNA in preparations corresponding to as few as 18 SSPE cells [189], or samples with 16 copies of the measles virus N gene transcript [180]. In our study, testing for the N gene was performed using 75-150 ng of total cellular RNA from patient samples, and 125-500 ng of RNA was used for M gene amplification. Therefore, the quantity of RNA from cultured osteoblast and bone marrow cells used in this study corresponds to at least 7,500 cells in the N gene assay, and 12,500 cells for the M gene assay. This suggests that the expression levels of these transcripts, if present, is at least 415- and 690-fold lower, respectively, than in SSPE in all of the samples tested [358].

7.5 Discussion

We found no evidence for the presence of measles virus in pagetic bone cells of either the osteoclast or osteoblast lineage. Our bone marrow samples contained osteoclast precursors, as demonstrated by the expression of *CD14*, *RANK* and *TRAP*. These samples are similar to some of those used by Reddy *et al.* who have found measles virus transcripts in bone marrow and peripheral blood from many Paget's patients using a less sensitive RT-PCR technique [165-168]. Both the bone marrow and the bone outgrowth cultures in this study expressed the marker genes alkaline phosphatase, bone sialoprotein and osteocalcin indicating the presence of osteoblastic cells. This osteoblastic RNA was also found to be negative for measles virus. Some in-situ hybridization studies have detected viral RNA in osteoblasts [164, 169, 170], but RT-PCR studies in osteoblastic or stromal cells have been negative [167, 177].

While some authors have never detected evidence of paramyxoviruses in pagetic tissue [175-179], other groups have found evidence for the presence of several different viruses. Apart from measles, positive results have also been reported for canine distemper virus, respiratory syncytial virus, simian virus 5, parainfluenza virus type 3 and mumps, and in a number of these reports up to three different viruses have been detected in one sample [163, 169-173].

Studies using in situ hybridization have often identified presence of viral RNA in osteoblasts, osteocytes and bone marrow cells, in addition to osteoclasts [164, 170, 171].

The primers used in the present study target the same region of the N gene as those used in the other studies where measles virus RNA has been detected (base pairs 1198-1630 for the first amplification in our study, compared with base pairs 1269-1450) [165, 167]. These primers successfully amplify measles virus RNA from SSPE samples, a condition associated with long-term measles virus infection. In a recently published study [180], samples spiked with measles virus were analyzed for measles virus sequences in five laboratories. The RT-PCR nested-PCR used in the present study proved to be the most sensitive technique, detecting as few as 16 copies of the measles virus N gene. Testing of 12 samples of RNA from pagetic bone showed no evidence for the presence of measles virus [180]. Given the sensitivity of this assay, and the relatively large number of samples in the present study, it seems unlikely that amplification of both N and M genes in all samples would fail due to the presence of mutations in the primer sites.

Most samples in our study were from patients who had received bisphosphonate treatment at some stage. Although bisphosphonates control Paget's disease, they do not cure it, suggesting that if the virus were a causative factor it should still be present. Measles virus mRNA and canine distemper virus have both been detected by RT-PCR in bisphosphonate-treated patients by other laboratories [162, 168, 169].

Nuclear inclusions are a feature of pagetic osteoclasts reported by various groups, including those who are unable to detect viruses in these cells [105, 106, 178]. These inclusions have been suggested to show features of a paramyxoviral infection [359]. However, similar inclusions have also been found in osteoclasts or macrophages in cases of osteopetrosis [185], pycnodysostosis [360] and oxalosis [361], none of which are attributed to viral infection. Thus, they might represent a non-specific stress response in osteoclasts. Nuclear inclusions are also seen in brain cells from patients with SSPE, a fatal condition caused by a long-term measles virus infection of the brain [161]. While the inclusions are similar in size to those in Paget's disease, their organisation appears different, as shown in Figure 1.18 [178]. In the past, measles virus has also been implicated in other conditions, such as inflammatory bowel disease [186-188], multiple sclerosis, and autism [214, 362]. These links are not currently thought to be etiologically important [189, 190]. Such studies of disease association are

complicated by the fact that there can be persistence of measles virus RNA in human tissue long after an acute infection, without evidence of ill-effects [362].

Epidemiological evidence suggests a decline in prevalence and severity of Paget's disease in New Zealand [83]. While this suggests environmental factors are involved, this decline is significant between cohorts born before 1910 and those born after 1930, whereas vaccination of children against measles did not begin until 1971. Therefore, the decline in prevalence of Paget's appears to precede the introduction of the vaccine.

A further strand of evidence for a viral aetiology of Paget's disease is the demonstration that infection of osteoclasts with paramyxoviruses or measles virus N protein produces Paget-like changes in these cells. Thus, human bone marrow cells expressing measles N gene produce increased numbers of large, highly nucleated osteoclasts, and IL-6 levels are increased [181]. Infection of mouse bone marrow with measles virus produces similar results [182]. Similarly, infection of human osteoclast precursors with canine distemper virus stimulates osteoclast formation and resorption, and increases the size and number of nuclei in the cells [183]. Mice expressing the measles N gene under the control of the *Trap* promoter develop a Paget-like phenotype that worsens with age [184]. Although these data suggest that paramyxoviral infection may reproduce some features of Paget's disease, there is no evidence that this response is specific for a particular virus, and it might be mediated by virus-induced increases in cytokines. Large, highly nucleated osteoclasts have also been identified in non-pagetic bone treated with bisphosphonate, again suggesting a non-specific response [363].

In conclusion, the present study has not detected evidence of measles virus infection in bone cells from a large cohort of patients with Paget's disease and control subjects. The virus detection method used has been shown to be highly sensitive, specific and robust. This finding, together with the similar recent report from Ralston *et al.* [180], raises major doubt regarding the role of measles virus infection in the pathogenesis of Paget's disease of bone.

CHAPTER 8: DETECTION OF SOMATIC SQSTM1 MUTATIONS IN PAGETIC CELLS

8.1 Introduction

Paget's disease sometimes runs in families, and about 15% of pagetic subjects are reported to have an affected relative [4]. Several genetic loci have been associated with the development of Paget's disease, including mutations in the gene for *SQSTM1* [147]. Although a number of other focal diseases, including Crohn's disease and polycystic kidney disease are caused by germline mutations, a mutation which is present in all cells can only provide a partial explanation for a focal condition and a 'second hit' that is present in the microenvironment may be involved. Both the genetic and environmental hypotheses associated with this disease fail to account for the focal nature of the condition. Somatic mutations in the *SQSTM1* gene in cells within the pagetic lesion could provide an explanation for the focal nature of the condition, particularly in monostotic sporadic cases, and have previously been suggested to be common [364]. We have tested the RNA samples collected from pagetic osteoblasts and bone marrow cells from 23 patients for the presence of somatic mutations in *SQSTM1*. Three of these patients have a confirmed family history of Paget's disease, nine reported no known family history, and for the remaining patients this information was not ascertained. Seven of the patients had monostotic disease.

8.2 Sequence analysis of exons 7 and 8 of SQSTM1

Sequencing of exons 7 and 8 of *SQSTM1* was carried out in cDNA from the pagetic RNA samples. Sequence analysis of *SQSTM1* showed the wild-type sequence in 27 out of 28 samples. The marrow sample from one patient was heterozygous for the P392L mutation (Figure 8.1(a)). This patient was 65 years of age when Paget's disease was diagnosed and had extensive disease of low activity, involving the vertebrae, pelvis and legs. Sequencing of DNA from peripheral blood in this subject showed the same heterozygous missense mutation (Figure 8.1(b)), indicating that this was a germline mutation. The patient had no known family members with Paget's disease but all three of his offspring were found to be carrying the mutation, further confirming that he had a germline mutation [365].



Figure 8.1: Electropherograms showing the heterozygous 1215C/T mutation SQSTM1 sequences of the patient's cDNA from bone marrow cells (a), and DNA from peripheral blood (b) are shown. An example of a wild-type sequence from another patient is shown in (c).

8.3 Allelic discrimination

8.3.1 Development and validation of methodology

When testing for a somatic mutation, it is possible that only a fraction of the cells contain the mutation and sequencing reactions might not be sensitive enough to identify that change. We therefore performed allelic discrimination to detect the most common *SQSTM1* mutation, P392L. Allelic discrimination is generally used to distinguish polymorphisms in genomic DNA using a real time PCR-based technique with two fluorescently labelled probes. It is designed to distinguish between two alleles, and produces different signals for the heterozygous and each of the homozygous forms. This technique can also be used to detect smaller quantities of a mutation, and the protocol used in this study was based on the method of Singh *et al.* [366].



Figure 8.2: Allelic discrimination standard curve amplification plots Amplification plots are shown of standard curves for wild-type (a, b) and P392L (c, d) plasmids at seven concentrations (highest concentration 10 pg/ μ l, then 1:5 serial dilutions). VIC plots (a, c) show specific amplification of wild-type, but FAM plots (b, d) show amplification of the mutant (d) and some background signal in the wild-type (b).

We utilised a custom made primer-probeset that identified the wild-type sequence with a VIC-labelled probe and the mutant sequence with a FAM-labelled probe. The assay and analysis were validated using plasmid mixes of known proportions. The allelic discrimination experiments were performed in a similar way to the other real time PCR experiments, and the changes in fluorescence between the baseline and endpoint were used for analysis. Our data consistently showed background FAM signal (which represents the mutant) in samples containing only wild-type sequence (as shown in Figure 8.2). The FAM signal is very sensitive, and the background signal may be a result of low levels of hybridisation with the mismatched probe. The endpoint signal data showed a much clearer distinction between the

non-specific amplification and low levels of mutant than Ct values generated from the amplification plots, so endpoint data was used for the analysis. Standard curves showed good target amplification at a wide range of plasmid concentrations, although the level of signal increased as the concentration of the template increased (Figure 8.3(a)). Testing of different proportions of wild-type and mutant plasmid indicated that as little as 5% (Figure 8.3(b)) or 2.5% mutant (Figure 8.4) could routinely be distinguished from wild-type. Mouse cDNA spiked with plasmid mixes grouped well with the plasmid mixes alone indicating that the presence of other non-specific DNA targets did not interfere with the assay. Samples with 1% or less mutant could not be reliably distinguished from the wild-type (Figure 8.3(b)). In order to perform statistical analysis the data were transformed as described in Section 2.3.4.2.



Figure 8.3: Allelic discrimination standard curve endpoint results

Results are presented with the measurements of the fluorescence of wild-type VIC-labelled probe on the x axis and the FAM-labelled P392L probe on the y axis. (a) Endpoint data showing standard curves of wild-type (red) and mutant (blue) plasmids at different concentrations. The NTC is shown in white. This is the same experiment as shown in Figure 8.2. (b) Endpoint data showing different plasmid mixes including 100% mutant (blue), 100% wild-type (red), 50% wild-type 50% mutant (gray), 40% mutant (dark purple), 30% mutant (pink), 20% mutant (orange), 10% mutant (green), 5% mutant (yellow), 1% mutant (light blue), 0.5% mutant (purple). The black diamonds show mouse cDNA spiked with the same concentration of plasmid mixes, from top to bottom, 100% mutant, 50% mutant, 10% mutant, 100% wild-type. The NTC is shown in white.

8.3.2 Testing of pagetic samples

Since *SQSTM1* was expressed at high and fairly stable levels in all the cDNA samples it was possible to use them, rather than genomic DNA, to perform allelic discrimination. All samples from patients with Paget's disease, whether from the pagetic lesion or unaffected tissue, were tested at least three times in quadruplicate. Each experiment included plasmid standards containing 100% wild-type, 100% mutant, 50% mutant, 10% mutant, 5% mutant and 2.5%

mutant. They also contained at least five samples from control patients. The bone marrow sample with the known mutation was consistently grouped with the 50% mutant (Figure 8.4(b)). All other samples showed no significant difference from the wild-type control, indicating they contained less than 2.5% mutant (Figure 8.4).



Figure 8.4: Allelic discrimination results in patient cDNA samples Non-transformed results are shown for osteoblast (a) and bone marrow (b) samples. The circle symbols show the following standards; NTC (white), 100% mutant (blue), 50% mutant (gray), 10% mutant (green), 5% mutant (yellow), 2.5% mutant (purple) and 100% wild-type (red). The triangles represent patient samples from pagetic sites, non-pagetic sites, and control patients. In (b), the sample grouped with the 50% mutant standard, is from the patient who had a germline mutation. All other patients' samples are not significantly different from the 100% wild-type standard.

8.4 Discussion

The present findings do not support the suggestion that somatic mutations in *SQSTM1* occur commonly in the bone lesions of Paget's disease. With the combination of osteoblast and bone marrow samples we have been able to test for the presence of mutations, in particular P392L, in cells from both the osteoblast and osteoclast lineages. Since osteoblasts and their precursors are less mobile than haematopoietic cells, somatic mutations in osteoblastic cells could explain the focal nature of the disease. It is possible that a mutation other than P392L expressed in a sub-population of cells, or P392L mutation in less than 5% of the cells might have been missed in our study. We only investigated the P392L mutation using the allelic discrimination technique since it is much more common than any of the other mutations, and was the only mutation detected in the report of somatic *SQSTM1* mutations [367]. Within the group of patients with Paget's disease that carry *SQSTM1* mutations, 64% of families and 85.5% of sporadic cases carry the P392L mutation [350].

P392L heterozygous somatic mutations in patients with Paget's disease have been reported previously. Merchant et al. studied homogeneous populations of cells isolated by laser capture microdissection from pagetic bone, and identified the SOSTM1 P392L mutations in bone samples, but not peripheral blood of two out of five patients examined [367]. Earlier the mutations were reported in abstract form to be in cells of the osteoblast and osteoclast lineage, although it is difficult to envisage a mechanism for this to occur [364]. The present study has not directly assessed the gene sequence in pagetic tissue, but has sequenced cDNA from primary cultures from this tissue. While we cannot be certain that the culture conditions did not preferentially select the wild-type phenotype, the sequencing and allelic discrimination results from the patient with the germline mutation suggest approximately 50% of the cDNA is the mutant, implying there is no difference in RNA expression or stability of the mutant within the cells. In addition, in vitro experiments using transfected RAW 264.7 cells and mouse mutant bone cells have suggested that the presence of SQSTM1 mutants may result in unchanged or slightly increased proliferation, without affecting cell survival [133, 156-158]. This is consistent with our data that suggests osteoblastic cells transfected with wild-type or mutant SOSTM1 have unchanged rates of proliferation.

Somatic mutations could certainly be a plausible cause of Paget's disease, particularly sporadic and monostotic disease, since germline mutations do not explain the focal nature of the disease. Disease-causing somatic mutations are present in some types of cancer. For example, *APC* mutations are found in 80% of colorectal cancers. Patients with familial adenomatous polyposis have one mutated *APC* allele, and generally develop colorectal cancer between the ages of 20 and 30 when a mutation occurs in the other allele [368]. Like the polyps in this condition, pagetic lesions occasionally progress to osteosarcoma.

Germline mutations in *SQSTM1* predispose patients to Paget's disease in an autosomal dominant fashion, although with incomplete penetrance. The few patients reported with homozygous mutations do not appear to have a dramatically worse phenotype, suggesting further mutations in the gene may not be necessary for the disease to occur [137, 138]. Overall, *SQSTM1* mutations were rare in this study, with only 1/23 patients (4.3%) having the P392L mutation. However, the numbers in this study are fairly small, and many of the patients have no known family history of the disease, and low disease activity. In a recent publication, meta-analysis of *SQSTM1* mutation rates produced an estimate of 28.8% in

familial Paget's and 6.1% in sporadic Paget's [134]. In our centre, 16/53 families (30%) with Paget's disease have been identified with *SQSTM1* mutations. There are certainly other genetic factors yet to be identified, the 10p13 locus in particular has recently been reported to show strong linkage in patients without *SQSTM1* mutations [140]. However, the late age of onset, focal nature of the condition and its waning incidence suggest that environmental factors are involved in disease initiation.

In conclusion, we have assessed 28 tissue samples affected by Paget's disease from 23 patients, and failed to find evidence of somatic mutations in the *SQSTM1* gene. While somatic mutations in susceptible patients are a plausible explanation for the focal nature and late onset of this disease, these results indicate that somatic mutations of *SQSTM1* are uncommon.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

9.1 Discussion

Paget's disease of bone has often been described as a disease of the osteoclast [369]. However, there are a number of lines of evidence that suggest the osteoblast may also be involved. Firstly, bone turnover remains coupled in Paget's disease, although bone formation can be delayed in lytic lesions. Bone formation is also abnormal, and the bone produced tends to be disordered woven bone. Given that osteoblasts play such an important role in the control of osteoclast differentiation and activity, both through the production of RANKL/OPG and M-CSF, and via other factors that modulate osteoclast precursor recruitment and bone resorption, a role for these cells in the development of the disease is certainly plausible. Previously, others have identified changes in non-collagenous protein distribution in the extracellular matrix, and in the proteins secreted from pagetic osteoblasts which also suggest there are alterations in the phenotype of the osteoblasts within the pagetic lesion [108, 121].

One intriguing aspect of Paget's disease is its focal nature, which has not been adequately explained by any of the work to date. Osteoclasts are derived from circulating cells that are much more mobile than osteoblast precursors. Local changes in the osteoblast population may explain why the disease does not tend to spread to new skeletal sites. Pagetic lesions occasionally progress to osteosarcoma. While this is an unusual complication, it is believed to be specific to Paget's disease, as osteosarcoma is otherwise very rare in elderly people, and the progression to a malignancy of osteoblastic origin further implicates osteoblast abnormalities in this disease [370].

Ideally, we would have liked to identify the major trigger that causes changes in the osteoblasts within the pagetic lesion resulting in up-regulation of bone turnover. There are many candidates for this hypothetical factor, including changes in a regulatory switch, an imbalance of important factors, a somatic mutation or mutations, or an environmental factor such as a local viral infection. These possible mechanisms would all be expected to change the osteoblast phenotype through changes in protein levels which in turn are usually caused by changes in levels of gene expression. Given the difficulty in obtaining patient samples,

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gene expression was an ideal target to measure, as it is possible to quantitatively examine the expression of many genes using small quantities of RNA.

This is the first study to extensively characterise gene expression in pagetic tissue. A number of earlier studies examined specific candidate genes, particularly osteoclastogenic cytokines such as IL-6 which has often been shown to be up-regulated in pagetic tissue [116-118], and RANKL, which some authors report to be up-regulated [113, 115]. One of the advantages of this study is the fairly large number of patient samples included (14 osteoblast and 14 bone marrow samples derived from 23 patients with Paget's disease), and the use of microarrays meant that identification of changes in gene expression was performed in a relatively unbiased manner, although many of the candidate genes were chosen because of their known roles in bone biology.

The microarray analysis performed in this study identified overall changes in pagetic osteoblasts compared to controls. Pagetic samples consistently clustered separately from control samples when analysed using all expressed transcripts, or the most highly differentially regulated transcripts. These results also indicated that there may be changes in extracellular matrix constituents and interactions. A number of cell signalling pathways with important roles in osteoblast biology including Wnt and TGF β /BMP signalling and G protein-coupled receptor signalling were also significantly altered suggesting there may be changes in signal transduction in pagetic osteoblasts. This analysis also identified many candidate genes that were confirmed to be up- or down-regulated in pagetic osteoblasts using real time RT-PCR.

Like a number of other reports [116-118, 279], we have found up-regulation of cytokine expression in Paget's disease. Most of these studies used bone marrow cultures, or measurements in patient serum meaning it is unclear which cells contributed to enhanced cytokine production. In our study, these changes were identified in osteoblastic cells, showing that osteoblasts either cause or contribute to the changes. A novel finding from the current study is the up-regulation of the Wnt signalling pathway inhibitor *DKK1* in both osteoblast and bone marrow cultures. This finding is particularly interesting in light of recent reports that DKK1 levels are increased in lytic bone lesions in multiple myeloma bone disease, and breast and prostate cancer bone metastases [74, 231, 252, 340]. The effects of both DKK1 and IL-6 on bone cells are complex, and appear to depend on the stage of osteoblast differentiation and

interactions with other factors. Therefore, DKK1 could interact with the interleukins to contribute to the formation of both lytic and sclerotic lesions. IL-6 can have positive effects on osteoblast proliferation and differentiation, and although DKK1 inhibits differentiation in osteoblast precursors, it can stimulate terminal differentiation and mineralisation in more mature cells [32, 280, 325]. A schematic diagram showing how the interleukins and DKK1 could interact to alter the bone microenvironment in Paget's disease, and stimulate osteoclastogenesis is shown in Figure 9.1. It is not clear from this study whether these are primary or secondary changes, and there are likely to be other factors, possibly including CCL2 which was also significantly up-regulated in the pagetic osteoblasts, and BMP2 which was down-regulated, which are likely to contribute to changes in osteoblast and osteoclast behaviour in the pagetic lesion.



Figure 9.1: Schema showing possible effects and interactions of changes in interleukin and DKK1 expression identified in this study

The overproduction of IL-6 and IL-1 by the pagetic osteoblast will result in osteoclast precursor proliferation and differentiation leading to more IL-6 production in the bone marrow microenvironment. Increased production of DKK1 by the pagetic osteoblast will further increase IL-6 levels by stimulating proliferation of cytokine-producing mesenchymal stem cells, and reduce osteoblast proliferation and early differentiation. This combination of effects could account for the development of lytic lesions in early phase Paget's disease. Over time, both excess DKK1 and IL-6 will result in increased terminal differentiation of osteoblasts, thus promoting mineralisation. This could contribute to the development of sclerosis in longer-standing pagetic lesions.

One of the unique findings of this work is down-regulation of a number of important genes involved in osteoblast differentiation and activity in cells from pagetic lesions. Downregulation of the transcription factor, RUNX2, which is critical in the early and mid stages of osteoblast differentiation, and decreases in DLX5 and SATB2, which are also transcription factors that positively influence osteoblast function, suggest that osteoblast differentiation and function is abnormal. Transcriptional targets of these proteins, bone sialoprotein and osteocalcin are also significantly down-regulated, while alkaline phosphatase and matrix gla protein are up-regulated, suggesting that the pagetic osteoblasts are less differentiated than both the cells from control patients, and the cells from unaffected tissue in patients with Paget's disease. There were also other changes in transcript abundance that suggested there may be changes in signalling pathways that are important for osteoblast function. Downregulation of BMP2 suggests that BMP signalling may be reduced, and reduced BMP signalling could lead to the observed decreased expression of RUNX2, DLX5, osteocalcin and bone sialoprotein. FGFR2, a receptor for FGF ligands was also down-regulated, suggesting this pathway could also be altered. As mentioned earlier, up-regulation of DKK1 suggests there may also be a change in Wnt signalling, which is also critical for osteoblast differentiation, and can interact synergistically or antagonistically with BMP signalling, depending on the context.

Unlike other studies [113, 115], we have found no evidence that changes in *RANKL* and *OPG* expression are contributing to the development of enhanced bone turnover in Paget's disease. However, consistent with the increased *OPG* expression in the bone marrow cultures, two studies have reported elevated serum OPG levels in patients with Paget's disease [118, 120]. If anything, the *RANKL/OPG* ratio tended to be decreased in both pagetic osteoblast and bone marrow cultures in this study. This occurred, despite the fact that the pagetic osteoblasts appeared to be less differentiated than the controls, and less differentiated osteoblasts would be expected to express higher *RANKL* and lower *OPG* levels [265, 266]. These results suggest that RANKL and OPG levels are not major drivers of the increased bone turnover in Paget's disease, and the *RANKL/OPG* ratio may be decreasing in order to compensate for increased osteoclastogenesis stimulated by other mechanisms. Reduced *BMP2* expression may contribute to these changes, as BMP signalling stimulates *RANKL* and suppresses *OPG* expression, and a recent publication suggests that the overriding effect of osteoblastic BMP signalling in developing bone may be stimulation of osteoclastogenesis [288]. The increased bone mass in the mice lacking osteoblastic BMP signalling in this study was characterised by

woven bone formation, suggesting reduced BMP signalling could also contribute to the disordered bone formation in pagetic lesions.

We have identified a number of other changes that are difficult to interpret at this stage, but may be more meaningful in future. For example, it will be interesting to determine if RGS4 plays a role in modulation of G protein-coupled receptor signalling in osteoblasts, and whether it has the negative effect on osteoblast differentiation consistent with downregulation by RUNX2 and during osteoblast differentiation [267]. It will also be interesting to see if GATA6, which is known to interact with various transcription factors to facilitate celltype specific gene expression [262], has a role in osteoblasts, as it does in many other endocrine tissues [318]. Up-regulation of SGK1, which can occur in response to many cytokines and growth factors [323], may be another marker of increased production of osteoclastogenic factors. We have investigated the role of keratin 18, which was up-regulated in the pagetic osteoblasts and bone marrow, in osteoblast biology. We developed techniques to transduce primary human osteoblasts with adenoviral vectors and found that keratin 18 over-expression resulted in some changes in gene expression, particularly up-regulation of BMP6, and altered cell morphology. However, these investigations have so far failed to provide convincing evidence that this protein has an important role in osteoblast biology and the development of Paget's disease.

The patient samples and cell culture methodology used in this study have both advantages and limitations. The patients tended to have fairly low disease activity, with most (14/23) showing serum alkaline phosphatase measurements within the reference range at the time of sample collection. This suggests that some samples might have been obtained from advanced sclerotic, or 'burnt out' lesions, although this was not specifically determined. Additionally most had received treatment for Paget's disease, with 19/23 receiving bisphosphonate treatment at some stage. Although this means that the samples collected were not generally from sites of highly active disease, the fact that there were still many significant changes in gene expression suggests there is an underlying abnormality in the osteoblastic cells, and the changes observed are not simply a side effect of excessive bone turnover. However, this may have made it difficult to identify changes that trigger the original development of the lesion. Bisphosphonates can also have effects on osteoblastic cells, although the demonstration that many of the changes in gene expression persist in the comparison between paired pagetic and non-pagetic samples from the same patient (that would have been exposed to the same

treatment regimen) suggests that bisphosphonate treatment is not the cause of these changes. While some of the changes in gene expression, such as up-regulation of *OPG*, are consistent with the effects of bisphosphonates on osteoblasts [94, 95, 371], bisphosphonates are also reported to stimulate osteoblast differentiation, and osteocalcin expression which does not appear to be occurring in the cultures in this study [93, 94]. The limited numbers of patients in this study meant analysing the effects of the presence or absence of bisphosphonate treatment, or time since bisphosphonate treatment on gene expression was not feasible.

The cells in this study had been cultured before RNA extraction, generally for a period of 2-3 weeks, and in most cases primary cells were used to ensure their phenotype did not change. This meant that cells had been separated from the pagetic microenvironment for some time, but still maintained changes, suggesting an intrinsic abnormality in the osteoblastic cells, rather than the effects of overactive or abnormal osteoclasts, or other cells within the bone microenvironment.

There are a number of limitations associated with using RNA transcript analysis alone to examine changes in disease. mRNA levels are certainly affected by changes in transcription, but they can also be affected by changes in RNA degradation by mechanisms including microRNA. Changes in microRNA expression are now recognised to contribute to a number of diseases [372]. Although we have generally considered factors that may be affecting transcription in the possible mechanisms for the observed changes, effects on RNA degradation may also be important. mRNA is generally translated into protein, but RNA levels do not always correlate closely with protein levels, and levels of translation can be enhanced independently of RNA levels. In the two genes where we did measure protein levels in this study, DKK1 and OPG, changes in RNA levels were mirrored by changes in protein levels in the conditioned media. Protein levels are not always indicative of protein activity either. Posttranslational modifications are very important for the activity of numerous proteins, including many signalling molecules, and changes in protein degradation do not necessarily affect RNA levels. However, changes in cells do not tend to affect single genes or proteins, but alter abundance and activity of many targets. Therefore changes in protein levels and activity may be reflected in changes in mRNA of a different gene or genes. This is the advantage of performing global analysis using microarrays. Despite this, there are likely to be changes in pagetic osteoblasts that we were unable to identify in the analyses in this study,

and it is possible that some of the changes we have identified may not result in functional changes within the cells.

We have also attempted to identify and characterise aetiological factors that may be involved in Paget's disease. Mutations in the UBA domain and C-terminal region of *SQSTM1* are definitely associated with the disease, and appear to account for about a third of families with genetic predisposition to developing Paget's disease [134]. Wild-type and mutant *SQSTM1* had different effects on NFkB signalling in osteoblastic cells, but we were unable to identify any downstream changes as a result of this. However, data from other studies suggests that the mutations can have functional effects on osteoblasts as well as osteoclasts [158], suggesting that over-expression of these genes in osteoblast-like cell lines may not have been the ideal experimental system in which to determine functional effects of the *SQSTM1* mutations. These mutations do not appear to make a significant contribution to the changes in gene expression we have identified in pagetic osteoblasts. However, as only one of the patients in this study had a *SQSTM1* mutation, we were unable to analyse whether the presence of this mutation affected expression of the differentially regulated genes. Additionally, there were no significant changes in *SQSTM1* gene expression in the pagetic cells.

In contrast to another recent study [367], we were unable to identify somatic mutations in SQSTMI in any of the patients in this study, suggesting somatic SQSTMI mutations are not a major cause of Paget's disease. This is not particularly surprising given that the mutations only predispose patients to developing the disease, showing incomplete penetrance in many families [139]. Somatic mutations in other genes are a plausible cause for a focal disease like Paget's disease, particularly in cases that are sporadic, and only involve one disease site. The challenge will be identifying candidate genes that could be mutated. Brandwood *et al.* found mutations in the *BCL2* promoter in some bone biopsy samples from patients with Paget's disease [373], although the possibility that these were somatic mutations was not confirmed by testing in other tissues. Unlike this study, we found no evidence that *BCL2* expression was up-regulated in our pagetic samples. Identification of further mutations that predispose patients to developing Paget's disease, such as the mutations that result in linkage to the 10p13 locus [140], may help clarify the role of SQSTM1 in Paget's disease as it is a protein with a very diverse array of functions, and an increasingly large number of binding partners [148, 374]. The involvement of *VCP* mutations in an autosomal dominant manner in IBMPFD

suggests alterations in proteasome function may be involved. Identification of further mutations in proteins involved in this process would support this hypothesis.

In agreement with a number of other publications, we failed to find measles virus RNA in any of our RNA samples derived from patients with Paget's disease, despite using a highly sensitive RT-PCR technique [175-180]. There is an extensive body of evidence that suggests measles and canine distemper viruses can stimulate formation of pagetic-like osteoclasts [181-184]. It is possible that these findings are coincidental. Osteoclasts are terminally differentiated cells and only have a limited range of responses to stimuli. They are also derived from immune cells, so it is not surprising that they respond to the presence of viral antigens. Highly nucleated osteoclasts are also found in hyperparathyroidism and bisphosphonate-treated bone, and nuclear inclusions are also found in other conditions such as osteopetrosis and FEO that are not associated with environmental agents such as paramyxoviral infection [185, 192, 363]. The nature of the nuclear inclusions found in pagetic osteoclasts are therefore not convincingly identified, although they have been shown to be degraded by proteases [375]. It is possible that they are made up of protein aggregates, and it will be interesting to determine if SQSTM1 is present in these structures.

We have used primary human osteoblasts cultured from bone obtained from surgery extensively in this study. Large inter-patient variability was often apparent, which made interpretation of results challenging in some cases. Gene expression for some genes was highly variable among patients, and the microarray analysis indicated that, when most genes were included, similarities between patients prevailed over disease-related changes. However, many of the changes in gene expression identified in the pagetic osteoblasts were replicated in the paired samples, and using human patient samples is necessary to advance our understanding of Paget's disease as there is currently no accepted animal model. The human cells from different patients also performed differently in *in vitro* assays, particularly the mineralisation assay, although proliferation results using the human osteoblasts did not always show agreement either. Although it can be beneficial to use a human primary cell model, it appears to be much more difficult to achieve reproducible results using these cells than with cell lines or primary rodent cells.

9.2 Future directions

The results of this study strongly suggest a role for the osteoblast in the development of Paget's disease, but there are still many unanswered questions about the pathophysiology and aetiology of the disease. Experimental evidence is required to demonstrate that pagetic osteoblasts can stimulate increased osteoclast formation and resorption, and the formation of larger, more highly nucleated osteoclasts. If increased osteoclastogenesis did occur, it would be interesting to examine whether it was still inhibited by OPG in a similar manner to control cultures, and whether IL-6 neutralising antibodies could partially or fully block the effects, as has been reported previously in bone marrow cultures [111, 116]. Demulder et al. demonstrated that pagetic stromal cells were equally effective at stimulating enhanced CFU-GM formation in normal or pagetic CD34-positive haematopoietic cells [109]. Unfortunately these authors did not examine osteoclastogenesis. Sun et al. have also performed co-culture experiments using pagetic osteoblasts, and while osteoclast numbers and resorption tended to increase when pagetic cells were used, this was not statistically significant [115]. The pagetic cells also supported osteoclast formation without direct cell-cell contact while the control cells did not. The osteoclast numbers formed in these experiments were very low suggesting the culture conditions were not ideal for osteoclastogenesis, so repetition of this type of study in a better culture system may provide a more conclusive result.

It would also be beneficial to examine other features of pagetic osteoblast cells. This could include the levels of Wnt, BMP, and FGF signalling, as well as RUNX2 activity, both generally and in response to various stimulators and inhibitors of these pathways. These assays could be performed using both cultured cells, and using immunohistochemistry in bone biopsies from pagetic lesions. Measuring gene and protein expression levels of some of the differentially expressed genes identified in this study in pagetic bone biopsies would also be very helpful for confirming our results. It would also be interesting to compare changes in these genes in lytic and sclerotic lesions, as well as changes with disease progression and after treatment. However, given the invasive nature of bone biopsies, and the declining numbers of patients with Paget's disease, obtaining appropriate samples for this type of study would be a major challenge. It would also be interesting to assess the ability of pagetic osteoblasts to form matrix and mineralise *in vitro*, and to determine if the matrix composition is changed. This will require development of better methods of human osteoblast differentiation, which may require the use of three dimensional scaffolds so that cells are cultured in a more 'life-like' environment. We are currently comparing the ultrastructure of pagetic osteoblasts to

control cells using transmission electron microscopy to investigate whether there are changes in microscopic cell structure to accompany the changes in gene expression.

In recent years, the role of the osteocyte in the maintenance of bone mass has been a major focus of investigations in bone biology. Osteocytes are the most abundant cell in bone, and play an important role in sensing the need for bone repair, as well as critical functions in phosphate metabolism. It would be interesting to see if the osteocytes in pagetic lesions are abnormal. This could be assessed by examining expression of important osteocyte genes using *in situ* hybridisation in bone biopsies.

There are further questions about the role of keratin 18 in osteoblasts. Firstly, it would be interesting to assess whether it is expressed in all cells within the human osteoblast cultures or in a subset of cells, and if so, to confirm that they are a subset of osteoblastic cells. Knock-down experiments could be performed to assess if it is necessary for normal osteoblastic proliferation and differentiation, and it would be particularly interesting to assess whether over-expression of keratin 18 influences the ability of osteoblasts to support osteoclastogenesis.

It would also be interesting to investigate the roles of some of the other genes that were differentially regulated in Paget's disease in osteoblast biology, including their effects on osteoblast proliferation, differentiation, gene expression and ability to support osteoclastogenesis. Given that several important regulators of bone metabolism such as PTH signal through G protein-coupled receptors, the effects of RGS4 in osteoblasts will be interesting to determine, especially considering that it is suppressed by RUNX2 and inhibits chondrocyte differentiation [267, 322]. Determining a function and cell type specific binding partners for the transcription factor GATA6 would also be of interest, as would the role of IFI27, which appeared to be down-regulated over time in differentiating MC3T3-E1 cells. Although we attempted to characterise the expression of these genes with unexplored function during osteoblast differentiation, the results tended to be variable in the different culture systems used. It would be helpful to repeat these experiments in a more reliable primary culture system, either using rodent cells, or using an improved method for stimulating human osteoblast differentiation that shows less interpatient variation.

There are certainly more questions to be addressed about the role of *SQSTM1* mutations in osteoblasts. We did not determine whether SQSTM1 forms protein aggregates in osteoblast cells as it does in other cell types, and whether the mutants affect this process in the same manner as previously described. The effects on support of osteoclastogenesis and osteoblast differentiation would also be interesting to determine. It would also be fascinating to compare the phenotype of a transgenic mouse with osteoblast-specific expression of mutant *SQSTM1* to the results in mice with mutations in all cells to determine if mutations in the osteoclastic cells are also required for the development of the localised lytic lesions described in some of the animal models [159, 160].

The likelihood that the environmental factor that triggers Paget's disease is a long-term infection with a paramyxovirus appears doubtful, and it may be very difficult to identify an environmental factor that is disappearing. However, it would be interesting to determine if paramyxoviruses other than measles and canine distemper virus, and other viruses that are not implicated in the aetiology of Paget's disease still stimulate enhanced osteoclastogenesis to determine if this is a non-specific effect.

9.3 Conclusions

The analysis of gene expression in pagetic osteoblasts has shown that osteoblasts derived from pagetic lesions are phenotypically distinct from osteoblasts cultured from normal bone tissue. The results also suggest that osteoblasts may be a driver of enhanced and disordered bone turnover in Paget's disease. Further investigations suggested that mutations in *SQSTM1* do not make a major contribution to this altered osteoblast phenotype, nor does up-regulation of keratin 18. We have also found that somatic *SQSTM1* mutations are uncommon in Paget's disease, and the disease does not appear to be associated with long-term measles virus infection.

We were unable to identify the major trigger that results in the initiation of Paget's disease. There are a number of possible reasons for this. There may not be one trigger, but a number of events that occur to trigger the disease, similar to many cancers, where a combination of processes eventually leads to malignancy. The patients that participated in the study represented a mixed population at varying stages of Paget's disease, although it is likely that most undergoing surgery had longstanding and advanced disease associated with osteoarthritis. The pagetic lesion changes over time, with initial lytic appearance that

gradually becomes sclerotic. Investigating whether changes in osteoblast function precede the accelerated bone resorption by over-active osteoclasts may require sampling of lesions at very early stages. The treatment of the patients with bisphosphonates may have been another confounding factor. Despite this, we have identified a number of abnormalities in pagetic osteoblasts that suggest they produce enhanced levels of some factors to increase osteoclastogenesis independently of RANKL, as well as changes that suggest the osteoblasts may differentiate and function abnormally. These changes were still present despite the fact that the tissue was obtained from patients with low disease activity and cultured outside the pagetic microenvironment for a number of weeks, suggesting they are not an epiphenomenon of accelerated bone turnover. Although it has often been overlooked, the osteoblast is likely to play a major role in the development of Paget's disease.

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