

The Role of BMP Signalling in Multiple Myeloma

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This thesis is dedicated firstly to the memory of my mother Jenny, who died during the first year of my DPhil. She taught me to love science, gave me the support to embark on this venture with two children under 5, and would have been very proud to know I got there in the end.

Secondly, to my husband Paul. His support and patience in the completion of this project have been endless; without his willingness to equally share the tasks of home and parenting I would not have managed.

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Declaration of authorship

The great majority of the work presented in this thesis was developed and performed by myself under the supervision of Professor Hal Drakesmith at the Weatherall Institute of Molecular Medicine, and Professor Claire Edwards at the Botnar Research Centre, University of Oxford. Experiments performed by facilities staff or laboratory members are clearly indicated in the acknowledgements section above. No part of this thesis has been submitted for examination for a degree at this or any other university.

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Abstract

Multiple myeloma is an incurable, bone marrow-dwelling malignancy of terminally differentiated plasma cells. It disrupts bone homeostasis causing lytic lesions, skeletal damage and pain. Mechanisms underlying myeloma-induced bone destruction are poorly understood and current therapies do not restore lost bone mass. In this project, I performed transcriptomic profiling of isolated endosteal cell types from a murine myeloma model, which had not previously been described. I found bone morphogenetic protein (BMP) signalling to be upregulated in mesenchymal progenitor cells of the bone lining niche in myeloma-bearing mice.

BMP signalling is not previously reported to be dysregulated in myeloma bone disease. Inhibition of BMP signalling in vivo using either a small molecule Type 1 BMP receptor antagonist (LDN193189) or a solubilized Alk3-Fc (Bmpr1a-Fc) receptor ligand trap prevented trabecular and cortical bone volume loss caused by myeloma, without altering tumour burden. These inhibitors directly reduced osteoclast formation and activity, increased osteoblast number and new bone formation, and suppressed bone marrow levels of sclerostin, a Wnt inhibitor implicated in mediating the failure of bone repair in myeloma sufferers. Conversely, specific blockade of BMP6 in myeloma-bearing mice decreased levels of the iron regulatory hormone hepcidin and improved iron availability and haemoglobin, but did not alter bone disease or tumour burden, indicating this myeloma-expressed BMP is not responsible for altered bone homeostasis. In summary this work describes a novel technique in the investigation of myeloma-niche interactions, shows its use in revealing a novel pathway involved in the pathogenesis of myeloma bone disease, and demonstrates that the therapeutic targeting of the BMP pathway has both anti-resorptive and anabolic benefits to bone metabolism, which could have a role alongside anti-tumour treatments.

Abbreviations

Abs(log2Fc)	Absolute log2 fold change
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ActRlla	Activin Receptor type 2a
ANOVA	Analysis of variance
ASCT	Autologous stem cell transplantation
BAMBI	BMP and activin membrane-bound inhibitor
BLC	Bone lining cell
BM	Bone Marrow
BMP	Bone Morphogenetic Protein
BMPR	BMP Receptor
BMPR1a	BMP Receptor 1a
BMSC	Bone Marrow Stromal Cell
BV/TV	Bone surface density
CBRG	Computational Biology Research Group
CD	Cluster of differentiation
cDNA	Complementary DNA
CSR	Class switch recombination
CT	Computed tomography
Dkk1	Dickkopf1
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphate
eCyPA	Extracellular cyclophilin A
EDTA	Ethylenediaminetetraacetic acid
ERCC	Excision repair cross-complementing
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FCS file	Flow cytometry standard file
FDR	False Discovery Rate
FISH	Fluorescence in situ hybridization
FMO	Fluorescence minus one
GC	Germinal Centre
GEP	Gene Expression Profiling
GFP	Green fluorescent protein
Hb	Haemoglobin
Hbmp6	Human Bone Morphogenetic Protein 6
HIF-1	Hypoxia Inducible Factor-1
HSC	Haematopoietic Stem Cell
Ig	Immunoglobulin
IgG	Immunoglobulin Gamma
IL-6	Interleukin-6
IFN α	Interferon α
IFN γ	Interferon γ
IP	Intra-peritoneal
IV	Intra-venous
KD	Knockdown
KO	Knock out
LDN	LDN-193189
LeptinR	Leptin Receptor
LogCPM	Log counts per million mapped reads
Log FC	Log fold change
MDS	Myelodysplastic syndrome

MDSC	myeloid-derived suppressor cell
MGUS	Monoclonal gammopathy of uncertain significance
MicroCT	Micro computed tomography
MIP-1a	Macrophage inflammatory protein- 1 alpha
MM	Multiple Myeloma
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
NIST cert	National Institute of Standards and Technology
NK	Natural killer
NS	Not significant
OB	Osteoblast
OC	Osteoclast
OPG	Osteoprotegerin
PET CT	Positron emission tomography–computed tomography
(p)SMAD	(Phosphorylated) Small Mothers Against Decapentaplegic
PBS	Phosphate buffered saline
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear-Factor kappaB ligand
RGM	Repulsive Guidance Molecule
R-ISS	Revised international staging system
RNA	Ribonucleic acid
RPKM	Reads Per kilobase of transcript, per Million mapped reads
SEM	Standard error of the mean
SHM	Somatic hypermutation
Sost	Sclerostin
SD	Standard deviation
TAM	Tumour Associated Macrophage
TRAP	Tartrate-resistant acid phosphatase
T-reg	Regulatory T cell
TGFb	Transforming growth factor beta
VEGF	Vascular Endothelial Growth Factor
VOI	Volume of Interest
WIMM	Weatherall Institute of Molecular Medicine

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Chapter 1 Introduction

1.1 Multiple Myeloma: an introduction

1.1.1 Epidemiology of Myeloma

Multiple myeloma is a malignancy of terminally differentiated B-lymphocytes, called plasma cells, the function of which in health is antibody secretion as part of the adaptive immune response. It is the second-most common haematological malignancy, representing 2% of all cancers. The UK incidence is approximately 5500 new cases a year, with an overall UK prevalence of around 18000 (2014 figures). Survival has steadily improved with better treatment in recent years, with a third surviving over 10 years and a half surviving over 5 years (2011 figures) (Cancer Research UK, 2016). It is still essentially an incurable disease however, with treatments achieving remissions but not cures, other than in a small proportion of those receiving allogeneic stem cell transplants. The incidence is rising; although the full cause for this is unknown, it is probably explainable by increased life expectancy and improved diagnostics (Kumar *et al*, 2017). The rarity of the disease makes studies of risk factors difficult, but obesity has been identified as such, with some immune disorders and farming or industrial exposures potentially implicated in a small number of cases (Sergentanis *et al*, 2015). Cases have risen by 15% over the past decade, with a further projected increase of 11% by 2035 (Fig. 1.1).

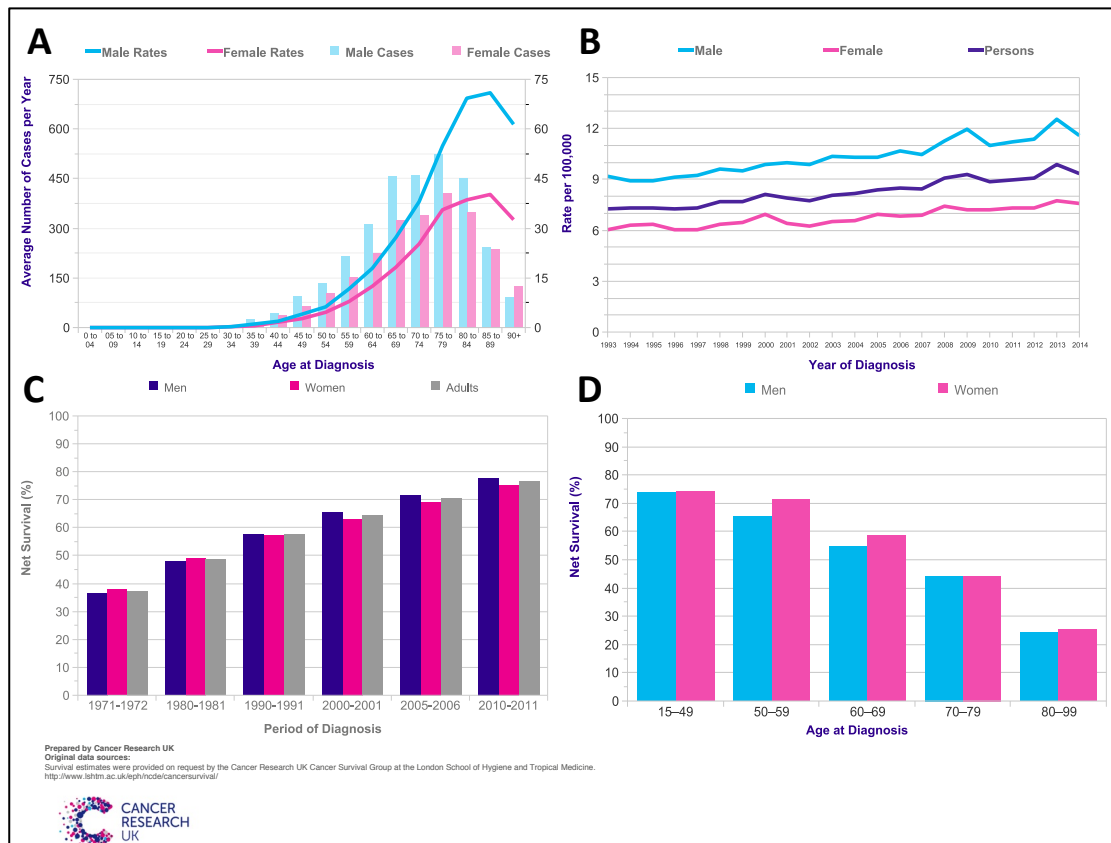


Figure 1.1: Statistics of myeloma in the UK (2014 data unless otherwise stated)

A: Incidence of new myeloma cases in the UK per year according to sex and age. Incidence left y-axis, rate per 100 000 right y-axis.

B: Overall rate per 100 000 from 1993 to 2014 showing increased incidence over time.

C: One-year net survival over past 40 years from 1971 to 2011.

D: five-year net survival according to age at diagnosis in 2014.

Myeloma is a disease highly associated with ageing; 45% of cases occur in the over 75s, with the highest incidence being in those aged 85-89 (Cancer Research UK). Frailty is therefore a major factor affecting survival, as it dictates the treatments that can be safely delivered without compromising side effects.

1.1.2 Pathogenesis of Myeloma

Normal B cell development

Long-lived plasma cells, which have the capacity to proliferate in response to antigenic stimulation, are a vital part of the adaptive immune response. Immature B cells initially undergo VDJ gene rearrangement in the bone marrow to generate a wide range of

immunoglobulin B cell receptors. If a functional cell-surface B cell receptor results, the B cell will migrate to a germinal centre in the lymphoid system where it encounters presented antigen. In the dark zone of germinal centres, B cells undergo intense proliferation and somatic hypermutation (SHM) of the Ig genes; if the B cell receptor remains functional they then enter the light zone where, if their affinity for presented antigen is sufficiently high following SHM (affinity maturation), T cell help may be provided and a long-lived plasmablast fate, dictated by a specific transcriptional programme, initiated (Kometani & Kurosaki, 2015) (Fig. 1.3).

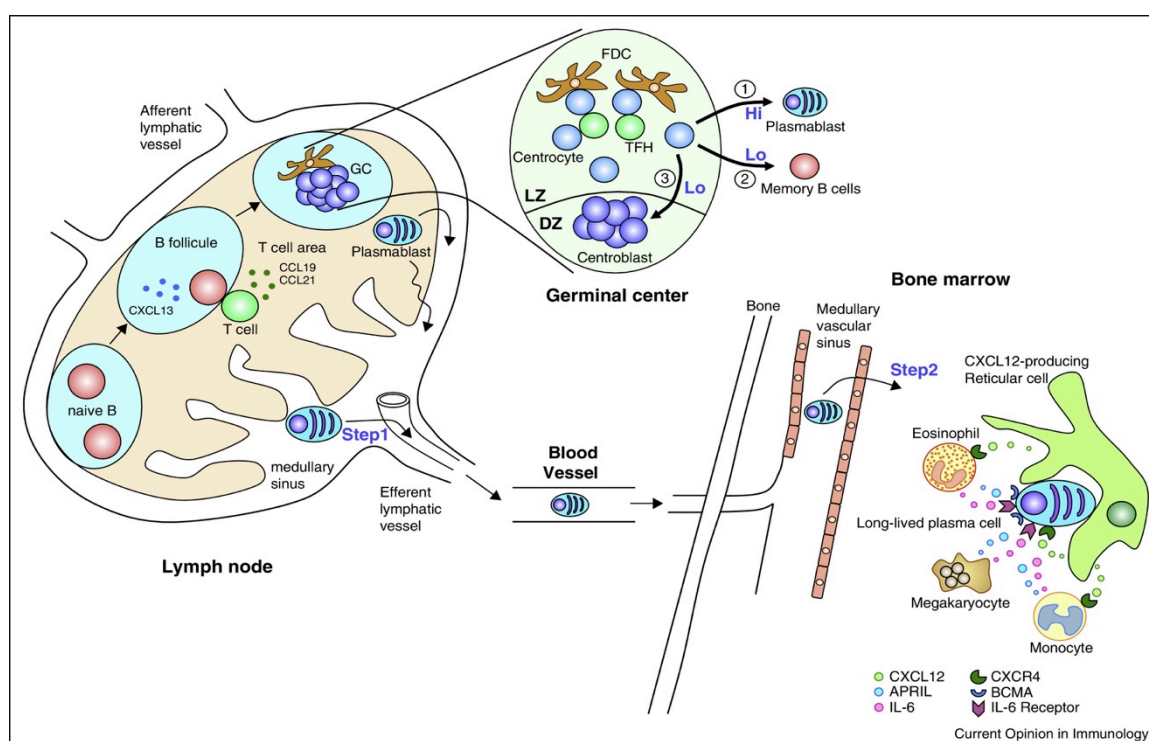


Fig. 1.3: Plasmablasts migrate to bone marrow and become long-lived plasma cells.

In the lymph node, B cells differentiate to short-lived plasmablasts residing in extrafollicular or germinal center (GC) B cells. High affinity light zone (LZ) GC B cells (centrocytes) have a high propensity to differentiate to plasmablasts (1). Low affinity LZ GC B cells either apoptose, give rise to memory B cells (2), or to re-enter the dark zone (DZ) for further rounds of SHM (3). GC-derived plasmablasts downregulate CXCR5 and leave GC. These plasmablasts move to the medullary sinus and enter efferent lymphatic vessels (Step1). After moving into the lymphatic system, plasmablasts go through blood vessels and migrate to the bone marrow. Medullary vascular sinuses in the bone marrow are surrounded by endothelial cells. Plasmablasts enter bone marrow probably by transmigrating through endothelial cells (Step2). In the bone marrow, CXCL12-expressing reticular cells recruit CXCR4-expressing plasma cells, eosinophils, and monocytes. Eosinophils, megakaryocytes, and monocytes secrete APRIL and IL-6. Plasmablasts that receive these survival factors differentiate into long-lived plasma cells and maintain continuous antibody production. Reprinted from Current Opinion in Immunology, 33, Kometani, K & Kurosaki, T. Differentiation and maintenance of long-lived plasma cells, page 65, Copyright 2015, with permission from Elsevier.

There are many stages throughout this process where B cells may be damaged or their receptors rendered less functional. Apoptosis levels are extremely high in the germinal centre, with only the most functional cells which also manage to gain T cell help surviving to gain either plasmablast or memory cell function (Mayer *et al*, 2017). As shown in figure 1.3, once the transcriptional decision towards plasmablast fate is made, cells migrate to the bone marrow, attracted to a niche high in CXCL12 expression. Only if the correct combination of growth and survival factors produced by this niche is present, will cells finally complete their differentiation into long-lived plasma cells.

Primary oncogenic genetic events

Despite the high levels of apoptosis of germinal centre B cells, it is possible that an unintended survival advantage can be gained if, during the double stranded DNA breaks of class switching and SHM, an oncogene is aberrantly placed under the control of an IgH enhancer. This may allow protection from germinal centre apoptosis. This is the means by which certain recurrent translocations involving the IgH genes are believed to be initiating genetic events in monoclonal gammopathies (Fig. 1.4, and Fig. 1.5) (Kumar *et al*, 2017; Manier *et al*, 2016). In addition to IgH translocations, the other main category of initiating event is chromosome number aberrations (primarily hyperdiploidy), which can also result during the proliferative process of SHM. While these initiating events are associated with different prognoses and gene expression profiles (Table 1.1), their uniting feature is CyclinD1, 2 or 3 aberrant overexpression (Bergsagel *et al*, 2005), allowing cell cycle progression at the G1/S transition. These pre-plasmablast events confer a survival advantage before arrival in the bone marrow and terminal differentiation to plasma cells. A premalignant plasma cell clone is produced, which can exist as MGUS for a highly variable period of time before secondary oncogenic events occur to drive proliferation forward.

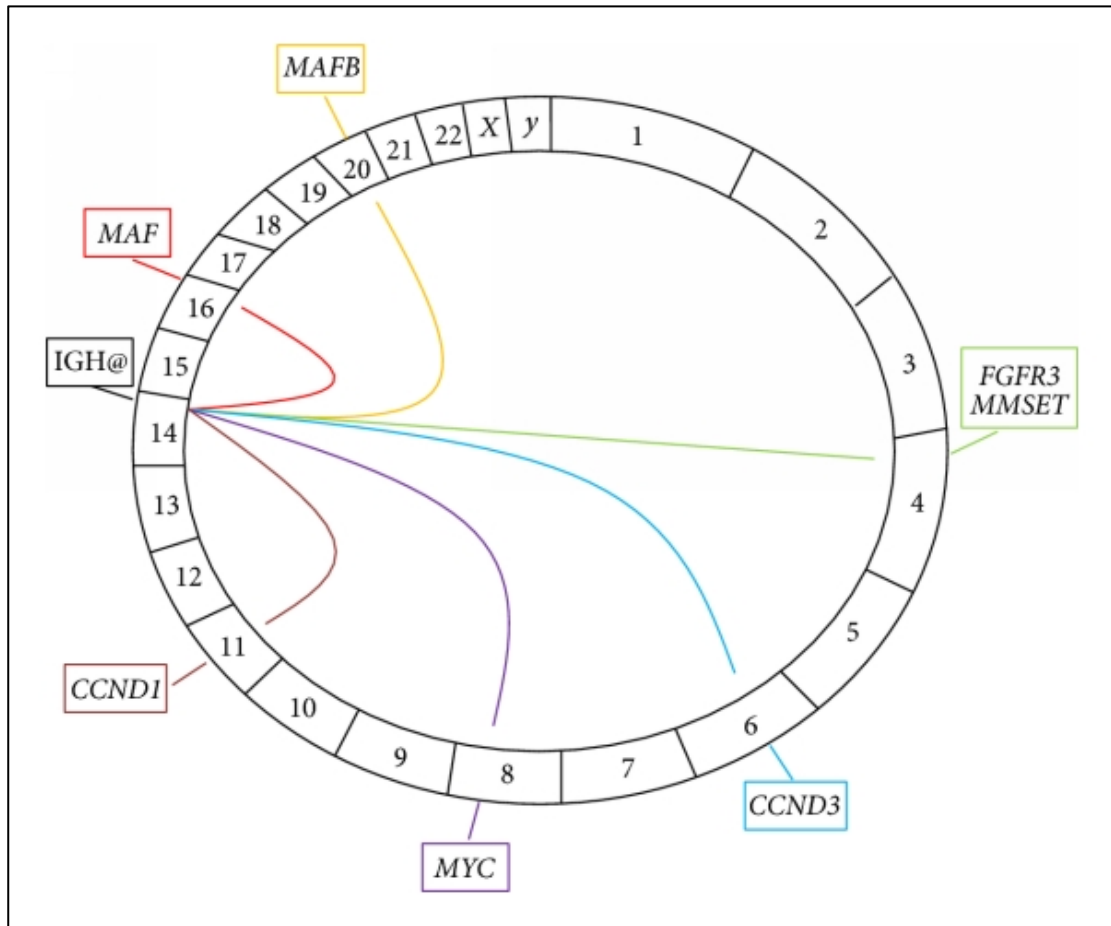


Fig 1.4: Chromosomal translocations in myeloma.

Chromosomes arranged in a clockwise direction to demonstrate the key translocations in myeloma. Translocations represented as lines emerging from the Immunoglobulin Heavy Chain locus on chromosome 14 to respective partner chromosomes. The genes involved are represented in boxes outside the plot. All translocations represent primary events except $t(8;14)$ involving *MYC* which is a secondary translocation.

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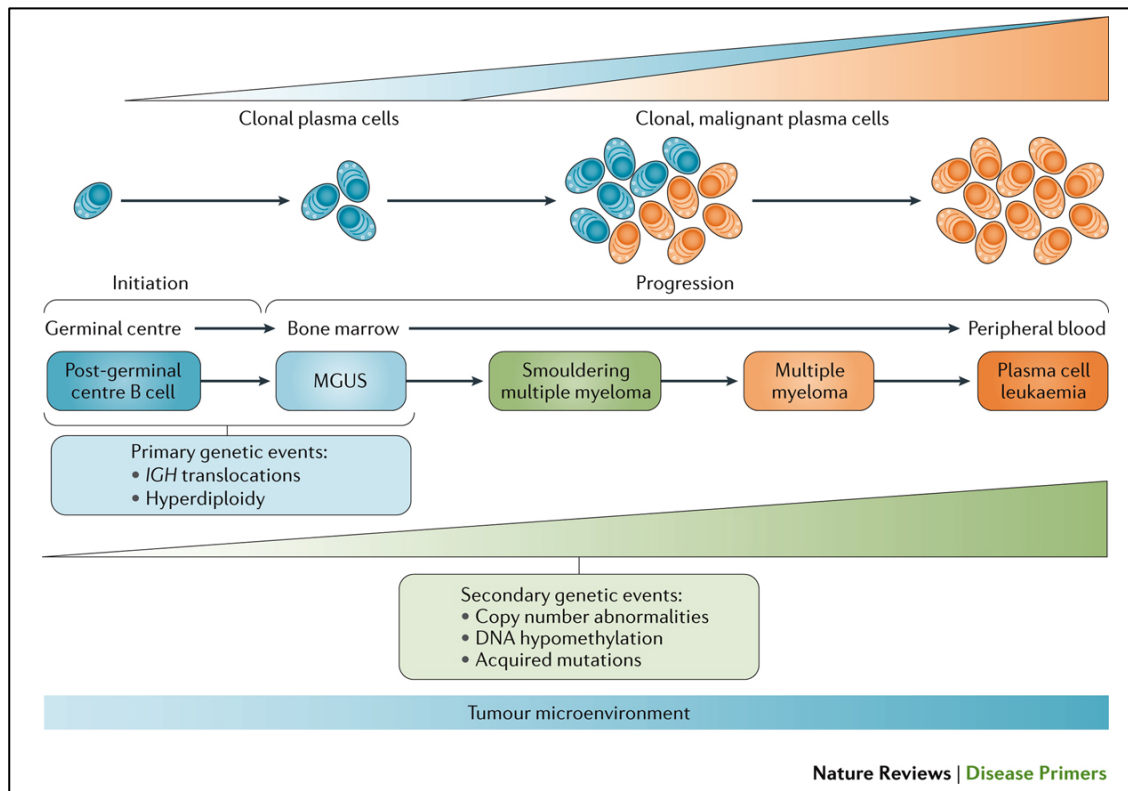


Fig 1.5: The development of monoclonal gammopathies

The development of multiple myeloma is a multistep process, which starts with precursor disease states, such as monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM). Primary genetic events in the development of MGUS, SMM and multiple myeloma include chromosomal translocations involving the immunoglobulin heavy-chain genes (IGH) and aneuploidy (with hyperdiploidy as the most frequent entity). The number of secondary genetic alterations increases from MGUS to SMM and then to multiple myeloma.

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Secondary oncogenic genetic events

The survival advantage of the primary genetic events described above are accompanied by an increased likelihood of genomic or transcriptomic instability, particularly if they involve aberrations of DNA methylation, as for example with t(4;14) leading to MMSET overexpression (Martinez-Garcia *et al*, 2011). This allows the acquisition of secondary mutations (Fig. 1.5 and Table 1.1) and drives progress towards overt multiple myeloma. This may occur in a linear fashion, but more usually results in clonal heterogeneity, with different combinations of secondary mutations co-occurring in different sub-clones (Lohr *et al*, 2014; Rasche, L. *et al*, 2017). These sub-clones may have different sensitivities to

chemotherapeutic agents and have been shown to respond to sequential therapies with alternating dominance (Keats *et al*, 2012).

Genomic event	(Potential) driver genes involved	Frequency in patients with MM	Prognostic value
Primary events			
Translocations: driver genes	t(11;14): <i>CCND1</i> (REFS 8,29,31)	15%	Neutral or adverse*
	t(4;14): <i>FGFR3/MMSET</i> ²⁷⁻³¹	15%	Adverse
	t(6;14): <i>CCND3</i> (REF. 45)	2%	Neutral
	t(14;16): <i>MAF</i> ^{29,42,46}	5%	Neutral or adverse†
	t(14;20): <i>MAFB</i> ⁴⁶	1%	Adverse
Copy-number variations	Hyperdiploidy: tri 3, 5, 7, 9, 11, 15, 19 or 21 (REFS 64,65)	50%	Favourable
	del13q: <i>RB1</i> , <i>DIS3</i> , <i>mir15a</i> or <i>mir16.1</i> (REFS 53,69) (potential drivers)	40%	Neutral§
Secondary events			
Chromosome gains: potential driver genes	1q: <i>MCL1</i> , <i>CKS1B</i> , <i>ANP32E</i> or <i>BCL9</i> (REFS 9,66,70)	40%	Adverse
	8q: <i>MYC</i> ⁸	15%	Neutral
	11q: <i>CCND1</i> (REF. 77)	15%	Neutral
Chromosome losses: potential tumour suppressor genes	1p: <i>CDKN2C</i> or <i>FAM46C</i> ^{53,75,77}	30%	Adverse
	12p: <i>CD27</i> (REF. 77)	15%	Adverse
	14q: <i>TRAF3</i> (REF. 53)	10%	Not determined
	16q: <i>CYLD</i> or <i>WWOX</i>	30%	Neutral
	17p: <i>TP53</i> (REF. 85)	10%	Adverse
Translocations	Affecting <i>MYC</i> ^{8,9}	15%	Adverse
Somatic mutations ⁹	MAPK pathway: <i>KRAS</i> , <i>NRAS</i> or <i>BRAF</i>	45%	Neutral
	NF-κB pathway: <i>CYLD</i> , <i>TRAF3</i> , <i>LBT</i> or <i>NIK</i>	15%	Neutral
	RNA metabolism: <i>DIS3</i> or <i>FAM46C</i>	15%	Neutral
	DNA-repair pathway: <i>TP53</i> , <i>ATM</i> or <i>ATR</i>	10%	Adverse
	Plasma cell differentiation: <i>IRF4</i> or <i>PRDM1</i>	10%	Favourable

Table 1.1: Main genomic alterations in multiple myeloma and their clinical impact

del, deletion; MM, multiple myeloma; t, translocation; tri, trisomy.

*The prognostic value of t(11;14) is considered neutral, but this translocation is associated with poor prognosis when it co-occurs with *CCND1* mutation.

†t(14;16) has been defined to have both adverse and neutral effects on prognosis.

§del13q has been traditionally associated with a poor prognosis, which is now believed to be a surrogate of its association with other high-risk lesions.

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Please note references referred to in table do not relate to this thesis but source material.

The most frequently occurring secondary mutations in patients with multiple myeloma are in *KRAS* (in 23% of patients), *NRAS* (20%), *FAM46C* (11%), *DIS3* (11%) and *TP53* (8%) (Kumar *et al*, 2017). There are others that feed in to, or interact with, critical shared pathways involved in proliferation and the cell cycle; aberrant activity of these pathways is often

present even if mutations of their members are not identified, NF- κ B being a classical example of this (Annunziata *et al*) (Fig. 1.6). *MYC* is the commonest secondary driver gene involved by chromosome gain or translocation, and its resultant upregulation is associated with advanced disease, plasma cell leukaemia, and poor prognosis (Walker, Brian A. *et al*, 2015).

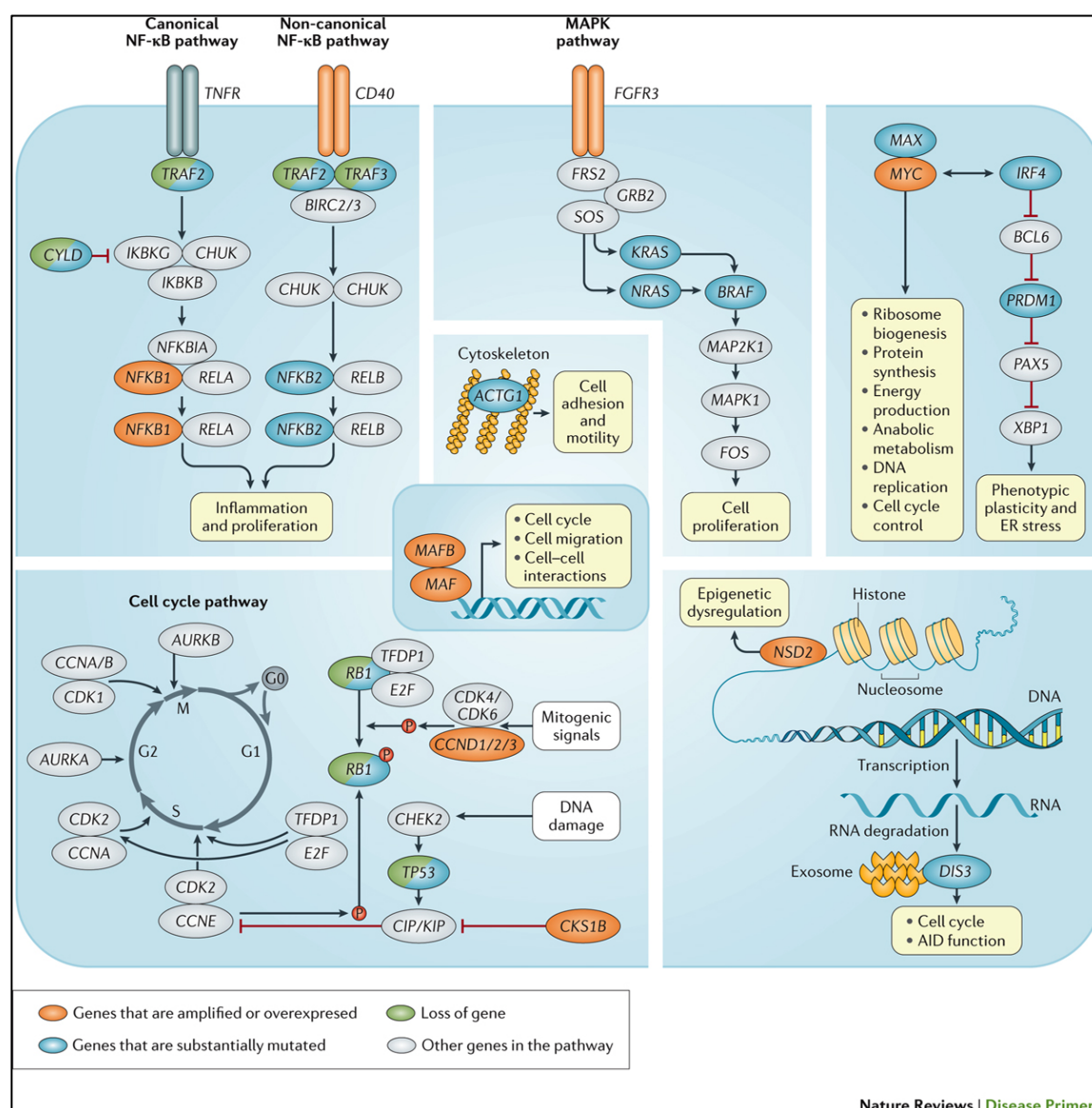


Figure 1.6: Signalling pathways affected in multiple myeloma.

Genetic alterations in multiple myeloma can affect several cellular signalling pathways. Frequent genetic aberrations in patients with multiple myeloma include *MYC* rearrangements, mutations in *KRAS* and *NRAS*, translocation t(11;14) and translocation t(4;14). Several chromosomal translocations indicate a central role of the cyclin D proteins in multiple myeloma; t(11;14) leads to overexpression of *CCND1* (which encodes cyclin D1) and t(6;14) leads to overexpression of *CCND3*. *CCND2* overexpression is also frequently observed in multiple myeloma. Other recurrent mutations include *DIS3*, *TP53*, *BRAF*, *TRAF3*, *PRDM1*, *CYLD*, *RB1*, *MAX* and *ACTG1*, of which (putative) functional links are indicated. Nuclear factor- κ B (NF- κ B) signalling is a central pathway in B cells, and this pathway is deregulated in most B cell malignancies. As opposed to some other types of B cell malignancies,

multiple myeloma is mostly characterized by the activation of the non-canonical NF- κ B pathway; alterations that affect the activation of this pathway include overexpression of CD40, mutations and deletions of TRAF2 or TRAF3 and mutations in NFKB2 (also known as P100), although aberrations that affect the canonical pathway have also been described, such as CYLD mutations. Signalling in the canonical pathways occurs through several receptors, including B cell receptor, Toll-like receptors and tumour necrosis factor receptor (TNFR).

Furthermore, for the non-canonical pathway, multiple receptors are involved including CD40 (also known as TNFRSF5), lymphotoxin- β receptor (also known as TNFRSF3) and also TNFR. Several genetic alterations indicate an important role for cell cycle deregulation in multiple myeloma, including overexpression of CKS1B, deletion and/or mutation of TP53 and frequent deletion of RB1. Other pathways of interest are the interaction with multiple myeloma cells and the microenvironment through overexpression of MAF oncogenes (in patients with t(14;16) or t(14;20)), which results in integrin deregulation. Mutations in ACTG1 are putatively involved in a cytoskeletal defect. AID, activation-induced cytidine deaminase; ER, endoplasmic reticulum; MAPK, mitogen-activated protein kinase.

Reprinted from *Nat. Rev. Dis. Primers*, doi:10.1038/nrdp.2017.46, Kumar, S. K. *et al.* Multiple Myeloma, copyright 2017, with permission from Springer Nature. Previously had been adapted with permission from Otto, T. & Sicinski, P. Cell cycle proteins as promising targets in cancer therapy. *Nat. Rev. Cancer* **17**, 93–115 (2017) and Manier, S. *et al.* Genomic complexity of multiple myeloma and its clinical implications. *Nat. Rev. Clin. Oncol.* **14**, 100–113 (2017), Macmillan Publishers Limited

The tumour microenvironment

The interplay between multiple myeloma and its bone marrow microenvironment is critical to disease pathogenesis, as it is niche support that allows slowly dividing monoclonal gammopathies to proliferate, mutate and gradually progress to multiple myeloma; only the most advanced tumours can survive without this bone marrow niche support. This relationship will be discussed extensively in section 1.2.

1.1.3 Clinical features of Myeloma

Myeloma has a set of ‘classical’ pathological features, which derive either from the monoclonal antibody (paraprotein) or light chains that the malignant clone produces, or from the inflammation and haematopoietic suppression in the bone marrow microenvironment that it infiltrates and subverts for its maintenance and growth. Although myeloma can grow outside the bone marrow in almost any location (‘extra-medullary disease’), this is usually a late disease feature, occurring when the tumour has mutated enough to survive without the bone marrow niche sustenance it more commonly depends upon; it is a feature associated with poor survival. Far more frequently, especially in early

disease, myeloma is an obligate dweller in the bone marrow, occupying the niche of healthy plasma cells, and haematopoiesis more generally (Noll *et al*, 2012).

An infiltrate of malignant plasma cells may be present, without apparent effect, in the bone marrow for many years, defined either as MGUS if the infiltrate is <10% of total marrow cells, or smouldering myeloma if more. The progression to multiple myeloma (Table 1.2) is defined as the point at which the clone causes end-organ damage, or more than 60% of the marrow is infiltrated, as this is associated with inevitable rapid progression to damage (Rajkumar *et al*, 2014). The features that summarise the end-organ damage are known by the acronym 'CRAB' criteria, namely Calcium (for hypercalcaemia, caused by bone matrix degradation), Renal (for renal failure, caused by immunoglobulin and free light chain-induced renal toxicity), Anaemia (caused by haematopoietic suppression as well as inflammatory suppression of erythropoiesis, and Bones (for lytic bone disease, as discussed in detail later) (Table 1.2). Clinical symptoms, related to the pathology described above, include fatigue and dyspnoea due to anaemia, renal failure or hyperviscosity (caused by high paraprotein levels), recurrent infections due to immunoparesis and/or leukopenia, bone pain or nerve compression pain and symptoms due to lytic skeletal damage, and the emergency symptoms of spinal cord compression which can result from vertebral collapse. Further wide-ranging symptoms can result from hypercalcaemia, paraprotein-associated autoimmune or viscosity-induced phenomena, or amyloid deposition (Kumar *et al*, 2017). The renal failure can be multi-factorial, but is most frequently caused by 'cast nephropathy': light chain accumulation in the distal nephron where they combine with the Tamm-Horsfall urinary glycoprotein and precipitate to form casts, causing obstruction (Heher *et al*, 2013).

Revised International Myeloma Working Group diagnostic criteria for multiple myeloma and smouldering multiple myeloma (Rajkumar *et al*, 2014)

Definition of multiple myeloma

Clonal bone marrow plasma cells $\geq 10\%$ or biopsy-proven bony or extramedullary plasmacytoma^{*}

^{*}Clonality should be established by showing κ/λ -light-chain restriction on flow cytometry, immunohistochemistry, or immunofluorescence. Bone marrow plasma cell percentage should preferably be estimated from a core biopsy specimen; in case of a disparity between the aspirate and core biopsy, the highest value should be used.

and any one or more of the following myeloma defining events:

- Myeloma defining events:
 - Evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
 - Hypercalcaemia: serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2.75 mmol/L (>11 mg/dL)
 - Renal insufficiency: creatinine clearance <40 mL per min[†]
†Measured or estimated by validated equations. or serum creatinine >177 μ mol/L (>2 mg/dL)
 - Anaemia: haemoglobin value of >20 g/L below the lower limit of normal, or a haemoglobin value <100 g/L
 - Bone lesions: one or more osteolytic lesions on skeletal radiography, CT, or PET-CT[‡]
‡If bone marrow has less than 10% clonal plasma cells, more than one bone lesion is required to distinguish from solitary plasmacytoma with minimal marrow involvement.
- Any one or more of the following biomarkers of malignancy:
 - Clonal bone marrow plasma cell percentage^{*} $\geq 60\%$
 - Involved: uninvolved serum free light chain ratio[§]
§. These values are based on the serum Freelite assay (The Binding Site Group, Birmingham, UK). The involved free light chain must be ≥ 100 mg/L.
 - >1 focal lesions on MRI studies[¶]
¶. Each focal lesion must be 5 mm or more in size.

Definition of smouldering multiple myeloma

Both criteria must be met:

- Serum monoclonal protein (IgG or IgA) ≥ 30 g/L or urinary monoclonal protein ≥ 500 mg per 24 h and/or clonal bone marrow plasma cells 10–60%
- Absence of myeloma defining events or amyloidosis

PET-CT = ¹⁸F-fluorodeoxyglucose PET with CT.

Table 1.2: The Revised International Myeloma Working Group diagnostic criteria for multiple myeloma and smouldering multiple myeloma

Reprinted from *Lancet Oncology*, Vol. 15, Rajkumar, SV, Dimopoulos, MA, Palumbo, A, Blade, J, Merlini, G, Mateos, M-V *et al.*, International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma, pages e538-e548, copyright 2014, with permission from Elsevier.

In recent years there has been a renewed interest in risk stratification in myeloma. This applies both to those with premalignant disease (MGUS or smouldering myeloma), to identify a cohort who may benefit from earlier initiation of treatment or intensity of monitoring because their risk of progression is higher, and to those with established disease who may, particularly based on their genetic profile as discussed in section 1.1.2, carry a particularly poor or good prognosis. Early identification of such individuals should enable appropriate, individually tailored intensification or de-escalation of therapy ('risk-adapted therapy'), as already occurs in other haematological malignancies including acute leukaemias. Whereas the International Staging System (ISS) has been used for many years to divide patients into high, intermediate or standard risk, a more recent revised version (R-ISS) incorporating the highest-risk genetic features has been shown to be of greater benefit (Table 1.3) (Palumbo *et al*, 2015).

Prognostic Factor	Criteria
ISS stage	
I	Serum β_2 -microglobulin < 3.5 mg/L, serum albumin $\geq$ 3.5 g/dL
II	Not ISS stage I or III
III	Serum β_2 -microglobulin $\geq$ 5.5 mg/L
CA by iFISH	
High risk	Presence of del(17p) and/or translocation t(4;14) and/or translocation t(14;16)
Standard risk	No high-risk CA
LDH	
Normal	Serum LDH < the upper limit of normal
High	Serum LDH > the upper limit of normal
A new model for risk stratification for MM	
R-ISS stage	
I	ISS stage I and standard-risk CA by iFISH and normal LDH
II	Not R-ISS stage I or III
III	ISS stage III and either high-risk CA by iFISH or high LDH
Abbreviations: CA, chromosomal abnormalities; iFISH, interphase fluorescent in situ hybridization; ISS, International Staging System; LDH, lactate dehydrogenase; MM, multiple myeloma; R-ISS, revised International Staging System.	

Table 1.3: Standard risk factors for myeloma and the R-ISS.

Patients with R-ISS stage I (approx. 40% total) have overall survival 82% at 5 years, but stage III (20% total patients) only 40% at 5 years.

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Palumbo, A *et al*: J Clin Oncol 33, 2015: 2863-2869

Various tumour gene expression profiles (GEPs) also deliver reproducible risk stratification, although there is no consensus on the best one to use (Chng *et al*, 2013).

1.1.4 Treatment of Myeloma

This is a large topic, which will be covered only briefly here. The aim of treatment in myeloma is to reduce tumour burden as low as possible ('measurable residual disease', MRD), manage symptoms such as renal failure, infections and bone disease, prevent further bone loss, and where possible delay disease relapse with consolidation and maintenance treatments. Historically there were few pharmacologic therapies with durable effects in myeloma. Induction of remission with steroids and chemotherapy (e.g. VAD, vincristine, doxorubicin and dexamethasone), followed by high dose melphalan with autologous stem cell rescue/transplant (ASCT), was the treatment associated with deepest remission and longest time to progression in those fit enough for intensive treatment, with a combination of oral steroids and alkylating agents in those not fit enough. Little could be achieved once patients relapsed following high dose melphalan.

However, in the late 1990s the efficacy of thalidomide, an immunomodulatory imide drug (IMiD) (Corral & Kaplan, 1999), was discovered in myeloma, with a range of direct antiproliferative and microenvironment-modifying effects (Görgün, 2010; Lopez-Girona *et al*, 2012). These effects are now known to be mediated through binding to the ubiquitin E3 ligase complex member cereblon (Ito *et al*, 2010), where thalidomide and analogues enhance the ubiquitination and degradation of key transcription factors Aiolos and Ikaros (Krönke *et al*, 2014; Lu *et al*, 2014). Thalidomide was found to be more effective in combinations with steroids and alkylating agents in inducing and maintaining remissions than chemotherapy alone. Newer and more potent IMiDs, with a better toxicity profile, including lenalidomide and pomalidomide, are now a mainstay of myeloma combination treatment regimes, at diagnosis, maintenance and relapses.

Proteasome inhibitors were added to the repertoire of effective myeloma therapies soon after IMiDs, with bortezomib the first in class (Hideshima *et al*, 2003; Rajkumar *et al*, 2005). Inhibition of the proteasome in myeloma results in elevation of the levels of certain inhibitory proteins usually rapidly destroyed by cancer cells to evade cell death; the result of this is inhibition of NF- κ B signalling, arrest of the cell cycle, and apoptosis due to increase in p53, JNK and damaged proteins that trigger cell stress.

While IMiDs and Proteasome inhibitors have transformed myeloma survival figures, and are the mainstay of treatment at induction, maintenance and subsequent relapses, they have not overcome the benefit of high dose melphalan ASCT consolidation, and critically, the myeloma clones do eventually become refractory to both classes of drug. Combination therapies and new-in-class alternatives delay but do not reverse this challenge. To this end, in recent years there has been intense research into new classes of anti-myeloma agents. The most promising of these include Daratumumab, an anti-CD38 monoclonal antibody (Lokhorst *et al*, 2015), and Elotuzumab, anti-SLAMF7 monoclonal antibody (Lonial *et al*, 2015). These antibodies are examples of immunotherapy in myeloma, and this field, discussed further in section 1.2.5, is generating considerable excitement (Rasche, Leo *et al*, 2017). An example of the cause of this excitement is the early evidence that CAR-T cells, autologous T cells engineered to recognise and destroy tumour cells carrying particular antigens (in the case of myeloma, most often BCMA) can induce complete remissions in heavily pre-treated patients with otherwise multi-drug resistant disease (Garfall *et al*, 2015; 2017). However this treatment, and other immunologic ones like it, are so new that no long-term follow up data exists to see whether they might indeed represent long term disease control or cure for patients.

New treatments in myeloma do not come without a high financial cost, and in the case of the drugs described above this continues to be a difficult and political issue (Cohen, 2017). At the time of writing, although IMiD – proteasome inhibitor – steroid triplets, even quadruplets including daratumumab, are commonly used upfront in resource-rich healthcare settings, the cost of this is prohibitive in many others, including the use of these drugs long term in a maintenance option. In the UK, due to healthcare budget constraints and the need to provide evidence of a clear increase in overall survival and/or life quality over a pre-specified level, the only upfront triplet combination approved by NICE is VTD (bortezomib thalidomide dexamethasone), with tight regulation at which point other drugs and combinations can be used (NICE, 2017). Daratumumab has been approved as a monotherapy for relapsed/refractory myeloma use in the NHS only in 2018, as overall survival data takes many years to accrue. The predicted rising costs of myeloma care, in particular if CAR-T and other individually tailored therapies become available, will pose an enormous challenge to the wide distribution of effective healthcare.

1.1.5 Why is Myeloma still incurable?

As discussed above, myeloma prognosis has improved greatly over past 20 years but it remains largely incurable. This is because no currently used combination of therapies can eradicate every tumour cell, and even from a tiny residual, disease propagation and repopulation will occur. It has however been shown that the tinier the fraction of residual disease, the longer patient survival (Munshi *et al*, 2017); so new therapeutic approaches must focus on ways to continually reduce, and eventually eradicate, the therapy-resistant residual tumour fraction.

There are two main reasons for the existence of residual disease:

1. Clonal heterogeneity and evolution of resistant populations. Exposure to treatments selects for the survival of the minority fraction that, whether at that point composed of

‘cancer stem cells’ or not, can tolerate the successive drug combinations experienced. This process has been described in myeloma and other cancers (Paino *et al*, 2015; Shaffer *et al*, 2017). Biomarkers of drug sensitivity that enable design of drug combinations based on an individual disease’s clonal characteristics are required to minimise resistance evolution, but may never overcome it completely.

2. Microenvironmental protection of tumour cells from pharmacological or immune-mediated killing. Niche-mediated drug resistance and immune system evasion is well described in myeloma, and will be discussed in detail in the next section.

1.2 The role of the tumour microenvironment in myeloma

1.2.1 The dependence of cancers on their environments

Understanding the cellular environment in which cancers arise and proliferate is vital in understanding how they evade killing and metastasize to new sites. The cancer stem cell has traditionally been defined as a tumour subpopulation which is chemoresistant, often with a stem/progenitor phenotype or de-differentiated, and capable of both self-renewal and tumour repopulation (Clarke *et al*, 2006). This concept is gradually evolving to accommodate evidence that suggests, to a variable extent in different malignancies, these characteristics are not cell-intrinsic but externally induced (Batlle & Clevers, 2017). Thus the cancer stem cell niche can be seen as an environment enabling, in any member of a clone, drug resistance and the ability to evade immune surveillance, self renew and (re)populate. As niche-induced, these characteristics are plastic, and can be lost or gained according to access to, and characteristics of, the stem cell niche (Plaks *et al*). It therefore follows that niche manipulation may hold the key to the eradication of residual resistant disease. The

immense pharmacological potential held in tumour-niche interactions and signalling networks lies in causing their disruption to a degree where a survival advantage is no longer afforded; thereby exposing protected dormant cells, restoring chemosensitivity and precipitating immune-mediated destruction, whilst allowing normal tissue function to continue.

A tumour that metastasizes through bone has usually been rendered incurable. As a cancer niche, specific areas of the bone marrow and bone surface lining afford drug resistance (Meads *et al*, 2008) and protection for stem cell quiescence, whilst still encouraging outgrowth of the bulk (Wang *et al*, 2015). As the cradle of the haematopoietic system, the bone marrow (BM) has evolved to support the growth of rapidly dividing cells, so it is perhaps unsurprising that a tumour cell with a capacity for clonal renewal would have the potential to thrive on arrival in such a niche. It is a mixed but highly spatially organized tissue comprising bone and fibrous extracellular matrices, mesenchymal stem cells, fibroblasts, cells committed to osteoblast or adipocyte fate, mature osteoblasts, adipocytes and osteocytes, as well as dense vascularity with adapted endothelial cells and innervation, and cells of haematopoietic origin including macrophages, osteoclasts, and T cells. To effectively hijack such an environment to maximum capacity however, mere proliferation is insufficient; the tumour must also suppress the features that keep normal checks on the proliferation and differentiation of the haematopoietic system usually nursed here, the cells whose role it is to control growth, maintain quiescence or exert immune-mediated control (Fig. 1.7). Therefore tumours destroy where haematopoiesis does not; bone matrix is lysed or laid down inefficiently; normal haematopoietic development is suppressed and immune mediation disrupted. Abnormal angiogenesis disrupts oxygen tension gradients and stromal components are harnessed to proliferate, apoptose, or alter their expression patterns.

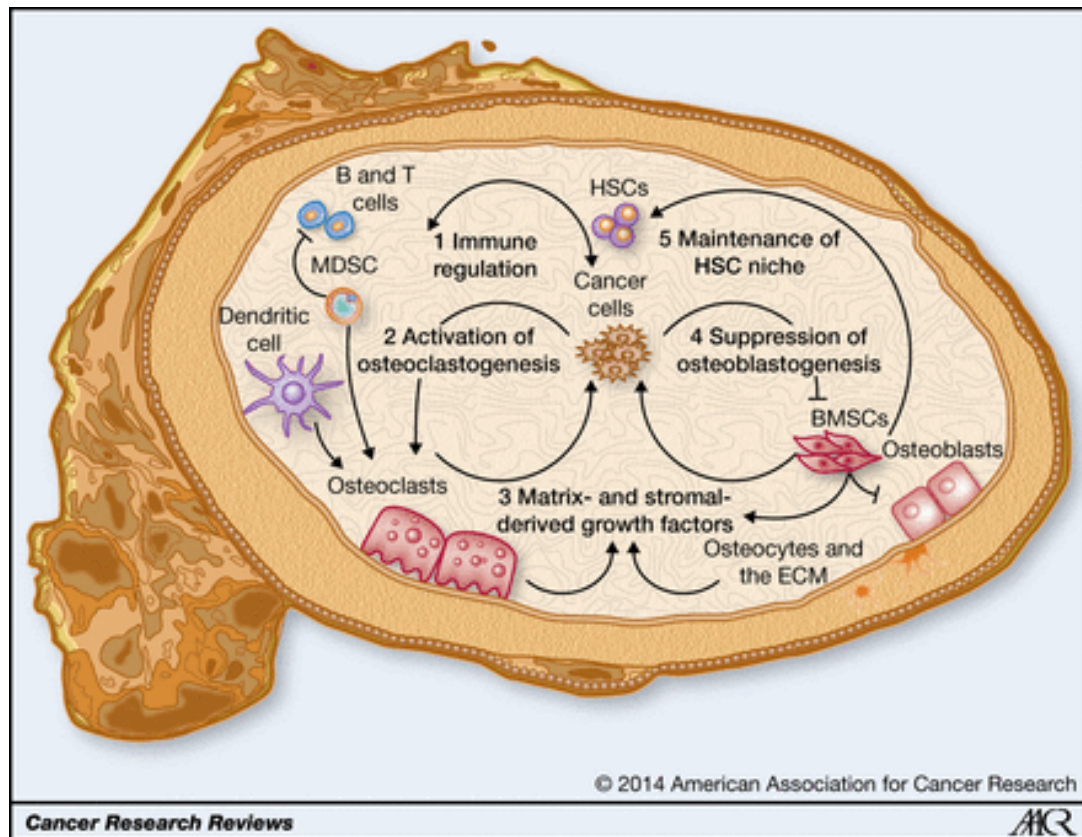


Figure 1.7: Host–tumour interactions within the bone marrow microenvironment.

Mechanisms by which specialized cell types within the bone marrow can support tumour growth: 1: regulation of the immune system. Immune cells have been shown to have both positive and negative effects on osteoclast formation and activity, and to suppress tumour growth, whereas MDSCs are increased in bone metastases and contribute to both immune suppression and osteoclastic bone disease. 2: activation of osteoclastogenesis. Immune cells, dendritic cells, and MDSCs all contribute to osteolytic bone disease, at least in vitro, mediated by RANKL, IL-17, and TNF- α . 3: matrix- and stromal-derived growth factors. The host microenvironment is an essential source of tumour growth factors, including TGF- β , IGF-1, and IL-6. 4: regulation of osteoblastogenesis. Osteoblast formation in cancer-induced bone disease is tightly controlled by tumour and stromal cells, mediated by factors including Dkk1, activin A, BMPs, and noggin. 5: hematopoietic stem cell niche components. The endosteal HSC niche is maintained by osteoblasts and stromal cells, and controlled by factors including GCSF and CXCL12.

Reprinted from Cancer Research, 2014, **74**, 1625-1631, Olechnowicz, SW & Edwards, CM, Contributions of the host microenvironment to cancer-induced bone disease, with permission from AACR

Multiple myeloma has a remarkable ability to manipulate its environment to optimise its own survival (Kumar *et al*, 2017). The irreversible alteration of microenvironment components is the root of many of the pathological features discussed above. The lytic bone disease and hypercalcaemia are caused by disruption of bone homeostasis; the anaemia, fatigue and weight loss are due to high levels of inflammatory cytokines and haematopoietic

suppression; recurrent infections are due to immunosuppression. All result from a manipulated bone marrow niche.

1.2.2 Myeloma-stromal cell interactions

In myeloma bone disease, the differentiation and survival of osteoblasts, which have a role in slowing tumour growth (Lawson *et al*, 2015a), is reduced, but their precursors mesenchymal stem cells (MSCs), which support myeloma growth, are expanded (Noll *et al*, 2014b). It has been shown that experimental infiltration of myeloma-exposed bone marrow stromal cells (BMSCs) can modify a healthy, non-permissive bone marrow microenvironment to render it permissive to myeloma development (Fowler *et al*, 2012). Although *in vitro*, either primary BMSCs or stromal cell line contact support myeloma growth, myeloma-exposed and modified BMSCs provide better stromal support than non-exposed (de la Puente *et al*, 2016), and are transcriptionally altered by the tumour contact (Dotterweich, Julia *et al*, 2016; Garcia-Gomez *et al*, 2014). These modified BMSCs promote myeloma proliferation and reduce apoptosis through the production of certain adhesion molecules, cytokines and exosomes (Fowler *et al*, 2012; Reagan & Ghobrial, 2012; Roccaro *et al*, 2013). Both myeloma cells and the BMSCs to which they adhere produce exosomes, containing mRNAs, miRNAs and proteins, which are transferred between the cell types. The exosomes of normal BMSCs are tumour suppressive, however in myeloma-BMSCs their content is altered to be tumour supportive (Roccaro *et al*, 2013), and mediate chemoresistance (Wang, 2014). No therapy yet exists that targets exosome release, but such an agent could potentially disrupt such tumour-niche interaction. A host of adhesion molecules mediate the binding of myeloma cells to stromal cells, including VCAM-1, VLA-4 and CD44. CD44 expression has been linked to stemness and drug resistance, and its blockade has been proposed as a mechanism for restoring drug sensitivity (Bjorklund *et al*, 2014; Canella *et al*, 2015).

Particular signalling pathways are consistently implicated in the relationship between myeloma and bone marrow stromal cells. These include the CXCL12-CXCR4 stem cell-stromal homing interaction, the proangiogenic HIF-1/VEGF pathway, IL6 and MIP-1 α , osteoclast activation mediated by RANKL, and the developmental pathways Wnt, TGF β , Hedgehog and Notch (Noll *et al*, 2012; Olechnowicz & Edwards, 2014). Putative drug targets to subvert niche-tumour support have included IL6, although neutralizing antibody siltuximab has recently not improved outcome in a combination therapy trial (San-Miguel *et al*, 2014), and VEGF inhibition, although bevacizumab has reached phase 2 trials (White *et al*, 2013), and unlike in some other malignancies, also showed no benefit. Bone marrow adipocytes and secreted adipokines may also support myeloma growth (Falank *et al*, 2016), especially noting the observation that a high-fat diet created a myeloma-permissive microenvironment reminiscent of MGUS (Lwin *et al*, 2015). This is supported by clinical data showing obesity is linked to a higher incidence of myeloma.

TGF β , and TGF β superfamily member Activin A are overexpressed by myeloma BMSCs, increased in the bone matrix in myeloma and other bone-invading malignancies, and therefore released from the matrix when it is lysed. Both contribute to inflammatory cell recruitment and have roles in osteoblast dysfunction. TGF β inhibition restored osteoblast function and reduced disease burden in a murine myeloma model (Takeuchi *et al*, 2010). Sotatercept, an Activin receptor type IIA (ActRIIA) ligand trap that binds Activin A/B and other TGF β super-family members, has been shown to improve osteolytic bone disease and anaemia in myeloma (Abdulkadyrov *et al*, 2014).

1.2.3 Tumour homing to the niche

In health, post-germinal centre plasmablast B cells accurately home to the bone marrow, there completing their maturation to plasma cells, to reside long term. This residency, as

that of haematopoietic stem cells (HSCs) (Ding & Morrison, 2013; Greenbaum *et al*, 2013; Hanoun & Frenette, 2013), is dependent on CXCL12 signalling from specialized stromal cells (Tokoyoda *et al*, 2004), and plasma cells continue to require contact with marrow CXCL12^{hi} reticular cells for survival, although other cell types are also required (Kometani & Kurosaki, 2015). Plasma cells without the CXCL12 receptor CXCR4 cannot home (Hargreaves *et al*, 2001). In keeping with their cell of origin, myeloma cells express high levels of CXCR4 and require stromal expression of CXCL12 for homing and niche maintenance (Alsayed *et al*, 2007). There is accumulating evidence for the potential of targeting CXCR4. A CXCR4 antagonist plerixafor (AMD3100), clinically used for HSC mobilization, has been proposed as a means of inducing chemosensitivity in myeloma (Azab *et al*, 2009). In addition, the CXCR4-neutralising antibody ulocuplumab has been shown to reduce disease dissemination in mice (Roccaro *et al*, 2015).

The interaction between bone marrow endothelial cell eCyPA and CD147 on myeloma cells has also recently been proposed as a key homing mechanism in myeloma, with an antibody to CD147 recently shown in murine models to abrogate homing, and have anti-MM activity (Zhu *et al*, 2015).

The bone marrow has long been presumed to be hypoxic, due to low perfusion and activation of hypoxia pathways, and this has recently been confirmed on direct measurement (Spencer *et al*, 2014; Winkler *et al*, 2010). Hypoxia benefits tumour invasion; it has been shown to promote both the epithelial to mesenchymal transition (EMT) required for bone invasion, and stem cell maintenance. Both effects are described in myeloma (Azab *et al*, 2012; Ikeda *et al*, 2017). Inhibition of HIF -1 α signalling is in clinical trials in other malignancies, but there are no reports of its success in myeloma to date.

1.2.4 Myeloma and the endosteal niche

Recent evidence suggests that there may be a subtle difference in the precise niche of the dormant (possibly the ‘cancer-propagating’) myeloma fraction compared to that of either pluripotent haematopoietic stem cells (HSCs) or healthy plasma cells, with non-dividing myeloma cells being located on the endosteal surface, in contact with mature osteoblasts which have relatively lower CXCL12 expression (Chen *et al*, 2014; Lawson *et al*, 2015a) (Hanoun & Frenette, 2013). A ‘purpose’ of the bone lining niche disruption caused by myeloma cells has been proposed to be that of niche-modification for deregulation of the dormant tumour fraction, in favour of proliferation (Croucher *et al*, 2016). It has been shown *in vivo* that the myeloma cells in contact with an intact BLC layer not only enter dormancy, but also exhibit a reversible drug resistance (Chen *et al*, 2014; Lawson *et al*, 2015a) and may therefore be the cell fraction during disease remission later responsible for relapse. In contrast, during phases of active disease proliferation, osteoclast disruption of this layer mobilises dormant adherent myeloma cells so allowing them to proliferate and lead to disease relapse. It could be argued that either pharmacological mobilization (to enhance chemosensitivity) or protection (to maintain dormancy) of these quiescent cells could have clinical benefit. The molecular nature of the interactions between myeloma and bone cells will be discussed in section 1.3.

1.2.5 The immune cell microenvironment of myeloma

The healthy bone marrow hosts varied immune system components, one of the functions of which is to recognise and destroy abnormal or malignant cells. This tumour suppressive environment must be overcome for tumours to proliferate, and evaded for tumour stem cells to survive. Macrophages, NK cells and cytotoxic T lymphocytes should recognize and initiate destruction of abnormal clonal proliferations. Myeloma, like other successfully

invading tumours, has been shown to employ many mechanisms to escape recognition by these cells. The mechanisms are both cell intrinsic, such as downregulation of surface proteins that engage immune recognition, and environment modifying, for example enlisting the support of immunosuppressive cells such as myeloid derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs) and T-regs, by inflammatory cytokine secretions (Guillerey *et al*, 2016; Kawano *et al*, 2015). It has been suggested that a key feature of the progression from MGUS to myeloma may be the transition from a tumour suppressive to a tumour promoting microenvironment (Bernal *et al*, 2009; Jinushi *et al*, 2008).

Several therapies, summarised in Fig. 1.8, have been developed or are under development that re-engage normal immune recognition and target destruction of cancer cells, in addition to the monoclonal antibodies and CAR-T cells already discussed in section 1.1.4. Lenalidomide and other IMiDs may modulate TAM activation, reduce MSDCs, reduce T-reg activity and stimulate NK cells. All these effects should enhance anti-tumour immunity, but it has been noted that the standard co-administration of dexamethasone blunts the beneficial NK cell stimulation (Hsu *et al*, 2010). Myeloma-induced downregulation of plasmacytoid dendritic cells' (pDCs) ability to present tumour antigen is another tumour immune evasion mechanism described: TLR-9 agonist C792 reactivates pDCs and enhances their anti-tumour effect. PDCs pre-treated with C792 significantly reduced tumour burden *in vivo* (Ray *et al*, 2014).

An immunomodulatory pathway attracting significant attention currently in anti-cancer therapy development is PD-1/PD-L1. Tumour cells express PD-L1, which binds PD-1 on T, NK and B cells, repressing CTL and NK cell-mediated killing. PD-L1 appears to be enriched in minimal residual disease (MRD) positive and relapsed myeloma disease (Paiva *et al*, 2015); myeloma microenvironment T cells express higher levels of PD-1 than normal BM. Clinical

trials of agents blocking this interaction have shown activity in many malignancies. However at the time of writing, clinical trials of three different PD-1 or PD-L1 inhibitors in myeloma, pembrolizumab, nivolumab and durvalumab, have recently been halted because of excess deaths in patients treated with pembrolizumab vs controls in phase III trials, for reasons not yet clear (Reuters, 2017). Therefore the future of these drugs in myeloma remains to be determined.

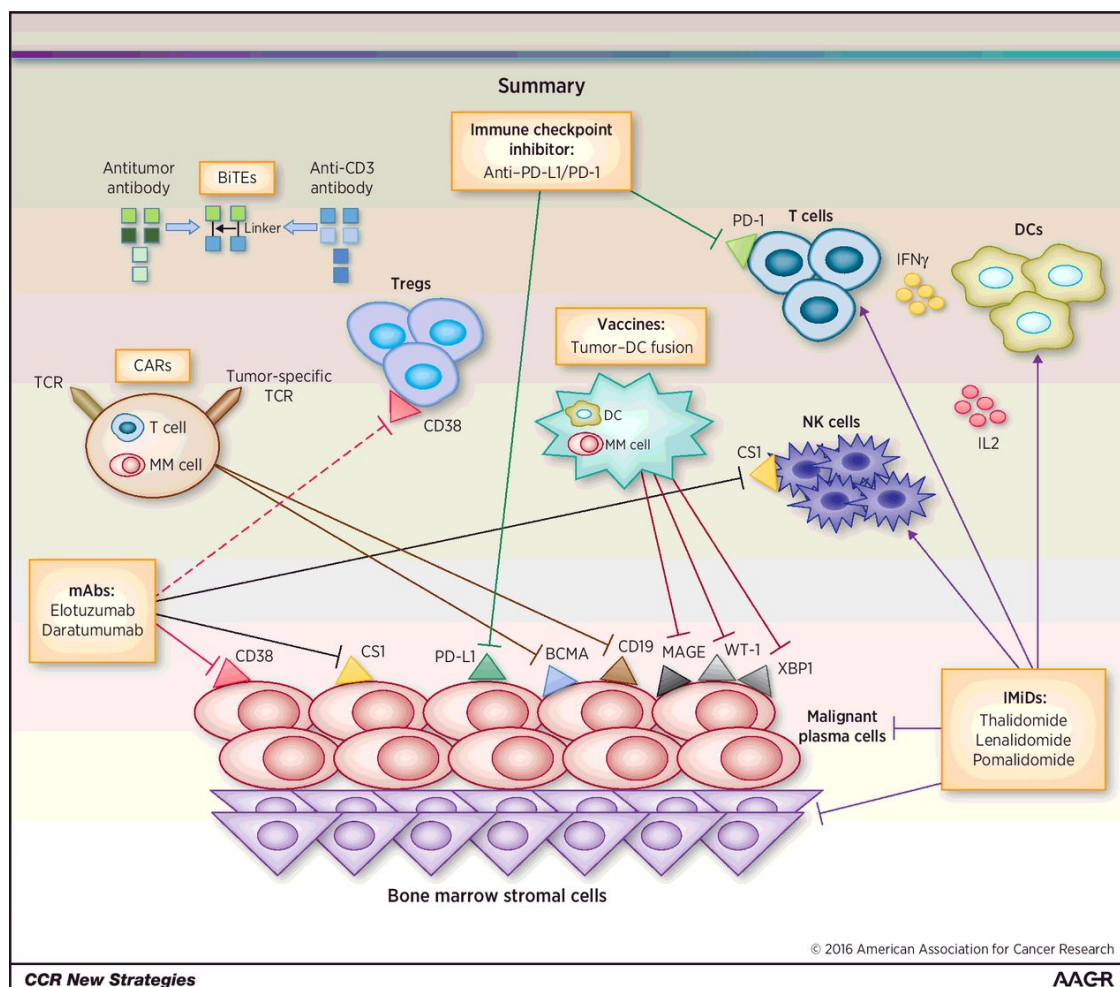


Figure 1.8: Overview of novel immunotherapeutic approaches in multiple myeloma.

Shown here are the cellular components of the bone marrow microenvironment and host immunity, and the novel immunotherapeutic targets that they present.

Reprinted from Clin Cancer Res, 'New Strategies in Multiple Myeloma: Immunotherapy as a Novel Approach to Treat Patients with Multiple Myeloma', 2016, (22) (24) 5959-5965, Neri, P. *et al*, with permission from AACR. (Neri *et al*, 2016)

1.3 Myeloma bone disease

Most myeloma patients (80-90%) either present with bone disease or develop it at some point during disease progression (Silbermann & Roodman, 2013). The characteristic lytic lesions result from an increase in uncoupled local bone remodelling, leading to the destruction of often-large areas of mature bone. Clinically, it is defined as the presence of osteolytic bone lesions or the presence of osteoporosis with compression fractures attributable to the underlying clonal plasma cell disorder (Rajkumar *et al*, 2014). These lesions cause the commonest myeloma symptom, bone pain, and can present with pathological fractures, spinal collapse or cord compression, and hypercalcaemia (Panaroni *et al*, 2017). It is standard practise to treat myeloma sufferers with bisphosphonate or equivalent therapies to protect against further bone loss, and myeloma therapies themselves halt the progress of bone destruction while they retain anti-tumour activity. In the case of proteasome inhibitors, there is some direct bone-anabolic effect (Mukherjee *et al*, 2008; Qiang *et al*, 2009; Zangari & Suva, 2016). However, at each disease relapse, if tumour growth is not halted promptly, many patients will have a return of their bone pain and progression of their lesions. No currently used therapy leads to significant replacement of the lost bone or repair of the damage with which many patients present. The battle against bone disease progression therefore currently lacks truly satisfactory tools.

The basis of myeloma bone disease lies in an expansion in number and activity of osteoclasts, coupled to maturation arrest and reduction in osteoblast numbers, leading to the normal balance between bone loss and bone formation being tipped decisively towards loss (Panaroni *et al*, 2017). This section will now consider the specific ways in which myeloma invasion impacts healthy bone homeostasis.

1.3.1 Healthy bone homeostasis

From foetal development through to early adulthood, bones grow and change continually and have huge remodelling capacity. Once adulthood is reached, bone cell homeostasis must alter to achieve maintenance rather than growth. While retaining the ability to repair what is lost or broken and respond to changes in body weight and use, overall bone mass and strength should remain constant. Maintenance requires a different cellular behaviour to that required for growth, with a requirement for highly subtle modulation and balance of signalling, so allowing matrix repair, but preventing its deposition in excess.

Bone homeostasis requires the ability to lay down new matrix, and to remodel existing matrix. Hence osteoblasts making bone matrix, and osteoclasts degrading it, must be balanced in their activities. The majority (approximately 80%) of trabecular and endocortical bone surfaces are in adulthood normally lined with quiescent 'bone lining cells' (Hauge *et al*, 2001) (BLCs), which differentiate from skeletal stem cells just like osteoblasts, and can become osteoblasts if required. Skeletal stem cells vary in origin, commitment and location at different stages of development (Ono & Kronenberg, 2016), but in adult long bones originate from LepR⁺ BMSCs that also express Prrx1 (Yue *et al*, 2016). Interestingly, the ability of these cells to become osteoblasts is determined by (lack of) exposure to the hormone Leptin, which is made by adipocytes and promotes adipocyte over osteoblast differentiation, an example of the negative reciprocity between osteo- and adipogenesis. When bone formation or repair is required, BLCs become activated to matrix-secreting osteoblasts (Kim *et al*, 2017; Kim *et al*, 2012) as part of the 'bone remodelling compartment' (Fig. 1.9) (Croucher *et al*, 2016).

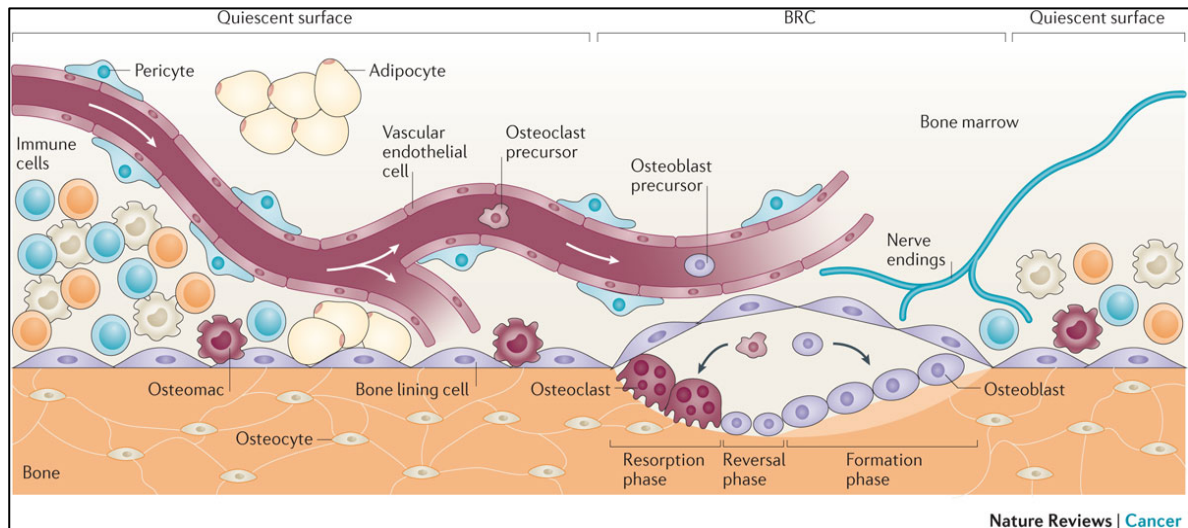
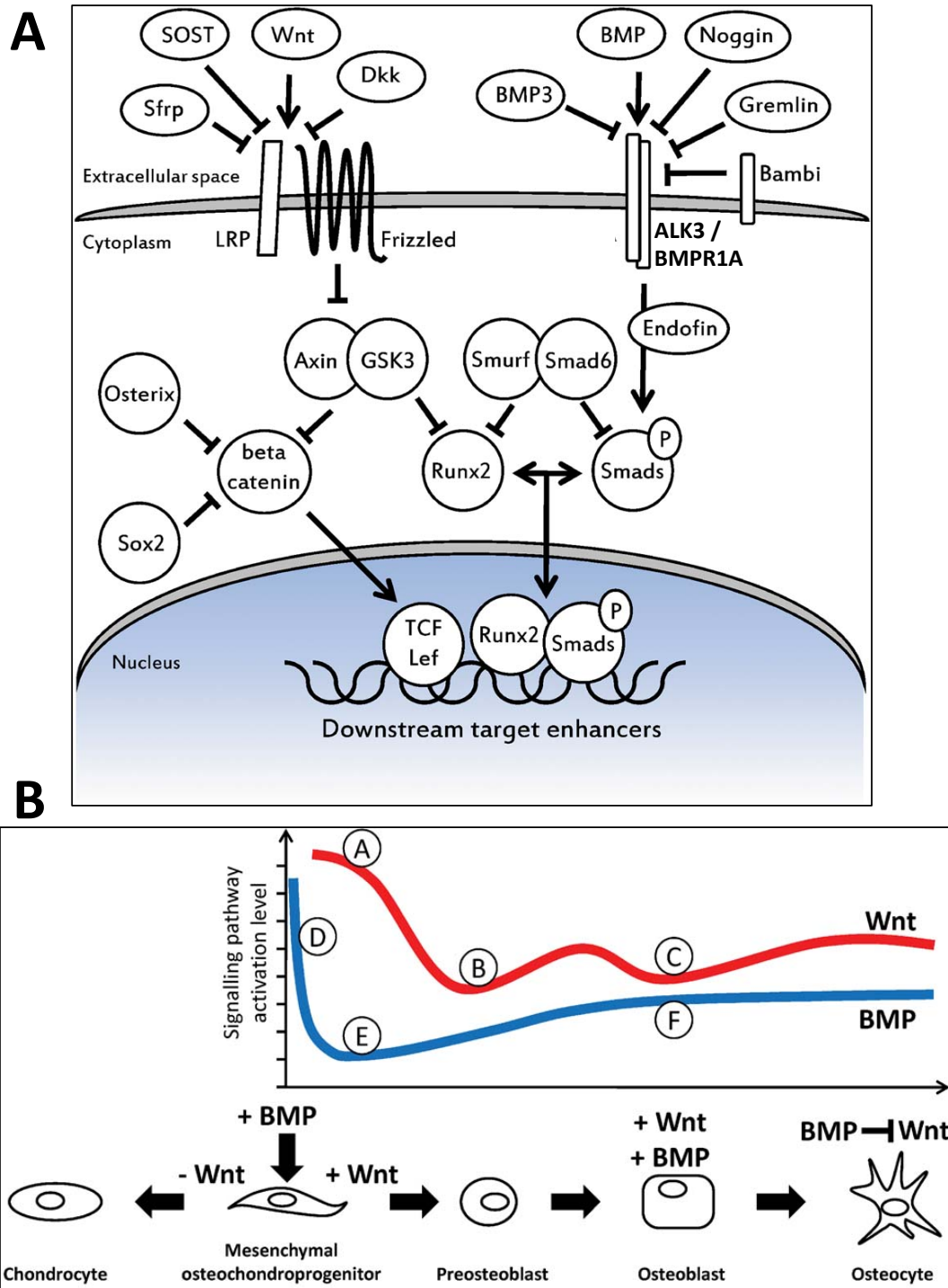


Figure 1.9: The endosteal bone surface in quiescence and remodelling

The majority (approximately 80%) of the endosteal bone surface is quiescent and covered by bone lining cells, adipocytes, osteomacs, cells of the immune system and neurons. Blood vessels, lined by vascular endothelial cells and pericytes, supply the quiescent bone surface. A minority of the endosteal surface (approximately 20%) undergoes active bone remodelling at any one time. This occurs in the bone remodelling compartment (BRC), which is separated from the bone marrow compartment by a canopy of bone lining cells or cells of the myeloid lineage. Adjacent to the BRC is a network of capillary blood vessels, which can provide nutrients and oxygenation. They contain osteoclast precursors and early cells of the osteogenic lineage that can differentiate into functional bone-resorbing osteoclasts and bone matrix-synthesizing osteoblasts, respectively (see text for details). The events leading to activation of bone remodelling and the formation of a BRC are complex and probably under the control of osteocytes embedded in the bone matrix.

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Osteoblast differentiation and maintenance critically depends on the co-ordinated activation of BMP and Wnt signalling pathways (Fig 1.10). Both pathways are required for osteogenesis but with dominance at different stages of development, exerting reciprocal control on the other, so achieving precise regulation of a cell's progression along the path of differentiation from progenitor to osteocyte (Baron & Kneissel, 2013; Genetos *et al*, 2010; Lin & Hankenson, 2011).



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<http://onlinelibrary.wiley.com/doi/10.1002/bies.201200061/full#fig1>

Figure 1.10: Control of osteogenesis by the canonical Wnt and BMP pathways *in vivo*

A: Simplified diagram of Wnt and BMP components relevant to their crosstalk in osteoblast differentiation. Wnt signalling stabilises B catenin and Runx2, allowing transcription of an osteoblast transcriptional program. P-Smad binding is required with Runx2 at osteoblast transcriptional enhancers, so Runx2 functions as an integrative hub for both pathways.

B: The relative activation level of the Wnt (red) and BMP (blue) pathways, at different steps of osteogenesis (NB level not comparative between pathways). A substantial activation of the Wnt pathway is required to drive osteochondroprogenitors towards the osteoblastic lineage (A), but its

activity must be negatively regulated twice, independently, by Sox2 and Osterix, to allow efficient proliferation (B) and differentiation (C), respectively. The BMP pathway is required to induce the osteochondroprogenitor fate (D), but its function regarding the acquisition of an osteoblastic fate is still under debate (E). At later steps, the BMP pathway positively contributes to the proliferation and differentiation of osteoblastic cells (F).

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Bone remodelling is performed by osteoclasts, which develop by fusion of modified monocytic-lineage cells into large, multinucleated cells that secrete acid to demineralise and digestive, proteolytic enzymes to degrade collagen in bone surfaces targeted for remodelling (Cappariello *et al*, 2014). Their recruitment and function is dependant on RANKL and OPG, secreted by osteoblasts and osteocytes, and the ratio between them; RANKL binds the osteoclast precursor cell receptor RANK to stimulate NF- κ B signalling and precipitate osteoclast development; OPG is a soluble decoy receptor for RANKL and prevents its binding and signalling. Osteoclasts are also extremely sensitive to inflammatory stimuli; for example, their formation requires MCSF and is enhanced by inflammatory mediators such as MIP-1 α (Fowler *et al*, 2011a) and GDF15 (Westhrin *et al*, 2015), although experimental data is often conflicting, with different responses to inflammatory mediators in homeostatic and pathological conditions (Brunetti *et al*, 2011). In keeping with their haematopoietic origins meaning they are essentially giant, specialised macrophages, osteoclasts also have roles in immunomodulation and function as antigen presenting cells. They have been found to stimulate regulatory T cell formation (Kiesel *et al*, 2009).

Although difficulties in obtaining and culturing osteocytes means understanding of their importance has lagged, they are now known to be critical mediators of the integrated cross-talk governing bone homeostasis (Bellido, 2014), as well as calcium and phosphate homeostasis. They far outnumber all other types of bone cell and are embedded in calcified matrix, communicating by numerous lacunae through which chemical signals may be exchanged. Osteocyte-specific deletions of RANKL and the Wnt inhibitor sclerostin have

shown that levels of osteocyte production of these two molecules in particular link bone formation and bone degradation. Whereas RANKL mediates osteoclast function, the main role of sclerostin is in the inhibition of osteoblast differentiation, dependant on active Wnt signalling as described above. This is demonstrated by rare inherited deficiency of sclerostin expression in humans, Von Buchem disease (sclerosteosis), with a phenotype of high bone mass (Balemans *et al*, 2001), and is recapitulated in mice with *Sost* deletion. The opposite, low bone mass, is seen in mice with *Sost* overexpression. By inhibiting the canonical Wnt signalling driving osteoblast differentiation from MSCs, sclerostin halts both the formation and maintenance of new osteoblasts (Delgado-Calle *et al*, 2016b) and the reactivation of bone lining cells (Kim *et al*, 2017). Anti-sclerostin antibodies have been shown to not only reverse bone loss but also to have an anabolic effect of bone gain in osteoporosis in clinical trials (Saag *et al*). Dkk1 is another Wnt inhibitor that has been shown to have a similar role in reducing bone formation, and is also produced by osteoblasts and osteocytes. Although its loss is not associated with such a dramatic phenotype, reduced expression in mice is also associated with increased bone mass (Pinzone *et al*, 2009). The control of osteocyte-secreted Sclerostin and Dkk1 is another example of Wnt and BMP reciprocal crosstalk; deletion of BMP receptor BMPRIa under osteoblast or osteocyte promoters has shown that BMP signalling controls expression of both Wnt inhibitors (Kamiya *et al*, 2010; Kamiya *et al*, 2016).

The RANKL/OPG ratio is also influenced by this crosstalk: their expression is tightly controlled as one means by which osteocytes regulate bone remodelling. Wnt signalling promotes OPG expression, so inhibiting osteoclastogenesis. By inhibiting Wnt signalling, sclerostin reverses this (Glass *et al*, 2005). BMP signalling by contrast, directly promotes osteoclastogenesis (Huntley *et al*, 2015), but is also reported to promote RANKL expression in osteocytes (Kamiya *et al*, 2016).

1.3.2 Enhanced osteoclastogenesis in myeloma

As described above, osteoclast formation is dependent upon available RANKL, the level of which is determined by the local RANKL: OPG ratio. The expression level of both these factors, secreted by osteocytes, osteoblasts and stromal cells, is tightly controlled. On myeloma contact with these cells RANKL secretion is enhanced, and OPG reduced: hence osteoclast formation increases. Increased inflammatory mediators in the myeloma microenvironment, such as TNF α , IL6, IL3, IL7, MIP-1 α , and GDF15, both stimulate RANKL production and enhance osteoclastogenesis directly (Fowler *et al*, 2011a). Cytokines and RANKL produced by T cells recruited to the tumour niche add to the pro-inflammatory environment. The combined effect is that resorptive function increases, as osteoclasts home to the endosteal surface and destroy both bone lining and matrix (Mansour *et al*, 2017). In myeloma, the inflammation also results in the increased recruitment of other monocyte-lineage cells; MDSCs and macrophages can represent osteoclast precursors in this environment (Asimakopoulos *et al*, 2013). Osteoclasts from myeloma patients have also recently been shown to modulate the immune microenvironment by protecting myeloma cells from T cell-mediated toxicity. They upregulate PD-L1, CD200, Galectin-9 and HVEM, all of which inhibit cytotoxic T cells, and APRIL, which induces higher PD-L1 expression in myeloma cells. All of this contributes to the defective T cell function in myeloma, allowing tumour escape from immune surveillance (An *et al*, 2016).

1.3.3 The effect of myeloma on osteoblast differentiation and bone formation

In the presence of active myeloma, the BLC layer is highly disrupted. Apoptosis is induced in differentiated cells already present, including BLCs, osteoblasts and osteocytes, and their replacement by maturing precursors is blocked. BLCs that survive are unable to activate to repair damage. The differentiation block causing the observed MSC/osteoblast imbalance

involves a host of transcriptional changes, and deregulation of the growth factors and adhesion molecules discussed above. It is reported to be mediated by Notch (Delgado-Calle *et al*, 2016a), Wnt (Edwards *et al*, 2008; Tian *et al*, 2003) and TGF β signalling pathways (Matsumoto & Abe, 2011; Nyman *et al*, 2016), among others (Toscani *et al*, 2015). These three are 'developmental pathways' of the early embryo, with roles in patterning and tissue commitment, controlling division versus dormancy and differentiation versus stemness, and are heavily implicated in the deregulated signalling in metastatic cancer (Giancotti, 2013; Sethi & Kang, 2011) that lead to niche alteration and invasion. Myeloma-induced Notch pathway activity in MSCs leads to IL6, VEGF and IGF-1 secretion, as well as deregulated adhesion, migration and bone resorption (Colombo *et al*, 2013). Drugs inhibiting Notch signalling, of which γ -secretase inhibitors are furthest developed, have activity in preclinical myeloma models (Mirandola *et al*, 2013) and have been clinically trialled in other malignancies (Yuan *et al*, 2015). Activin A has also been implicated in osteoblast differentiation block (Chantry *et al*, 2010; Scullen *et al*, 2013; Vallet *et al*, 2010). In recent years, Wnt inhibitors Dkk1 and sclerostin, discussed above to control osteoblast differentiation during normal adult bone homeostasis, have been shown to be present in high levels in the serum and bone marrow of myeloma patients, and their inhibition to improve myeloma bone disease pre-clinically (Eda *et al*, 2016; Heath *et al*, 2009; Reagan *et al*, 2015), although the anti-Dkk1 antibody BHQ880 did not significantly improve bone disease in a phase 1b study (Iyer *et al*, 2014).

Wnt and BMP pathway interactions in bone homeostasis have already been discussed. In some other cancers, notably demonstrated in colon cancer, a disruption of the reciprocal signalling relationship between BMP and Wnt signalling is a neoplastic event; where in health the inhibitors of one pathway both allow activity of, and are controlled by the other, thus allowing a tight balance between differentiation and renewal, in cancer the

deregulation of spatial gradients of this relationship allow uncontrolled proliferation (Davis *et al*, 2015). It would therefore be a hypothesis with precedent rationale, that if osteoblast differentiation impairment in myeloma is attributed to excessive Wnt inhibition, BMP signalling may also be deregulated. However this has never been shown in myeloma bone disease.

1.3.4 Current clinical management of myeloma bone disease and potential future treatments

No treatment currently exists that can rebuild the bone lesions that occur in myeloma. Myeloma cells recruit high numbers of osteoclasts to their endosteal niche, and it is this process that is targeted by current clinical therapies in use in the treatment of myeloma bone disease. Bisphosphonates, which kill osteoclasts directly, have been used in myeloma for many years, and have been shown in meta-analysis to reduce vertebral fracture, skeletal related events, and bone pain. Zolendronic Acid may also increase overall survival (Mhaskar *et al*, 2017). More recently, the anti-RANKL antibody Denosumab has been shown in solid tumour bone metastases to be non-inferior to standard-of-care treatment zolendronic acid for prevention of skeletal events in bone metastases (Henry *et al*, 2011). However in this mixed malignancy trial the myeloma cohort on denosumab had an unexpected higher mortality; repeat phase III trials are underway (NCT01345019). Newer drugs are still under investigation. The clinically approved glucosylceramide synthase inhibitor N-butyl-deoxynojirimycin (NB-DNJ), which inhibits RANKL-mediated osteoclast recruitment through lipid membrane synthesis disruption, has shown efficacy in myeloma osteolytic bone disease *in vivo* (Ersek *et al*, 2015). It is already in clinical use as miglustat for Gaucher disease, where it improves osteoporosis.

To effectively treat myeloma bone disease, not only should osteoclastogenesis be inhibited, but also the osteoblast differentiation block should be reversed and a bone anabolic effect produced. To this end, inhibitors of the Wnt antagonists Dkk1 and Sclerostin have been in development, although they do not have a marked inhibitory effect on osteoclast function. Anti-DKK1 antibody BHQ880 has undergone Phase 1b clinical trial in myeloma with possible benefit to bone density demonstrated (Iyer *et al*, 2014), but no reduction in fracture risk. Anti-Sclerostin antibody romosozumab has shown efficacy in improving bone mineral density in post-menopausal women (McClung *et al*, 2014), and in a mouse model of myeloma (McDonald *et al*, 2017), but has not yet entered clinical trials in myeloma.

An optimal drug targeting the metastatic bone niche would have dual activity, on tumour cells and the microenvironment, leading to both direct and indirect anti-tumour effects. One such class, a mainstay of myeloma treatment, is proteasome inhibitors: with direct tumour toxicity, proteasome inhibitors also stabilize β -catenin in pre-osteoblasts, so enhancing osteoblastogenesis and negatively regulating RANKL (Qiang *et al*, 2009). More recent examples of this dual activity include BTK inhibitors such as ibrutinib, which not only targets myeloma cells but also inhibits osteoclast activity and stromal cell growth factor secretion (Tai *et al*, 2012), and Pim2 kinase inhibitors, targeting Pim2 in tumour cells but also in osteoblasts, where it is a negative regulator of osteoblastogenesis (Keane *et al*, 2015). Phase II clinical trials of ibrutinib (Chari *et al*, 2015; Richardson *et al*, 2015) and LGH447 (Raab *et al*, 2014), a pan-Pim inhibitor, are ongoing in myeloma.

1.4 Bone Morphogenetic Protein (BMP) Signalling

As the BMP pathway forms the major investigational focus in this project, it will be introduced in more detail here. Its role in bone homeostasis has been introduced in section 1.3 and will again be addressed in chapter 4 (section 4.1.3) so will not be repeated here.

1.4.1 Introduction to the BMP signalling cascade

Bone morphogenetic proteins (BMPs), members of the TGF β superfamily, have fundamental roles in basic embryo patterning, cell proliferation and fate commitment, angiogenesis and bone formation. They are increasingly recognized to influence proliferation vs. differentiation decisions in a variety of tumours, with active BMP pathway signalling favouring quiescence and differentiation over proliferation (Giancotti, 2013; Wakefield & Hill, 2013). They were first described for their osteoinductive properties (Urist, 2002); BMP-controlled transcription of critical osteoblast differentiation genes such as Runx2 and osterix is demonstrated by the failure of skeletal development in bone and/or cartilage specific embryonic knockouts of pathway components (Chen *et al*, 2012). They are also pivotal for control of iron homeostasis, including the restriction of iron in inflammatory anaemias (Steinbicker *et al*, 2011b) (see below), and for regulation of muscle mass (Sartori *et al*, 2013), potentially implicating them in cancer cachexia.

BMPs usually bind receptors as dimers, with receptors comprised of two Type 1 receptor subunits and two Type 2 subunits (Fig. 1.11). Ligand and receptor subtype diversity, in addition to cofactors such as RGMs, pseudoreceptors such as BAMBI, and inhibitors such as follistatin, gremlin and noggin, as well as a range of inhibitory and regulatory cytoplasmic components and Smad-binding partners (for example, Smad6, Smad7, Evi1, Smurf proteins) enable enormous subtlety of signal transduction, despite the common Smad1/5/8

intracellular pathway. Smad1/5/8 phosphorylation, induced by ligand receptor binding, enables cofactor Smad4 binding and translocation to the nucleus where the Smad complex acts as a transcription factor, or to recruit chromatin-remodelling machinery for various genes. Smad6 and 7 inhibit the nuclear translocation of the phospho-Smad complex, and the intracellular components of other TGF β family member signalling, Smad2/3, (usually activated by TGF β or activin binding receptors), compete for the Smad4 co-factor. Despite the promiscuity of ligand-receptor interactions, where many ligands can apparently bind many receptors with similar affinities, data indicates that related ligands can exhibit highly variable agonist activity for a single receptor. How this is achieved is one of the poorly understood aspects of BMP signalling. For example, ligands such as BMPs and activins can compete for binding; if one of them may initiate signal transmission on binding and the other does not, this could determine response specificity according to thresholds of signal intensity reached in a particular cell, which in turn will have been determined by the density ratio of one extracellular ligand present to another (Aykul & Martinez-Hackert, 2016).

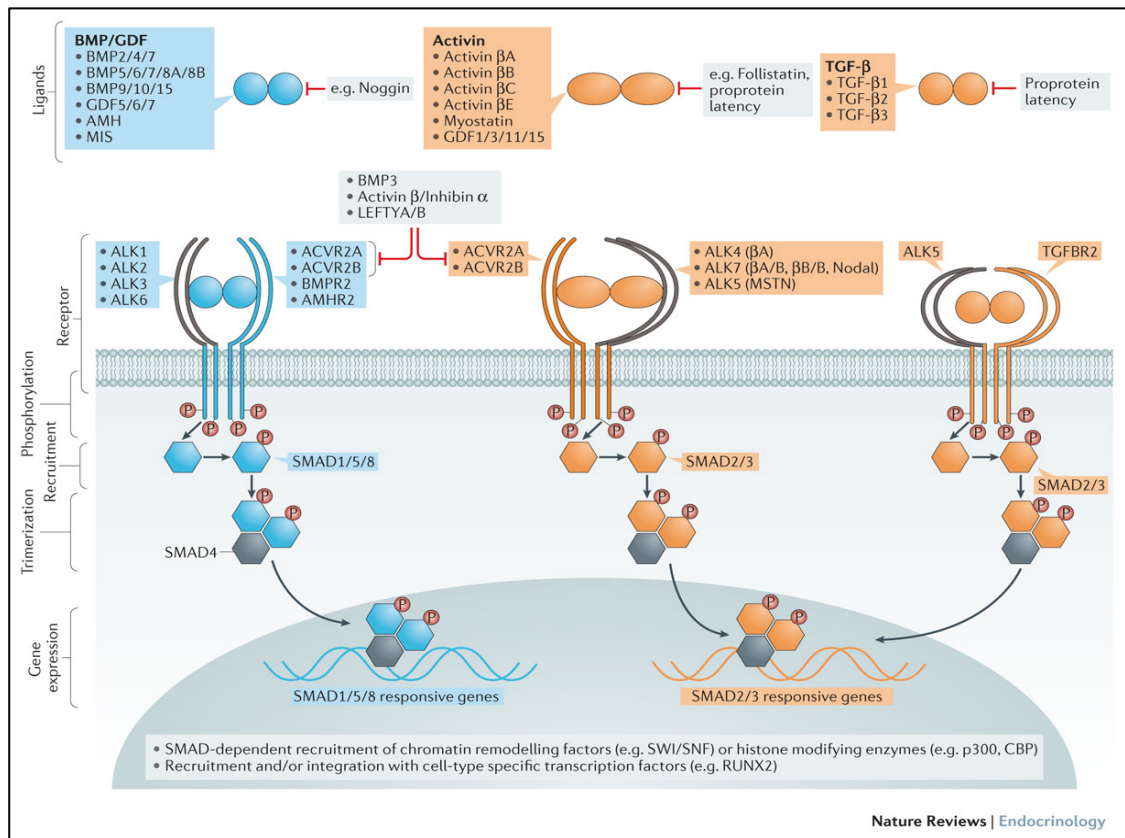


Figure 1.11: Fundamental mechanisms of canonical BMP superfamily signalling

BMPs signal through a multimeric cell surface complex consisting of two Type I receptors and two Type II receptors. Type I and type II BMP receptors are single pass transmembrane proteins with an intracellular serine/threonine kinase domain. After ligand binding, type II receptors phosphorylate (P) the type I receptors. Activated type I receptors recruit and phosphorylate pathway-specific R-SMADs (SMAD1, SMAD5 and SMAD8 (blue pathway), and SMAD2 and SMAD3 (orange pathway)), which can form trimers with SMAD4 and translocate to the nucleus. SMADs have intrinsic DNA-binding activity and are able to regulate gene expression by recruitment of chromatin-remodelling machinery and integration with tissue-specific transcription factors. SMAD8 is also known as SMAD9. The pathway can be antagonized by many mechanisms including neutralization of ligands by secreted traps such as noggin or follistatin, secretion of latent ligands bound to their propeptides, or via binding of receptors by non-signalling ligands such as BMP3, activin β/inhibin α dimers or LEFTY monomers.

Figure and adapted legend reprinted by permission from Nature Springer: Nature Reviews Endocrinology 12, 203-221. Salazar, VS, Gamer, LW, Rosen, V BMP signaling in skeletal development, disease and repair. Copyright 2016.

1.4.2 The role of BMPs in inflammatory anaemia and the anaemia of myeloma

Iron, required for haemoglobin synthesis, is absorbed from the gut, transported bound to transferrin or ferritin, and either used by cells, or stored in macrophages to be released when required for use in erythropoiesis. Once old red cells are degraded, haem is metabolised in the spleen and the liver, and the iron either recycled back into serum, utilised in the cell, stored in ferritin, or excreted in bile. This process is highly regulated;

firstly because adequate iron availability is so important for erythropoiesis and other cellular functions; secondly because unbound iron can generate highly toxic free radicals and must therefore be minimised and thirdly, because iron storage, and its corresponding removal from the circulation, is a mechanism evolved to protect the host against pathogen invasion, as nutrient iron availability is required for survival and growth of bacteria, viruses and protozoa (Drakesmith & Prentice, 2012). Iron homeostasis is regulated to achieve these aims by the hormone hepcidin. Hepcidin is synthesized by hepatocytes, predominantly under the control of BMP signalling. Hepcidin binds and causes degradation of the iron transporter ferroportin, so causing iron to be sequestered in macrophages, and preventing its absorption from the gut. In this way hepcidin prevents iron overload and limits iron availability to potential pathogens. Inflammatory stimuli, for example IL6, IL22 and type 1 interferon, by signalling through STAT3, modulate hepcidin expression in conjunction with BMP-Smad signalling, to ensure that hepcidin rises as part of the innate immune / inflammation response. If inflammation is chronic, persistently high hepcidin synthesis can result in the 'anaemia of inflammation' (AI): where functionally reduced iron availability limits erythropoiesis. When inflammation is not pathogen-induced, for example in cancer or autoimmune disease, this anaemia has no benefit, and is the cause of significant morbidity in chronic disease. Although anaemia in myeloma is multifactorial, with renal impairment and haematopoietic suppression often involved, hepcidin-mediated inflammatory anaemia is likely to be a prominent feature (Katodritou *et al*, 2009; Sharma *et al*, 2008).

The serum of myeloma patients has been shown to switch on the hepcidin promoter more than serum from control individuals; BMP2 was higher in myeloma serum, and the effect was abrogated by the addition of antiBMP2/4, suggesting BMP2 may be the mediator of hepcidin-mediated anaemia in myeloma (Maes *et al*, 2010). As has been shown with other

AI aetiologies (Steinbicker *et al*, 2011b), this leads to the hypothesis that BMP inhibition may have a role in mitigating anaemia in myeloma.

1.4.3 BMPs in myeloma

Findings reported in BMP signalling in myeloma tumour cells are discussed further in the introduction to chapter 4, but essentially are limited to observations that BMPs are toxic to myeloma cells *in vitro* (Hjertner *et al*, 2001; Holien *et al*, 2012; Kawamura *et al*, 2000), and certain ligands and receptors may be increased in myeloma marrow samples, particularly BMP6 (Grcevic *et al*, 2010; Seckinger *et al*, 2009). Myeloma BMP6 expression is proposed as a marker of better prognosis (Seckinger *et al*, 2009), and methylation of its promoter a marker of worse prognosis (Hashida *et al*, 2012). There is however to our knowledge, no understanding of the effect of myeloma on this signalling pathway *in vivo*, or in specific cells of the myeloma-bone marrow microenvironment.

1.5 Myeloma mouse models

Mouse models of disease are a basic preclinical tool. Diseases such as myeloma where pathogenesis relies on interactions of many cell types and tissues cannot be properly modelled *in vitro*, and drugs that have a particular activity on a tumour cell line *in vitro* may not achieve this effect *in vivo*. However, mouse models must recapitulate the features and cellular interactions of human disease if they are to enable correct conclusions to be drawn. For example, a tension exists between whether it is better to use primary human myeloma samples in a xenograft - but therefore requiring an immune deficient mouse and so failing to model tumour-immune interactions - or to use a fully murine tumour, either reliant on cell

lines, limited in their recapitulation of the heterogeneity of human disease, or at it's most accurate representation, reliant on mice spontaneously developing slowly progressive disease, logistically and financially limited in scope.

Radl *et al.* identified that 0.5% of ageing C57BL6/KaLwRij mice spontaneously develop myeloma (Radl *et al.*, 1988), developing the features of human myeloma including the associated bone disease. They have been shown to carry a mutation in *Samsn1*, likely to affect B cell development (Noll *et al.*, 2014a), but additionally have stromal defects, for example low adiponectin levels, that promote myeloma growth compared to the parent strain (Fowler *et al.*, 2011b). Myeloma, and associated osteolytic lesions, can be induced in synergistic young C57BL6/KaLwRij mice by inoculation of freshly isolated bone marrow cells from older mice with myeloma, also known as 5T Radl model or 5TMM murine model of myeloma bone disease. A further refinement of this model is the 5TGM1 cell line, derived from the 5T model but which can be cultured *in vitro* and inoculated back into young mice in precise quantities (Garrett *et al.*, 1997). However this cell line, like most others, represents a relatively aggressive tumour that leads to hindlimb paralysis within weeks, so is not suitable for study of MGUS-like states. Although the 5T-based model is the best characterised, another widely used syngeneic murine myeloma model is the Vk*Myc model, where *vk-Myc* is constitutively activated, meaning mice spontaneously but usually slowly develop an MGUS or myeloma-like disease over time (Chesi *et al.*, 2008).

Xenograft myeloma models require an immunodeficient strain, for example NSG (NOD/SCID/GAMMA), lacking functional B and T cells, and with low NK and innate immune function. In myeloma, such models have been widely used, both with human cell lines and primary samples, which delivers a varied disease phenotype (Lawson *et al.*, 2015b; Paton-Hough *et al.*, 2015). However, these models cannot be used to study tumour-immune

interactions, or relied upon to recapitulate all features of niche-tumour crosstalk. Recent developments in other cancers have allowed introduction of human immune systems into such immunodeficient mice to study tumour immunity, although the problem of GVHD generated by recognition of mouse antigens must be overcome. The deficient immune systems that allow cancers such as myeloma to develop ideally require successful xenograft of the autologous tumour, immune system and even stroma together, so that interactions promoted by defects in each component can be studied. Such models have been described in other tumours (Walsh *et al*, 2017), and recently also in myeloma as the MIS^(KI)-TRG6 model, which expresses six human growth factors, including IL6 to support myeloma cell growth, on an immunodeficient background (Das *et al*, 2016). The expression of these human growth factors enabled the growth not only of primary human myeloma samples but also normal human haematopoiesis, including innate immune cells, from the same donor. This enabled recapitulation of tumour-immune interactions.

Another approximation of a complete human microenvironment for tumour growth are models using ossicle or scaffold implants, where human tumour, mesenchymal and haematopoietic components can co-engraft local structures (Abarrategi *et al*, 2017; Reinisch *et al*, 2016). The closest approximations of such a technique in myeloma are the SCID-Hu, using foetal bone chips (Yaccoby *et al*, 2002), or SCID-Synth-Hu model (Calimeri *et al*, 2011), using a polymer scaffold structure, which are injected with primary whole myeloma bone marrow samples (including tumour, haematopoietic and stromal cells) and implanted subcutaneously in a SCID mouse. While neither model recapitulates myeloma bone destruction completely accurately, and all components of the tumour microenvironment have not been derived concurrently successfully as yet in such models, tumour-niche microenvironments are formed in such implants and have been successfully used to test drugs (Paton-Hough *et al*, 2015).

The KaLwRij 5T murine myeloma model was selected for this project due to its faithful recapitulation of the lytic lesions of human myeloma, combined with immunocompetence, which is critical to accurately model niche-tumour interactions.

1.6 Summary and development of experimental aims

1.6.1 Conclusions on the priorities in myeloma microenvironment research

The battle against cancers residing in the bone/bone marrow niche wields a number of ready weapons to manipulate niche interactions, with more being continually added to the armoury. As discussed in this introduction, myeloma provides an excellent example of the breadth of this field. Some agents, such as bisphosphonates, are already in widespread use to protect bones; however, they have not demonstrated the ability to repair damage already present. Others, such as proteasome inhibitors, and BTK inhibitors, have shown major disease reduction in haematological malignancies, and combine niche protection with tumour toxicity; but resistance inevitably occurs.

While increasing numbers of myeloma genomes have been sequenced (Walker, B. A. *et al*, 2015), and knowledge grows regarding the genetic changes that occur, both during B cell development to produce myeloma, and clonal evolution during sequential therapies (Bolli *et al*, 2014), the transcriptional changes that occur in bone marrow niche cells that surround and control this tumour as it develops have not previously been investigated *in vivo*. If alterations to the niche of this bone marrow-dependent cancer are as important to tumour survival as evidence suggests, the changes that occur when myeloma develops must be better understood to allow the possibility of therapeutic manipulation.

Based on the hypothesis that myeloma invasion would induce changes in niche gene expression profiles, we decided to performed bone lining cell transcriptome profiling in an *in vivo* myeloma model with two purposes: firstly to investigate whether BLC transcriptional changes might reveal novel therapeutic targets in myeloma bone disease, and to test these if so, and secondly whether discovery of novel niche-tumour interactions can lead to further hypotheses on the nature of niche-induced tumour persistence.

1.6.2 Aims of this project

- 1) To obtain transcriptional differences between healthy and myeloma-bearing mice in specific cell types of the bone lining niche.
- 2) To use this information to infer signalling pathways that may be dysregulated and contribute to myeloma bone disease.
- 3) To manipulate potential signalling pathways identified *in vivo*, with the aim of identifying potential therapeutic targets that may be of future clinical utility in myeloma bone disease or niche-tumour interaction.

In the event, BMP signalling was identified as being dysregulated in BLC from myeloma-bearing mice, and we subsequently studied the effects of BMP inhibition on bone disease, tumour burden and anaemia

Chapter 2 Materials and Methods

In this chapter general methodology for experimental work in this thesis will be described. Experiment details that are specific to one particular chapter have been presented as part of that chapter, and are therefore not additionally included here. Where methods were performed by individuals other than myself, this is stated in thesis acknowledgements and footnotes.

2.1 *In vivo* methods

As discussed in chapter 1, the KaLwRij 5T murine myeloma model was selected for this project due to its faithful recapitulation of the lytic bone lesions of human myeloma, combined with immunocompetence, which is critical to accurately model niche-tumour interactions. To detect a 2-fold change between experimental groups, power analysis using the probability of type I error $\alpha=0.05$ and the probability of type II error $\beta=0.20$ indicated that $n=10$ mice per group would be required to detect a significant change with an error of 5% and a power of 0.93. Standard sample size used for pharmacological intervention studies was therefore 10 per myeloma-bearing group and 5 per control group. In the case of LDN193189 treatment intervention, for microCT purposes only, the experiment was repeated once to give a total $n = 20$ per myeloma-bearing group. In the anti-BMP6 intervention study, there were only myeloma-bearing groups.

2.1.1 Mouse husbandry

Unless otherwise stated, animal procedures were performed under the authority of UK Home Office project (PL 30-2996 Claire Edwards) and personal license (I171CEFEF Sarah Gooding) in accordance with the Animals (Scientific Procedures) Act 1986, and were

approved by the University of Oxford ethical review committee. Mice were housed in individually ventilated cages and fed ad-libitum with standard diet and water. Mice were euthanized in increasing CO₂ concentrations confirmed by cervical dislocation.

6-week-old female C57BL/KaLwRijHsd mice were purchased from Envigo, Netherlands for experiments testing BMP inhibitors, and no experiment commenced before 8 weeks of age, to ensure skeletal maturity. A small colony of C57BL/KaLwRijHsd mice was maintained in the University of Oxford Biomedical Services breeding facility to provide smaller numbers of mice when required. These mice were used for both RNASeq experiments. Within each study, mice were age, sex and weight matched between treatments.

For all experiments using myeloma-bearing mice, myeloma development was induced by the intravenous (tail vein) inoculation of 8-10 week-old mice with 5TGM1-GFP tagged myeloma cells in 100µL of phosphate buffered saline (PBS). Control mice received 100µL of PBS only. 1.5×10^6 5TGM1-GFP cells (niche sorting experiments) or 2×10^6 5TGM1-GFP (LDN193189, BMPR1a-FC and anti-BMP6 experiments) were injected per mouse. Mice were monitored daily and, after day 21-post myeloma inoculation, twice daily. Experiments for bone lining niche sorting were ended prospectively on day 23. All other experiments were ended on the day the first mouse exhibited signs of hindlimb weakness, typically day 21-25. All drugs were injected from day 5 post tumour-inoculation until cull.

LDN193189 (Sigma) or vehicle (20% (2-hydroxypropyl)-beta- cyclodextrin) was injected IP daily at 3mg/kg.

BMPR1a-FC (Acceleron Pharma) was injected SC twice per week at 10mg/kg.

Anti-BMP6 (Kymab Ltd, fully cross-reactive with mouse Bmp6) was injected IP three times per week at 10mg/kg.

In one experimental replicate using LDN193189 (total n = 30), 0.4mg calcein was injected IP twice in the week preceding cull to enable measurement of bone formation rate by histomorphometry as below.

2.1.2 Bone lining niche sorting of endosteal niche components for RNASeq

The following methods are detailed in chapter 3:

1. Preparation of bone lining cell suspension from limb long bones
2. FACS sorting of mesenchymal progenitors, osteoblasts, endothelial cells and myeloma cells.
3. Generation of differential gene expression data and gene set enrichment analysis of RNASeq data.

However the SmartSeq2 protocol used for RNASeq material preparation is described below.

2.1.3 SmartSeq2 RNA-Seq of 100-cell samples of bone-lining niche cells.

The following adaptation of the SmartSeq2 protocol was used (Picelli *et al*, 2014)¹.

Sort into lysis buffer. Using a BD FACSAria III Cell Sorter (BD Biosciences), 100 cells per sample were sorted into 4µl lysis buffer (0.4% (vol/vol) Triton X-100 and 2 U/µl RNase inhibitor, 4 x10⁷ dilution of ERCC spike in control (a pre-formulated blends of 92 transcripts, derived and traceable from NIST-certified DNA plasmids), 2.5mM dNTPs (Thermo-Fisher), 2.5µM Oligo-dT30VN Oligonucleotide primers), and immediately frozen at -80°C.

Reverse transcription. First strand synthesis reverse transcriptase reaction buffer was prepared (Table 2.1). Samples were thawed on ice and heated at 72°C for 3 minutes before cooling on ice and then centrifuged. 6 µl of first strand synthesis buffer was added to each

¹ This part of the sample preparation was performed by Dr. Neil Ashley, Weatherall Institute of Molecular Medicine Single Cell Core Facility.

² Performed by Dr. Reza Morovat, Department of Biochemistry, John Radcliffe Hospital

sample, then samples incubated at 42°C for 90 minutes followed by 10 x 2 minute cycles of 50°C + 2 minutes 42°C, then 15 minutes at 70°C.

Reagent	Volume (µl per reaction)	Final Conc.
SuperScript II first strand buffer (5x)	2	1x
DTT (100mM)	0.5	5mM
Betaine (5M)	2	1M
MgCl ₂ (1M) (0.06ul in original protocol)	0.1	0.01M
Rnase Inhibitor (40 U/ul)	0.25	1U/ul
TSO (100 uM)	0.1	1 µM
Water (RT-PCR grade)	0.7965*	-
Superscript II RT (200 U/ul)	0.25**	50U
Total	5.9965	

Table 2.1: First strand synthesis reverse transcriptase reaction buffer components

*Volume of water added depends on sample size, based on a single droplet volume of FACS Aria.

**Superscript added last whilst denaturing the samples at 72°C

PCR Preamplification of cDNA. 15µl of PCR mix (Table 2.2) was added to each sample and PCR cycles (pre-optimised to 20 cycles for cell populations in this experiment) performed as follows:

Cycle	Denature	Anneal	Extend	Hold
1	98 °C, 3 min	–	–	–
2–20	98 °C, 20 s	67 °C, 15 s	72 °C, 6 min	4 °C

KAPA Hifi HotStart Ready Mix (2x)	12.5ul
ISPCR oligonucleotide primers (10uM)	0.125ul
Water	2.375ul
Total	15ul

Table 2.2: PCR amplification mix per sample

Purification, quality check and quantification of cDNA product. PCR products were cleaned using a 1:1 volume of Agencourt Ampure XP magnetic beads (Beckman Coulter) per sample, as per manufacturers instructions. The purified cDNA was then analysed for quality on an Agilent High Sensitivity DNA microfluidic chip using a BioAnalyzer, and quantified using the Quantit PicoGreen Kit, both as per manufacturer instructions.

cDNA Tagmentation to fragment and add adaptors to cDNA. DNA was diluted to 0.8 ng/μl in distilled H₂O, fragmented and tagmented with the Illumina Nextera XT Kit, using ¼ the manufacturer volumes. The tagmentation reaction was a single 55°C cycle of 5 minutes followed by 4°C hold.

Enrichment PCR of adaptor-ligated fragments. A PCR reaction to add barcodes and adaptors was then performed: DNA 5 μl, Nextera PCR master mix 3.75 μl, index 1 primers (N7xx) 1.25 μl, and index 2 primers (N5xx) 1.25 μl were mixed to total volume 12.5 μl.

12 PCR cycles were run:

Cycle	Denature	Anneal	Extend	Hold
1	–	–	72 °C, 3 min	–
2	95 °C, 30 s	–	–	–
3–12	95 °C, 10 s	55 °C, 30 s	72 °C, 30 s	4 °C (final cycle only)

Repeat purification, quality check and quantitation of cDNA libraries: as above step.

Pooling of library samples ready for sequencing. Using the concentration (ng /μl) obtained with PicoGreen and the average size obtained on the Bioanalyzer/ Tapestation, sample molarities were calculated. Libraries were pooled together aiming for a 2-10 nM solution for each pool. Finally, total pool concentrations (ng/μl) were re-calculated with PicoGreen, and the average size of the pools with Bioanalyzer/Tapestation, to calculate library molarities.

Sequencing of cDNA libraries. Using Illumina HiSeq2000 (first RNASeq experiment (chapter 3), 50bp single end reads) and Illumina HiSeq4000 (second RNASeq experiment (chapter 4), 75bp paired end reads)

2.1.4 Murine sample handling

Blood

Blood was taken by cardiac puncture immediately after euthanising mice. 150μl stored in BD Microtainer EDTA tubes (Beckton Dickinson) was used to measure plasma haemoglobin

concentration within 6 hours (Sysmex KX-21N automated haematology analyser) when sample quality permitted. Serum was prepared by the centrifugation of clotted blood at >6,000 g for 3-5 min in BD Microtainer SST tubes (Beckton Dickinson); serum aliquots were frozen immediately at -80°C. Serum was used for iron and specific protein measurements as follows:

Serum IgG2b ELISA (Bethyl laboratories, Inc.), used sample serum diluted 1:50 000 to assess global 5TGM1 tumour burden, according to manufacturers instructions, after dilution optimisations. All samples were measured in duplicate.

Serum hepcidin was quantified by competitive ELISA using the Hepcidin-Murine Compete Kit (Intrinsic Lifesciences) according to the manufacturer's protocol. After dilution optimisation, 6µl sample serum, diluted 1:40, was used per well and all samples measured in duplicate.

Serum iron² was quantified using the MULTIGENT Iron Kit on the Abbott Architect c16000 automated analyzer (Abbott Laboratories) at Oxford John Radcliffe Hospital, UK (BCSH, 1990).

Serum transferrin² was quantified using the Abbott Architect c16000 transferrin assay at Oxford John Radcliffe Hospital as above. Of note, this transferrin assay used an antibody to human transferrin and therefore, as its accurate quantitation and linear relationship to serum iron cannot be assumed in a mouse, transferrin saturation could not be calculated (discussed further chapter 5).

Serum TRAP5b activity (MOUSE TRAP assay, Immunodiagnostic Systems, Inc.) was quantified by competitive ELISA in duplicate for all samples, using 25µl sample serum, according to the manufacturer's protocol, to assess global osteoclast activity.

² Performed by Dr. Reza Morovat, Department of Biochemistry, John Radcliffe Hospital

Abdominal viscerae

Livers were harvested into RNAlater (Ambion) and/or snap-frozen. They were homogenized by TissueRuptor (Qiagen) for RNA extraction and *Hamp1* gene expression quantification (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control, as below)

Spleens were harvested into PBS containing 5% FBS. They were homogenised by gentle crushing, passed through a 70 μ m filter (Thermo Scientific), resuspended in red cell lysis buffer for 5 minutes, washed and resuspended in 10% neutral buffered formalin (Thermo Scientific). After 24 hours fixation, spleen cells were washed and analysed for GFP content by flow cytometry using a Cyan-ADP Flow cytometer (Beckman Coulter).

Bone marrow

Bone marrow cells were obtained by crushing dried and cleaned left femur and tibia by pestle and mortar in exactly 1.5ml sterile PBS. Bone marrow cells suspended in the PBS were filtered and spun to a pellet, from which the supernatant was snap-frozen in aliquots as bone marrow plasma. The bone marrow cell pellet was resuspended in red cell lysis buffer for 5 minutes, washed and resuspended in 10% neutral buffered formalin (Thermo Scientific). After 24 hours fixation, cells were washed and analysed for GFP content by flow cytometry using a Cyan-ADP Flow cytometer (Beckman Coulter).

Bone marrow plasma Dkk1 and Sclerostin levels were measured by Magnetic Luminex Screening Assay (R&D Systems) at a two-fold dilution, in duplicate, according to manufacturers instructions, on a Biorad Bio-Plex 200 System.

Bones

Right tibiae were fixed in 10% neutral buffered formalin (Thermo Scientific) and microCT performed and analysed as below.

Formalin-fixed, decalcified, paraffin embedded right femorae were used for histological analyses as below.

In one experimental replicate comparing treatment of myeloma-bearing and healthy mice with LDN193189 or vehicle, methyl/butyl methacrylate plastic embedded non-decalcified lumbar vertebral sections were used for measurement of calcein incorporation, as below.

2.1.5 MicroCT analysis of right tibiae

MicroCT analysis (Skyscan 1172 X-ray Microtomograph; Bruker MicroCT, Kontich, Belgium) was performed at 37 kV/228 μ A, with an isometric resolution of 9.94 μ m/ pixel using a 0.5-mm aluminium filter³. Cross-sectional TIFF images were reconstructed using NRecon, and imported to Syksan CT-Analyser (CTan) software (Bruker MicroCT, Kontich, Belgium) for integrated 3D morphometry calculations of surface-rendered volume models (Bouxsein *et al*, 2010; Bruker MicroCT manual). For 'trabecular' BV/TV (ratio of bone volume to total tissue volume) volume-of-interest (VOI) contours were drawn within the cortical endosteal surface, such that the total volume (TV) included only metaphyseal medullary trabecular bone. For 'total' BV/TV VOIs, cortical and medullary areas were included in TV. Cortical volume represented the difference between the total volumes of these two measurements ('total' and 'trabecular'), such that only the cortical area was included. 'Bone volume' (BV) included all tissue that had a material density greater than 438.7 mgHA/cm³, thereby giving a measure of BV/TV. The same threshold settings for bone tissue were used for all samples and the VOI was always taken from the distal margin of the growth plate for 50 consecutive cross-sectional images, such that the analysis area was consistent, and included the metaphysis and proximal diaphysis. Derived indices from measurements generated by CTAn

³ CT Scanning performed by Aude Vernet, Wellcome Centre for Human Genetics, University of Oxford

included trabecular thickness and trabecular separation (Bouxsein *et al*, 2010; Bruker MicroCT manual).

For analysis of cortical bone lesions, a 3-D reconstruction of the metaphyses and proximal tibial shaft surface was generated using a consistent threshold. On the 3D reconstructed image, osteolytic lesions on the curved medial tibial surface that completely penetrated the cortical bone and were greater than 100µm in diameter were counted.

2.1.6 Histology and histomorphometry

Decalcification and paraffin embedding

Right femorae were placed in histological cassettes and decalcified in 10% EDTA in distilled water (pH 7.2) for 2 weeks. Upon successful decalcification, the bones were dehydrated in an ethanol series, cleared in xylene and paraffin-coated (automated Leica ASP300 tissue processor). Bones were then immersed in hot 60°C paraffin within the labelled cassette and cooled on a cold plate (Leica), giving a block of tissue embedded in paraffin wax, which was sectioned at 5µm thickness and mounted.

Haematoxylin and Eosin staining

Tissue sections were de-paraffinised in Histoclear clearing agent for 5 minutes twice and hydrated through graded alcohols to water (100% ethanol for 2 minutes twice, 90% ethanol 1 minute, 70% ethanol 1 minute). The slides were then washed in water three times and immersed in Harris Hematoxylin solution (Thermo Scientific) for 1 minute. Slides were thoroughly rinsed in tap water until clear run-off, and nuclear stain enhanced by dipping the sections in 5% ammonium water for 10 seconds (Thermo Scientific) and washing again. Slides were counterstained with aqueous Eosin solution (Sigma-Aldrich) for 2 minutes and washed until clear run-off. Finally, slides were dehydrated through graded ethanol (1 minute

in 70% ethanol twice, 1 minute in 100% ethanol twice, 5 minutes in HistoClear twice), and mounted with a coverslip using DPX mountant (Thermo Scientific), before leaving to dry.

Tartrate resistant acid phosphatase activity (TRAP) staining

Tissue sections were de-paraffinised and rehydrated through graded ethanol and distilled water as described above. The slides were then incubated in a tartrate/napthol AS-BI phosphate substrate solution pH 5 (Sigma-Aldrich) at 42°C for 30 minutes. Immediately after the incubation period, slides were transferred directly to a pararosaniline/nitrite colour reaction solution (Sigma-Aldrich) for 10 minutes, until colour development observed, at room temperature. Slides were thoroughly rinsed in distilled water and counterstained with Harris Hematoxylin solution (Thermo Scientific) for 1 minute, and again washed until clear run-off. Finally, slides were dehydrated through graded ethanol as above, and mounted with a coverslip using DPX mountant (Thermo Scientific), before leaving to dry.

Methyl/butyl methacrylate plastic embedding

Formalin-fixed, intact but cleaned lumbar vertebral columns were dehydrated through graded ethanol (1 day 70% ethanol, 1 day 95% ethanol, 3 days 100% ethanol, changed daily). They were then processed, using rotating immersion, in 'monomer mix 1' for 2 days at room temperature, 'monomer mix 2' for 3 days then 'monomer mix 3' for 3 days, both in an explosion-proof container at 4°C. Finally they were placed ventral side-down on a pre-poured and solidified plastic base of 'mix for bases' in a glass scintillation vial, and covered in 10ml 'embedding mix', then placed in a -20°C freezer until solidified (2-5 days) (Table 2.3). After polymerisation, the glass vial was smashed to obtain the plastic block, and the block ground down to expose ventral vertebral surface (Buehler Beta grinder polisher). Blocks were sectioned at 5µm thickness (Leica RM2255 microtome), sections mounted, and dried under compression to cause plastic sections to adhere to slides.

Monomer Mix	Components	To note
Monomer Mix 1	Methyl methacrylate 300ml Butyl methacrylate 175ml Methyl benzoate 25ml Polyethylene glycol 400 6ml	Prepared and used in fume hood (applies to all below)
Monomer Mix 2	'Monomer mix 1' 500ml 2.5g wet benzyl peroxide (catalyst)	Mixed and filtered through gauze containing calcium chloride pellets to remove water.
Monomer Mix 3	'Monomer mix 1' 500ml 5.0g wet benzyl peroxide	As above
Embedding Mix	'Monomer mix 3' 100ml, cold 4,N,N-Trimethylaniline 400µl	Aliquoted and used only 100ml monomer mix 3 at a time, poured immediately on mixing
Mix for bases	'Monomer mix 3' 100ml, cold 4,N,N-Trimethylaniline 600µl	Add max. 5ml per vial and leave for 2 days to solidify.

Table 2.3: Composition of monomers for methyl/butyl methacrylate plastic embedding of calcified bony tissue.

Volumes as for 500ml total, enough for 30 blocks. Note monomer mixes made fresh at each stage and not reused.

Histomorphometry

Histomorphometric analyses were performed using OsteoMeasure software (OsteoMetrics, Decatur, GA, USA). Total subcortical tissue volume and trabecular bone volume (to generate BV/TV as with microCT analysis) were traced manually and measured on haematoxylin and eosin stained sections at 20x magnification. The area measured was five fields of view, starting directly below the growth plate but excluding the primary spongiosa. Using an identical area and magnification on TRAP and haematoxylin-stained sections, bone-lining cell number and thickness, and osteoclast number and surface contact with bone, were quantified. Bone lining cell data were expressed as cell density (N. Ob/T. Ar in mm³) and cell thickness (µm). Osteoclast data were expressed as number of cells per mm of bone surface (N. Oc. B. Pm), or an osteoclast surface/ bone surface ratio (OcS/BS).

Mineral apposition rate (MAR, µm/day) and surface referent/ bone formation rate (BFR/BS, µm³/µm²/day) were calculated using Osteomeasure software using fluorescence microscopy at 20x magnification, on plastic-embedded vertebral sections from calcein-injected mice.

Three fields of view per slide were measured, and bone formation measured where clear double or single calcein-labelled bone formation could be seen, and traced manually. MAR

was the mean interlabel distance divided by the time interval (3 days) between the two fluorochromes administered.

Image capture

Images of tissue sections for use in this thesis were photographed using a Zeiss AxioCam MRc digital camera for microscopy, and Olympus BX40 microscope (Olympus UK Limited).

2.2 *In vitro* methods

2.2.1 Cell line culture

HuH7 cell line was a gift of Prof Persephone Borrow, University of Oxford.

UMR-106.01 cell line was a gift of Dr Philippa Hulley, University of Oxford.

JJN3 and MM1-S cell lines were a gift of Prof Udo Oppermann, University of Oxford.

2T3 mouse preosteoblast cell line, ST2 mouse stromal cell line and HS5 human stromal cell line were obtained from ATCC.

5TGM1 and 5TGM1-GFP were a gift from Prof. Gregory Mundy, University of Texas Health Science Center at San Antonio.

Mouse 2T3 osteoprogenitor cell line, mouse ST2 stromal cell line, human HS5 stromal cell line, rat UMR106.01 osteosarcoma line and human hepatoma-derived HuH7 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin, unless otherwise specified.

Myeloma cell lines (5TGM1-GFP murine myeloma cells, and JJN3 and MM-1S human myeloma cell lines) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin, 1mM sodium pyruvate, and 1% 100X non-essential amino acids solution (Sigma-Aldrich), unless otherwise specified.

All cell lines were maintained at 37°C in 5% CO₂ atmosphere. All cryopreserved cell lines were stored in 10% DMSO (Sigma) in FBS in liquid nitrogen.

2.2.2 Mineralisation assay

48 hours after seeding at 5×10^4 /well (12-well plates), 2T3 cells were treated every 3 days with fresh osteogenic media comprising non-FCS supplemented Minimum Essential Medium

Eagle (alpha modification) (α -MEM), supplemented with 2% β -glycerophosphate, 50mM L-ascorbic acid and L-thyroxine 50ng/ml. Successful mineralisation was assessed at day 7 by Alizarin Red staining in representative control and treated wells (Fig. 2.1). On successful mineralisation, media was replaced with DMEM, supplemented as standard, other than with only 1% FCS, and transwells (Corning® Transwell® polyester membrane cell culture inserts) were added containing 1×10^6 5TGM1 cells also in DMEM 1% FCS. Incubation was for 24 hours before RNA extraction and qRT-PCR as below.

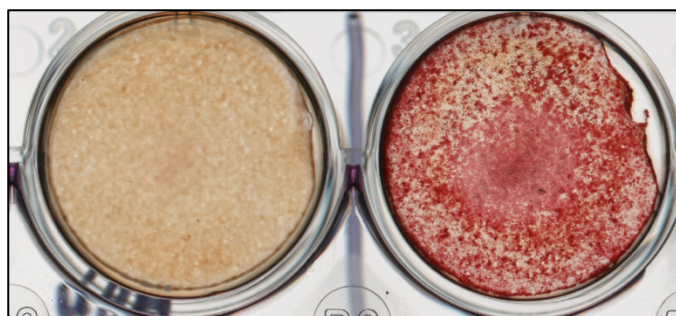


Figure 2.1: Example of 2T3 cell mineralisation after 7 days in osteogenic medium. Calcified matrix stains red with Alizarin Red stain, but non-calcification in control well does not stain.

2.2.3 Viability and proliferation assays

AlamarBlue® cell viability assays were performed on 4×10^4 cells in 100 μ l RPMI supplemented as above but only 2%FCS, as per manufacturer's instructions. After 72 hours incubation with human BMP6 (R&D Systems) and/or LDN193189 (Sigma) at doses specified in Figure 4.4, 10% AlamarBlue® (10 μ l) was added and red fluorescence measured after 3 hours. Plate fluorescence was measured with 554nm excitation filter and 590nm emission filter using FLUOstar Optima. All treatments were performed in quadruplet.

For fluorescence expressing cell contact experiment, HS5 cells were seeded for culture (2×10^4 cells/well, 96-well plate) in complete DMEM, and allowed to adhere for 4 hours. Media was replaced with phenol-free RPMI containing 1% FCS and additional experimental treatments and/or 4×10^4 5TGM1-GFP cells as specified in chapter 4 (Fig. 4.4D). Plate fluorescence was read at 72 hours as above. Data was normalised by subtraction of

background fluorescence from media-alone wells, then expression ratio of experimental well fluorescence to mean of control wells fluorescence. All treatments were performed in quadruplet.

2.2.4 siRNA-mediated gene knockdown experiments

For siRNA-mediated gene knockdown experiments, rat UMR-106.01 cells were plated at 50% confluency; after 8 h they were transfected with siRNA targeting rat *Acvr1* (*Alk2*) and/or rat *Bmpr1a* (*Alk3*) (siGenome Smartpool, Dharmacon) using Lipofectamine RNAiMAX (Thermofisher), Opti-MEM (Thermofisher) and antibiotic-free DMEM media. The total amount of siRNA added was 100nM. Cells were harvested after a further 48 h. 100nM LDN193189 was added to equivalent wells 24 h before harvest. RNA extraction and qRT-PCR were performed as below.

2.2.5 Gene expression analysis by quantitative real time PCR (qRT-PCR)

Gene expression in mouse liver tissue and *in vitro* assays was performed on RNA extracted using RNEasy Plus kit (Qiagen) and reverse transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative real-time PCR was performed using inventoried TaqMan Gene Expression assays (Table 2.4) and TaqMan Gene Expression Master Mix (Applied Biosystems). Changes in gene expression relative to endogenous controls (*Hprt1* for mouse and rat, *GAPDH* for human) were calculated using the $2^{-\Delta CT}$ method.

Species	Gene	Probe
Human	<i>GAPDH</i>	Hs999905
	<i>HAMP</i>	Hs00221783
	<i>POLR2A</i>	Hs00172187
	<i>DKK1</i>	Hs00183740
	<i>SMAD6</i>	Hs00178579
	<i>ATOH8</i>	Hs01031629
	<i>SMAD7</i>	Hs00998193
	<i>ID1</i>	Hs03676575
	<i>ID3</i>	Hs00171409
Mouse	<i>Hprt</i>	Mm03024075
	<i>Hamp</i>	Mm04231240
	<i>Smad6</i>	Mm00484738
	<i>Id1</i>	Mm00775963
	<i>Bmp6</i>	Mm01332882
Rat	<i>Hprt</i>	Rn01527840
	<i>Acvr1</i>	Rn01449846
	<i>Bmpr1a</i>	Rn01450292
	<i>Sost</i>	Rn00577971
	<i>Dkk1</i>	Rn01501537

Table 2.4. Taqman probes used in experiments included in this thesis.

2.2.6 Western Blot

Whole cell extracts were harvested using the RIPA lysis buffer (ThermoFisher) with protease and phosphatase inhibitor cocktail. Protein was quantified using the Pierce Micro BCA Protein Assay Kit (ThermoFisher). Proteins (20µg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis (Tris-HCl 4-20% gradient pre-cast gel, Bio-rad), accompanied by protein standard ladder (Precision Plus Protein™ Kaleidoscope™ Prestained

Protein Standards). After running, protein was transferred onto a 0.2µm nitrocellulose membrane using the TransBlot (Bio-Rad) transfer system. After blocking (5% w/v nonfat dry milk), the membrane was probed with rabbit anti-phospho-Smad1/5/9 (Cell Signalling Technology, #13820), HRP-conjugated anti-rabbit secondary (Cell Signalling Technology #7074S), and signal detected using Novex ECL HRP Chemiluminescent substrate reagent kit (Invitrogen). The membrane was then stripped (Restore™ Western Blot Stripping Buffer) and re-probed with rabbit anti-Smad1 (Cell Signaling Technology, #6944), secondary as above, and finally with mouse anti-β-Actin (Sigma A5316), and HRP-conjugated anti-mouse secondary (Cell Signaling Technology, #7076S) as a loading control. Blot densitometry was performed using FIJI (ImageJ) software.

2.2.7 Human stromal cell culture for gene expression

Patients with or under investigation for myeloma consented to the use of bone marrow aspirate samples for research purposes (patient characteristics, Supplementary Table 3). This work was approved by Oxford Clinical Research Ethics Committee (09/H0606/5 project 13/A238). Mononuclear cells were isolated from fresh bone marrow aspirates using Histopaque (Sigma), and seeded into T25 tissue culture flasks, in DMEM supplemented with 20% FBS, 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin. Adherent cells (BMSCs) were washed the next day to remove non-adherent debris, then grown to confluence for up to 3 weeks. At confluence, cells were reseeded into 12 well plates at 5×10^4 /well (same DMEM media), and expanded for 72 hours. Wells were then treated for 24 hours with fresh media alone, or containing LDN 100nM or BMPR1a-FC 10µg/ml, before RNA extraction and RT-qPCR as above.

2.2.8 Osteoclast differentiation assays

Peripheral blood mononuclear cells (PBMCs) were isolated from pooled human leucocyte cones purchased from NHS Blood and Transplant and osteoclasts differentiated following protocols described previously (Knowles *et al*, 2010). This work was approved by the Oxford Clinical Research Ethics Committee (C01.070). PBMCs were isolated from leukocyte cones diluted 1:1 in unsupplemented α -MEM using Histopaque (Sigma) density centrifugation. CD14⁺ monocytes were positively selected using Anti-CD14 magnetic bead sorting (Miltenyi), and were seeded at 0.25×10^6 cells in 200 μ l per well onto 96-well tissue culture plates in α -MEM culture media (without ribonucleosides/ deoxyribonucleosides, Lonza BE12-169F) containing 10% FCS, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulphate (50 μ g/ml). After overnight incubation, media were changed to contain M-CSF (R&D systems, 25 ng/ml), RANKL (gift Dr. J Dunford, 35 ng/ml) and in duplicate experimental wells, either OPG 500ng/ml (Peprotech), LDN193189 100nM (Sigma), human BMPR1a-FC 10 μ g/ml (gift Dr. E Martinez Hackert), or human ACVR2a-FC 10 μ g/ml (gift Dr. E Martinez Hackert). Media were changed every 3–4 days, with mature osteoclasts being formed by day 9. Cells were then fixed in 4% formalin and tartrate-resistant acid phosphatase (TRAP) was visualized using naphthol AS-BI phosphate as a substrate plus tartrate, with reaction of the product with Fast Violet B salt to stain. TRAP-positive multi-nucleated cells containing ≥ 3 nuclei were considered osteoclasts. Wells were photographed using a Nikon SMZ1500 stereomicroscope, and osteoclasts enumerated manually using FIJI software. The mean number of cells between duplicate wells was paired to the mean number in control wells for each experimental replicate, as inter-experimental variation existed.

2.3 Statistical methods

Statistical significance was determined using a student T test, or one way or two way ANOVA where appropriate, and considered significant if $p < 0.05$. Data are represented as mean $\pm$ SD unless otherwise stated.

Analysis was performed with GraphPad Prism Software (GraphPad Software Inc.). Where differences between particular treatment groups were experimentally hypothesized, means were compared using unpaired t-tests, with normal distribution assumed on grounds of biological likelihood. Multiple groupwise comparisons where differences between individual groups were not hypothesis driven, were made by ANOVA. Where difference from control condition only was sought, each group was compared to control condition using Dunnett's method. Šídák's multiple comparisons test was used to compare a defined set of conditions. Changes in osteoclast differentiation between paired untreated/ treated samples were assessed using paired t-tests, and in patient BMSC gene expression experiments, paired ratio t-tests with correction for false discovery rate by Benjamini & Hochberg method were used, with individual normalisation of each sample to control condition, due to high baseline variation in gene expression between patient samples. Significance was defined as $P < 0.05$. RNASeq statistical analysis is described in Chapter 3 Methods ('RNASeq data analysis').

Missing data values

MicroCT and histomorphometric data have occasional missing data values relative to total group n. This is due to broken/damaged bones at harvest, especially likely if severe bone disease is present. In Fig. 4.6 two GFP percentage data values are missing from the myeloma + LDN193189 cohort due to sample contamination.

Chapter 3 The Transcriptional Effects of Myeloma Invasion on components of the Bone Marrow Endosteal Niche

3.1 Introduction

In chapter 1, the concept of the bone marrow niche as a site for the specific nurture and growth /differentiation control of the developing components of the haematopoietic system was examined, including as the site of plasma cell homing and residence. When myeloma goes beyond the natural behaviour of its cell-of-origin, to hijack and damage its niche, and to pursue uncontrolled growth, it must overcome the microenvironmental checks and balances normally present to prevent this.

The examination of how the avoidance and subversion of these checks and balances lead to uncontrolled tumour growth is one approach to a better understanding of how cancer develops, in contexts wider than myeloma. Mechanisms by which myeloma cells avoid control, such as evasion of immune recognition or niche –induced dormancy, are discussed in chapter 1. Many of the changes that occur in the bone-lining niche in response to myeloma invasion are well described: for example the increase in RANKL and decrease in OPG production by osteocytes and their precursors, as well as by myeloma cells, which leads to osteoclast recruitment and enhanced bone lysis. This understanding has led to development of the drug Denosumab, anti-RANKL, widely used in osteoporosis and very recently FDA approved for fracture prevention in myeloma bone disease (Amgen, 2018; Henry *et al*, 2011).

Gene expression profiling of *in vitro*-passaged bone marrow stromal cells and/or preosteoblasts has shown that the RANKL/OPG axis is just one of many pathways altered in bone marrow stromal cells when they grow in contact with myeloma (Arnulf *et al*, 2007; Corre *et al*, 2007; Garcia-Gomez *et al*, 2014; Todoerti *et al*, 2010). It has been shown that stromal cells from myeloma patients acquire differences that persist beyond contact *in*

vivo, that they are intrinsically and potentially permanently changed from that point (Fowler *et al*, 2012; Hiruma *et al*, 2009). In addition, osteoblast differentiation and bone matrix secretion are reported to be irreversibly suppressed (Corre *et al*, 2007; D'Souza *et al*, 2011; Giuliani *et al*, 2005). As discussed in chapter 1 however, the assembled data on this subject can be confusing and in some cases contradictory. There is evidence that the early stages of MSC proliferation, and possibly early pre-osteoblast differentiation, may be enhanced by myeloma contact (Kassen *et al*, 2014; Noll *et al*, 2014b), while the changes that result in bone matrix formation block occur at a later stage in the progression from pre-osteoblast to osteoblast (Roodman, 2011).

I noted that the gene expression profiling and functional mineralisation assays that have been used to determine the differences in MSC or osteoblast differentiation in myeloma patients had only ever been performed on *in vitro*-passaged, heterogeneous populations of adherent stromal cells, which contain putative MSCs amongst a range of cells at other stages of differentiation. This might be an explanation for some of the variation in reports of the effect of myeloma on MSC and osteoblast behaviour. In this technique, cells are isolated by plastic adherence (Pittenger *et al*, 1999; Tondreau *et al*, 2004) then expanded through culture, with the potential to use osteoblast differentiation media (usually a combination of dexamethasone, β -glycerophosphate, and ascorbate) to induce an osteoblast-phenotype and bone matrix deposition (Pittenger *et al*, 1999).

3.1.1 Cell surface markers of bone marrow niche cells

FACS-assisted isolation of MSCs, pre-osteoblasts and/or endothelial cells has not been reported in any myeloma or cancer-induced bone disease mouse models, although this technique has been available for some years and is widely accepted, particularly in the haematopoietic stem cell niche field (Morikawa *et al*, 2009; Nakamura *et al*, 2010). It has

been reported in the generation of samples of particular niche components for RNA transcriptome profiling (Ramasamy *et al*, 2014; Zhang *et al*, 2014).

Whereas CD31 is a widely accepted and specific marker for endothelial cells (Rakocevic *et al*, 2017), specific cell surface markers for cells of the mesenchymal lineage are more challenging and controversial; indeed in humans, robust FACS-suitable markers for these cells still do not exist. However in the mouse bone marrow, much work has been done to validate markers for genuine mesenchymal 'stemness', (usually defined by the ability to differentiate down both an osteoblastic and adipogenic pathway, although there are plenty of other MSC fates) versus later stages of differentiation along the path to osteoblast maturity. Many of the best markers of stages of MSC-osteoblast differentiation, for example *sox9*, *osterix* or *osteocalcin*, are internal or secreted, and not cell surface markers, although are commonly used as sites for Cre insertion of fluorescent tags for marking cells at particular stages (Ono & Kronenberg, 2016; Yu, V. W. *et al*, 2015). How specific a population should be isolated depends on the experimental question: for example recent work on the identification of very specific sub types of early stromal cells marked by expression of CXCL12, Nestin or Leptin Receptor has demonstrated their very particular roles in supporting early stages of haematopoietic cell development (Mendez-Ferrer *et al*, 2010; Omatsu *et al*, 2010; Zhou *et al*, 2014). However, at this point of experimental progress in dissecting the myeloma microenvironment, there is as yet no evidence these sub-types are important in myeloma development, although one day they may be shown to be so.

In considering how to use a novel technique in a tumour model with the prominent feature of rapid bone-lining niche destruction (see chapter 1), I decided to start by trying to simply separate a less mature, more multipotent MSC population from a more committed pre-osteoblast/osteoblast one, with the purpose of seeing where the greater effect of myeloma

invasion was felt. For this reason I followed a well-respected, highly-cited protocol by Nakamura and colleagues where it was demonstrated that Sca1 positivity in CD45/Ter119/CD31- bone lining cells marked an stromal progenitor population (referred to as 'MSCs' henceforth for simplicity), enriched in cells capable of differentiating into osteoblast or adipocytic lineages, and CD166/ALCAM positivity marked a more mature population expressing markers associated with osteoblast differentiation (Nakamura *et al*, 2010). This protocol and the differentiation potential of cells according to these markers have since been validated by others (Hu *et al*, 2016).

3.1.2 Hypotheses of this chapter

1. Gene set enrichment analysis of the transcriptome differences between healthy and myeloma-bearing mice in stromal cellular components of the endosteal / bone-lining niche will reveal new pathways that are dysregulated a result of myeloma invasion.
2. Identification of dysregulated pathways in MSCs and osteoblasts in myeloma will enable new hypothesis generation regarding where, in the development from MSC to osteoblast, the important barriers to bone repair caused by myeloma occur. Thus novel targets for experimental pathway manipulation, and potentially therapeutic consideration, will be revealed.

3.2 Experimental design and specific methods

This section describes the sorting of bone-lining niche cells from myeloma-bearing and healthy Kalwrij mice, RNA sequencing, then analysis of gene expression differences. All steps were practiced and optimised in advance of the process described here. In particular the sorting of the bone-lining niche cells was a technically complicated procedure, due to the difficulty in maintaining these strongly adherent cells as viable, and in a single-cell suspension during processing, and was optimised over many months.

As in chapter 2, some of the methods described here were performed by others. These are listed in the acknowledgements section at the start of the thesis, and noted by footnote where applicable.

8 female 8-10 week old KaLwRij mice were injected IV with 1.5×10^6 5TGM1-GFP murine myeloma cells, and 8 with PBS for healthy control purposes, as described in chapter 2. Because use of the cell sorting facility had to be booked in advance, instead of waiting for the first mouse to exhibit signs of hindlimb weakness (the usual trigger for ending an experiment in this model), the date of cull had to be planned in advance. Based on optimisation experiments to predict a degree of disease that might correspond to early-stage bone destruction by tumour (the target was approximately 10% marrow tumour burden), day 23 was chosen. In addition, to minimise delays that might cause excess cell death or altered gene expression, only 8 samples were sorted per day, meaning that Day 0 IV infusion of myeloma cells or PBS, and then Day 23 cull, were staggered over two consecutive days.

3.2.1 Sorting of bone-lining niche constituents from healthy or 5TGM1-GFP

myeloma-bearing KaLwRij mice

Following cull, bilateral femora and tibiae were rapidly removed and cleaned in a sterile environment, and placed in ice cold PBS with 2% FCS. Bones were crushed, before thorough washing with repeated aliquots of PBS to remove all soluble marrow cells, so only white bone fragments remained. The easily dissociated marrow cells were retained separately to obtain the 'central marrow' myeloma fraction. Bones were then finely minced to a 'porridge-like' consistency, and transferred to a sterile 37°C solution of 3mg/ml Type 1 Collagenase (Worthington) in PBS with 5% FCS and 200U/ml DNase1, and incubated on rotation for 30 minutes to allow bone matrix digestion. At the end of this time, the cell suspension released from the bone fragments was filtered, washed and stored on ice, this time suspended in PBS with 5% FCS and 2mM EDTA (FACS buffer), while the bone fragments were subjected to a second 30-minute incubation in fresh Collagenase solution. Cells released from the second incubation were washed and added to those from the first incubation.

The collagenase-digested bone lining cells were first briefly incubated in FC block (anti-CD16/CD32: to reduce non-specific binding of antibodies to widely expressed Fc γ receptors) then stained with the antibody cocktail (Table 3.1). Cellular targets used were based upon markers previously described and validated, as discussed in the introduction to this chapter.

Following antibody incubation, samples were washed, resuspended in FACS buffer and aliquots of 100 cells sorted, using a BD FACSAria III Cell Sorter, into 4 μ l of lysis buffer (comprising 0.4% (vol/vol) Triton X-100, 2U/ μ l RNase inhibitor, 4 x10⁷ dilution of ERCC spike in control (a pre-formulated blends of 92 transcripts, derived and traceable from NIST-certified DNA plasmids), 2.5mM dNTPs (Thermo-Fisher) and 2.5 μ M Oligo (Oligo-dT30VN).

Cellular Target	Fluorochrome	Working dilution	Source	Clone
CD45	AF700	1/50	eBioscience	30-F11
Ter119	BV650	1/50	Biolegend	TER-119
CD31	APC	1/800	eBioscience	390
Sca-1	PeCy7	1/200	Biolegend	E13-161.7
ALCAM (CD166)	PE	1/10	R&D Systems	Polyclonal Goat IgG (FAB1172P)
Dead cells	7AAD	1/100		

Table 3.1: Antibodies used in FACS sort of bone-lining niche cellular components

Including the cellular target, fluorochrome on which they were sorted, working dilution, commercial source and clone.

Single stained control samples (using BD™ CompBeads) and ‘Fluorescence-minus-one’ (FMO) stained bone lining cell samples for each fluorochrome were run in advance to calculate compensation and set gates. The gating strategy was based upon that originally described for the sorting of this bone lining niche preparation (Nakamura *et al*, 2010) (Fig. 3.1). Cell types concurrently sorted⁴ were:

1. GFP-expressing 5TGM1 myeloma cells
2. CD45-Ter119-CD31+ endothelial cells
3. CD45-Ter119-CD31-ALCAM-Sca1+ mesenchymal stromal progenitor cells (‘MSCs’)
4. CD45-Ter119-CD31- Sca1- ALCAM+ committed osteoblast lineage cells (‘osteoblasts’)

Within 5 minutes of sorting into lysis buffer, 100-cell aliquots were spun and snap frozen, then stored at -80°C until use.

⁴ Sort operators Paul Sopp and Craig Waugh, WIMM FACS facility

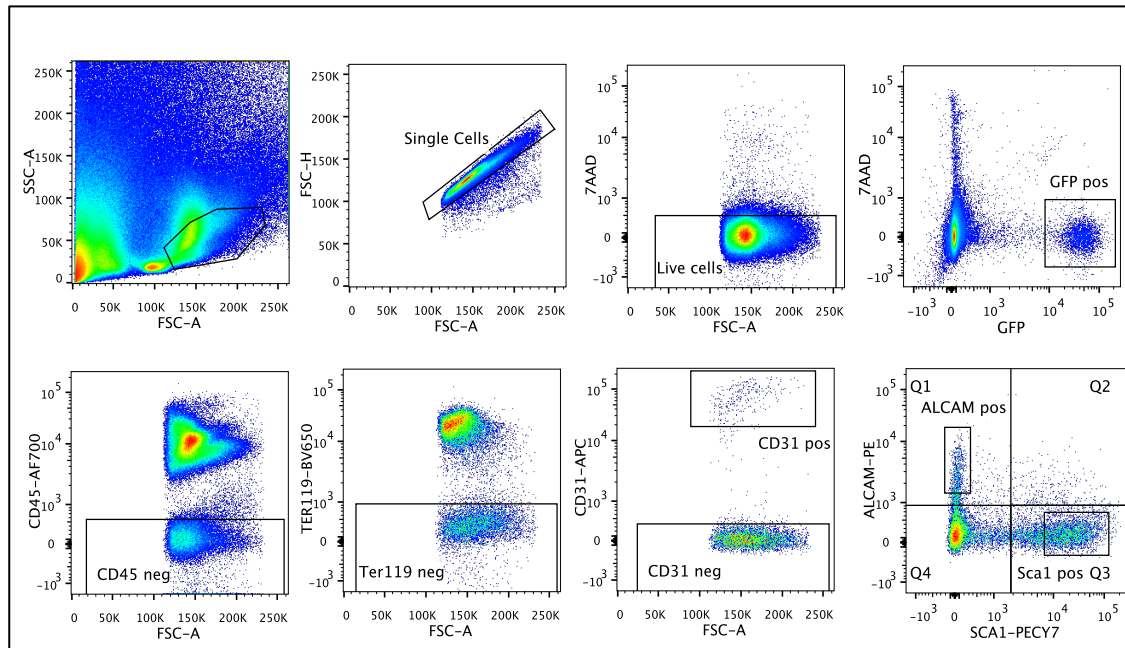


Figure 3.1: Example gating strategy used to FACS sort cell populations from bone-lining niche of healthy and 5TGM1-GFP myeloma-bearing KaLwRij mice.

Plots representing the full gating strategy used to sort GFP-tagged 5TGM1 myeloma cells, CD45-/Ter119-/CD31+ endothelial cells, CD45-/Ter119-/CD31-/Sca1+ mesenchymal stromal progenitor cells (MSCs) and CD45-/Ter119-/CD31-/ALCAM+ osteoblast cells.

Note: optimised image produced using FlowJo Application V 10.0.7, using FCS file from representative sample. For comparison, Supplementary Figure 1 shows sorting strategy gates generated in real time during experiment using BD FACSDiva V 8.0.1 Application.

3.2.2 SmartSeq2 RNA-Seq of 100-cell samples of bone-lining niche cells.

According to the more detailed protocol described in chapter 2, the following steps were performed according to the published SmartSeq2 protocol (Picelli *et al*, 2014)⁵:

1. Reverse transcripton
2. PCR Preamplification of cDNA
3. Bead purification of cDNA product
4. cDNA quality check and quantification
5. cDNA Tagmentation to fragment and add adaptors to cDNA
6. Enrichment PCR of adaptor-ligated fragments
7. Repeat purification, quality check and quantitation of cDNA libraries

⁵ SmartSeq2 protocol performed by Neil Ashley, WIMM single cell facility

8. Pooling of library samples ready for sequencing
9. 50bp Single-end sequencing of cDNA libraries using Illumina HiSeq2000 (Wellcome Trust Centre for Human Genetics, University of Oxford).

3.2.3 Data analysis of sequenced samples⁶

Following QC analysis with the fastQC package (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>), reads generated from sequencing as described above were aligned using STAR (Dobin *et al*, 2013) against the mouse genome assembly (Genome Reference Consortium GRCm38 (mm10) UCSC transcripts). Post mapping quality control was assessed using RNA-SeQC (DeLuca *et al*, 2012) (Supplementary Figure 2). Gene expression levels were quantified as read counts using the featureCounts function (Liao *et al*, 2014) from the Subread package (Liao *et al*, 2013) (<http://subread.sourceforge.net>) with default parameters. Only genes with more than 1 count per million in at least 4 samples were kept for any downstream analysis. RPKM values and the multidimensional scaling plot (MDS plot) (Fig. 3.2) were generated using the edgeR package (Robinson *et al*, 2010). The read counts were used for the identification of global differential gene expression between specified populations using the voom-limma method (Law *et al*, 2014; Ritchie *et al*, 2015; Robinson *et al*, 2010). Differential gene expression between healthy and myeloma-bearing mice was compared in CD45-Ter119-CD31-ALCAM-Sca-1+ MSCs, CD45-Ter119-CD31-Sca-1-ALCAM+ osteoblasts, and CD45-Ter119-CD31+ endothelial cells. Genes were considered differentially expressed between populations if they had an adjusted p-value (FDR)<0.1 and $\text{abs}(\log_2\text{FC}) > 1$. Heatmaps were generated using the package pheatmap (<https://CRAN.Rproject.org/package=pheatmap>). MDS plot (Fig. 3.2) and a panel of control genes known to be expressed differentially by the cell types that had been sorted (Fig 3.3)

⁶ All bioinformatics described in section 3.2.3 were performed by Nikki Gray and Emmanouela Repapi, Computational Biology Research Group, WIMM

were used to confirm the success of the sorting process in isolating the target cells of interest.

3.2.4 Gene Set Enrichment Analysis

The Broad Institute Gene Set Enrichment Analysis (GSEA) software (Mootha *et al*, 2003; Subramanian *et al*, 2005) (<http://software.broadinstitute.org/gsea/index.jsp>) was used to explore potential biological significance in the differential gene expression. Input data were RPKM values generated by edgeR as above. GSEA methodology determines a gene's mean expression value for each phenotype and then calculates the gene's differential expression with respect to two phenotypes (in this case, 'myeloma-bearing' vs. 'healthy'). Rather than Log2FC as per the voom-limma method above, it uses a ranking metric ('signal2noise') to rank genes into a list according to their differential expression. The 'signal2noise' metric is the difference of the means scaled by the standard deviation, where μ is the mean and σ is the standard deviation:

$$\frac{\mu_A - \mu_B}{\sigma_A + \sigma_B}$$

The larger the signal-to-noise ratio, the larger the differences of the means (scaled by the standard deviations); that is, the more distinct the gene expression is in each phenotype and the more the gene acts as a "class marker." When a set of genes is compared to the ranked list of all expressed genes, the set generates an enrichment score, dependent upon the sum of the ranking value of genes that appear in the set (explanatory figure: Supplementary Figure 3).

(http://software.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html?Run_GSEA_Page)

The Hallmark gene sets from the Molecular Signatures Database (Liberzon *et al*, 2015) were used as an initial broad analysis of differences in biological behaviour between myeloma-

bearing and healthy mice for each cell type, using FDR of <0.05 to identify sets with significant enrichment. The 50 hallmark gene sets summarise and represent specific well-defined biological states or processes and display coherent expression. These gene sets were generated by a computational methodology based on identifying gene set overlaps, and retaining genes that display coordinate expression, to reduce noise and redundancy (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>).

3.2.5 Novel gene set design

Existing gene set databases (MSigDB, GO, BioCarta, KEGG) were searched for gene sets of relevance to bone marrow stromal or bone cell response to myeloma or metastatic invasion. No ideal matches were found, although various GO gene sets existed for both catabolic and anabolic aspects of bone turnover, which had direct relevance.

I therefore decided to make three new gene sets to investigate the hypothesis that myeloma invasion altered gene expression in healthy MSC and osteoblast development, leading to pathogenic myeloma bone disease. These were designed as follows:

1. 'Bone remodelling' gene set

The above gene set databases were searched using the term 'bone remodeling' / 'bone remodelling'. Only genes expressed in MSCs and their derivatives, rather than those only expressed by osteoclasts or other inflammatory cells during bone remodelling, were included. The following gene sets were combined:

- a) GO 'Bone remodeling' (GO:0046849), including all descendent GO term gene sets – limited to genes with evidence in *Mus musculus* or *Rattus norvegicus*.
- b) MSigDB C2 gene set **ZAIDI_OSTEOBLAST_TRANSCRIPTION_FACTORS**, with additional genes added by consulting source material (Zaidi, 2007)

- c) Cancer Gene Anatomy Project (Biocarta) 'bone remodeling' pathway

(https://cgap.nci.nih.gov/Pathways/BioCarta/h_ranklPathway)

The final set comprised 151 members (Supplementary Table 1)

2. 'Bone formation' gene set

The above gene set databases were searched using the terms 'bone', 'ossification', 'mineralization' and 'osteoblast'. All gene sets concerned with the positive regulation of bone formation or negative regulation of bone loss (ie bone anabolism) were included, but individual genes within sets only included in the final list if the following criteria were met:

- a) Genes were expressed in MSCs or their osteoblast lineage derivatives, in *Mus musculus* or *Rattus norvegicus*.
- b) There was experimental evidence, ideally from animal knockout models, that bone formation was enhanced by (in any way and at any stage), or dependent upon, expression of the gene in question, in the MSC lineage.

Genes were not included if these criteria were not met.

The following gene sets were included:

GO:0002076 - osteoblast development

GO:0045669 - positive regulation of osteoblast differentiation

GO:0002051 - osteoblast fate commitment

GO:0033690 - positive regulation of osteoblast proliferation

GO:0001503 - ossification

GO:0045779 - negative regulation of bone resorption

GO:0045671 - negative regulation of osteoclast differentiation

GO:2001205 - negative regulation of osteoclast development

GO:0030501 - positive regulation of bone mineralization

In addition, genes in the 'bone remodelling' gene set described above were investigated for evidence of being 'pro-bone formation' or 'pro-bone loss'. Those that were 'pro-bone formation', that met inclusion criteria, were added to this gene set if not already present. The final set comprised 198 members. (Supplementary Table 1)

3. 'Bone loss' gene set

This gene set was designed using an identical process to the 'bone formation' set, but reversing the criteria, to include gene sets concerned with the negative regulation of bone formation or positive regulation of bone loss. Individual genes within sets were only included in the final list if the following criteria were met:

- a) Genes were expressed in MSCs or their osteoblast lineage derivatives, in *Mus musculus* or *Rattus norvegicus*.
- c) There was experimental evidence, ideally from animal knockout models, that bone loss was enhanced by (in any way and at any stage), or dependent upon, expression of the gene in question, in the MSC lineage.

Genes were not included if these criteria were not met.

The following gene sets were included:

GO:0045668 - negative regulation of osteoblast differentiation

GO:0033689 - negative regulation of osteoblast proliferation

GO:0045780 - positive regulation of bone resorption

GO:0030502 - negative regulation of bone mineralization

GO:0045672 - positive regulation of osteoclast differentiation

GO:2001206 - positive regulation of osteoclast development

GO:0090290 - positive regulation of osteoclast proliferation

In addition, genes in the 'bone remodelling' gene set that were 'pro-bone loss', that met inclusion criteria, were added to this gene set if not already present.

The final set comprised 154 members. (Supplementary Table 1)

4. BMP signalling gene set

Following identification of increased expression of BMP signalling member *Bmpr1b* as described in results section 3.3, a fourth gene set was designed, comprising members of the BMP signalling pathway. Although BMP signalling-based pathways were possible to find in gene set databases, they were small, not validated and not specific to MSC expression. For this reason a new set was designed, comprising 44 BMP pathway members (recognized ligands, inhibitors, receptors, intracellular signalling components and 6 well-recognized targets. Activin and TGF β receptors but not ligands were included, due to evidence for promiscuous binding being a means of competitive signalling inhibition (Aykul & Martinez-Hackert, 2016)), included only if expressed by murine MSCs (Supplementary Table 1).

5. Bone remodelling set with BMP pathway supplementation

Members of the BMP signalling pathway described above (but not already included) were added to bone remodelling gene set (Supplementary Table 1).

3.3 Results

3.3.1 Gene expression evidence that bone-lining niche cells were appropriately sorted according to phenotype

Firstly, RNASeq generated gene expression data was used to assess whether the 100 cell samples collected by FACS of bone lining niche cells did represent the *in vivo* populations predicted by their antibody binding profile. A multi-dimensional scaling plot of the RNA samples, in which distances correspond to leading log-fold-changes between each pair of RNA samples, was generated (Fig. 3.2). The leading log-fold-change is the average (root-mean-square) of the largest absolute log-fold-changes between each pair of samples.

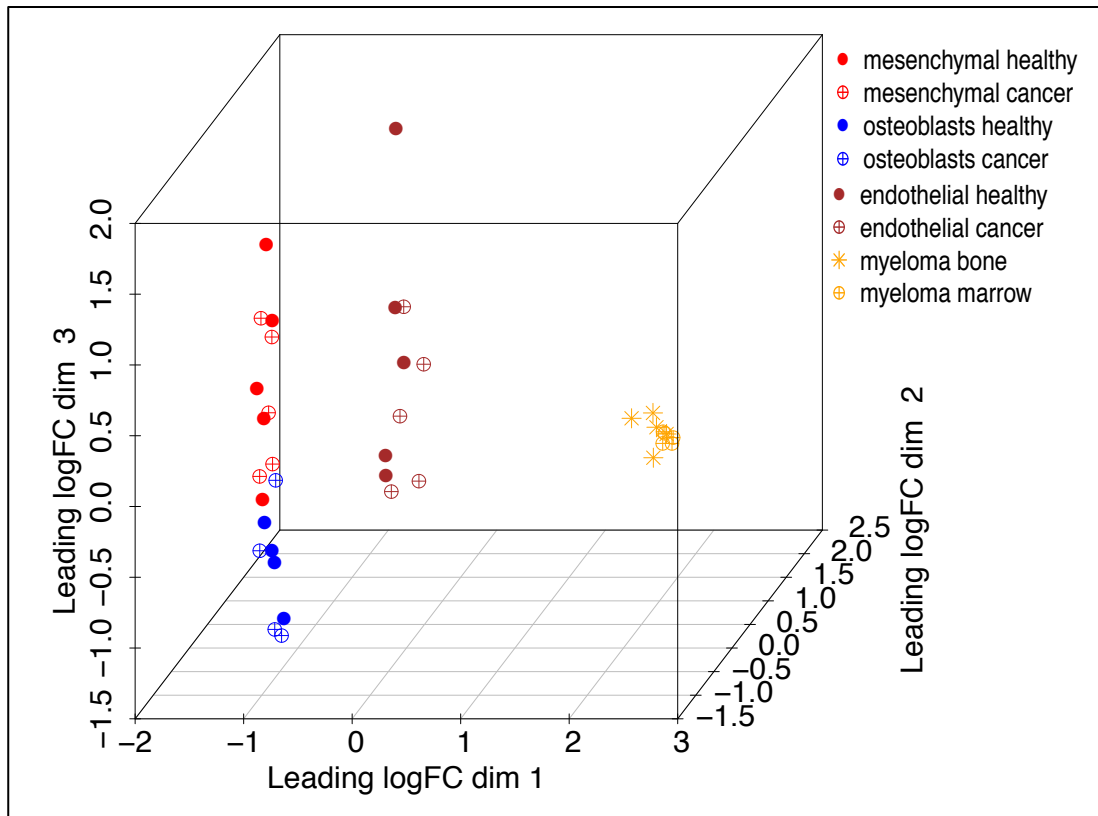


Fig 3.2: RNASeq-generated gene expression data visualisation by MDS plot, to demonstrate unsupervised clustering of bone-lining cell types.

Distances correspond to leading log-fold-changes between each pair of RNA samples, where the leading log-fold-change is the average (root-mean-square) of the largest absolute log-fold-changes between each pair of samples.

This plot can be viewed as a type of unsupervised clustering. It was not expected that the relatively subtle variation between healthy and myeloma-bearing examples of the same cell type would be enough to cause cluster separation, but was expected that the different cell types would separate if they had been sorted appropriately. Clustering of all cell types was demonstrated. The two stages of mesenchymal differentiation, MSCs and osteoblasts, are closer than others. These two cell types are of the same origin but differ only by stage of differentiation; their relative similarity in the context of the whole dataset is therefore to be expected.

Secondly, a small number of genes whose expression defines the cell types that had been sorted were used to assess the accuracy of the procedure (Fig. 3.3). A heatmap was made using logRPKM values to reflect relative expression of the genes included. Genes were not selected on the basis that their expression should or would be different between healthy and myeloma-bearing mice (indeed this possibility was avoided), but rather that their expression in a particular cell type would confirm its phenotype to be as predicted from the expression pattern of the markers used. Two endogenous control genes, *Hprt* and *B2m*, were also included.

1. 5TGM1-GFP myeloma cells uniquely expressed haematopoietic marker CD45 (*Ptprc*), B cell marker *Cd48*, malignant myeloma marker *Slamf7* and immunoglobulin J chain *Igj*. *Cxcl12* ligand *Cxcr4* was also enriched.

2. CD45- Ter119- CD31+ endothelial cells uniquely expressed P-selectin (*Selep*), VE-cadherin (*Cadh5*), and the angiopoietin-1 receptor (*Tek*), all markers of endothelial cells. CD31 (*Pecam1*) is also expressed by some haematopoietic cells including 5TGM1 myeloma cells, but on sorting these had been previously excluded by CD45 staining, as confirmed by *Ptprc*

expression negativity. Endothelial cell-associated genes *Vcam1*, *Eng* and *Fabp4* were highly enriched.

3. CD45- Ter119- CD31- ALCAM- Sca-1+ mesenchymal stromal progenitor cells expressed MSC markers Sca-1 (*Ly6a*), Leptin receptor (*Lepr*), *Pdgfra* and *Pdgfrb*, *Prrx1* and *Cxcl12*. There was also some expression of markers that suggested mixed early populations with a range of commitments may be present: osteoprogenitor markers *Sox9*, *Col1a1* and *Runx2* were all also expressed, although at lower levels than in ALCAM+ cells.

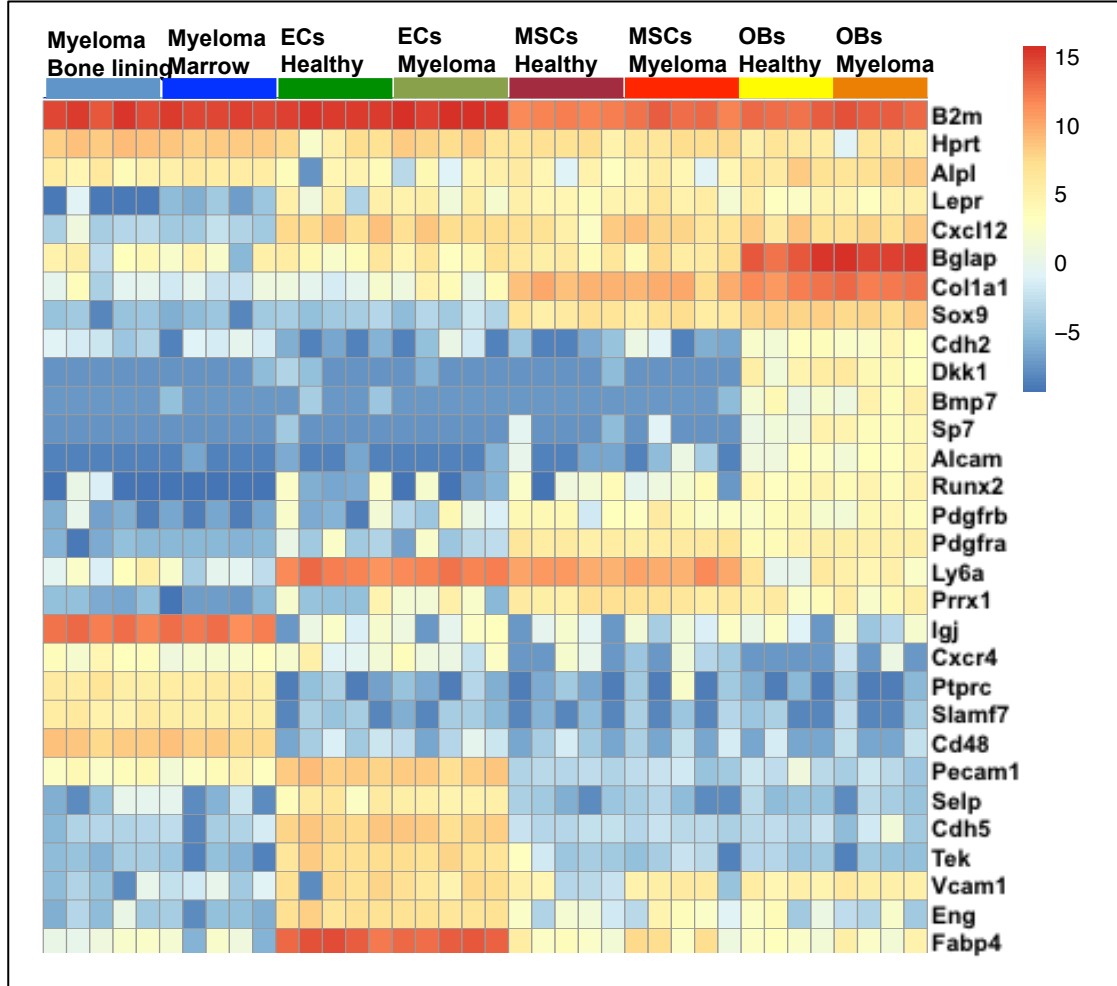


Figure 3.3: Heatmap of the relative expression of selected genes that identify the phenotype of sorted cell populations.
Each column represents a 100-sorted cell sample from an individual mouse, the top colour block legend providing reference of cell type.
Each row represents a particular gene, the colour of each block representing the expression of that gene (by logRPKM values) in that cell population, as per reference scale strip.
B2m and *hprt*: selected endogenous control genes.
ECs = endothelial cells; MSCs = mesenchymal stromal progenitor cells; OBs = osteoblasts.

4. CD45- Ter119- CD31- Sca-1- ALCAM+ osteoprogenitor-osteoblast cells expressed the same MSC markers as the Sca-1+ fractions, although there was lower expression of multipotency markers *Ly6a* (Sca-1), *Prrx1* and *Pdgfra*. Interestingly *Lepr*, *Cxcl12* and *Pdgfrb* were unchanged. However there was marked enrichment of markers associated with osteoblast commitment and maturity: *Sox9*, *Col1a1*, *Runx2*, Alkaline Phosphatase (*Alpl*) and Osteocalcin (*Bglap*). In addition certain genes only expressed as osteoblasts mature were present: *Alcam*, *Bmp7*, Dickkopf-1 (*Dkk1*), Osterix (*Sp7*) and N-cadherin (*cdh2*), all of which had absent expression in the Sca-1+ fraction.

In summary, expression of these gene markers confirmed that bone lining niche cells had been sorted into the anticipated constituents with a sufficient degree of accuracy to commence analysis of gene expression differences between myeloma-bearing and healthy mice.

3.3.2 Gene set enrichment analysis using GSEA MSigDB Hallmark Gene Sets reveals differential pathway activity in cells of the bone-lining niche on invasion by myeloma

As described in section 3.2.4, rpkm values were used by GSEA software to rank expressed genes in each cell type, by difference in expression between healthy and myeloma-bearing mice. Initially, the MSigDB Hallmark gene sets were examined for significant enrichment in either phenotype, for each cell type of the bone-lining niche. Expression of a gene set was considered significantly enriched if p (FDR) < 0.05 . This revealed common upregulation of interferon response sets in all cell types from myeloma bearing mice, and cell cycle-related sets in MSCs and endothelial cells (Figs. 3.4-3.6).

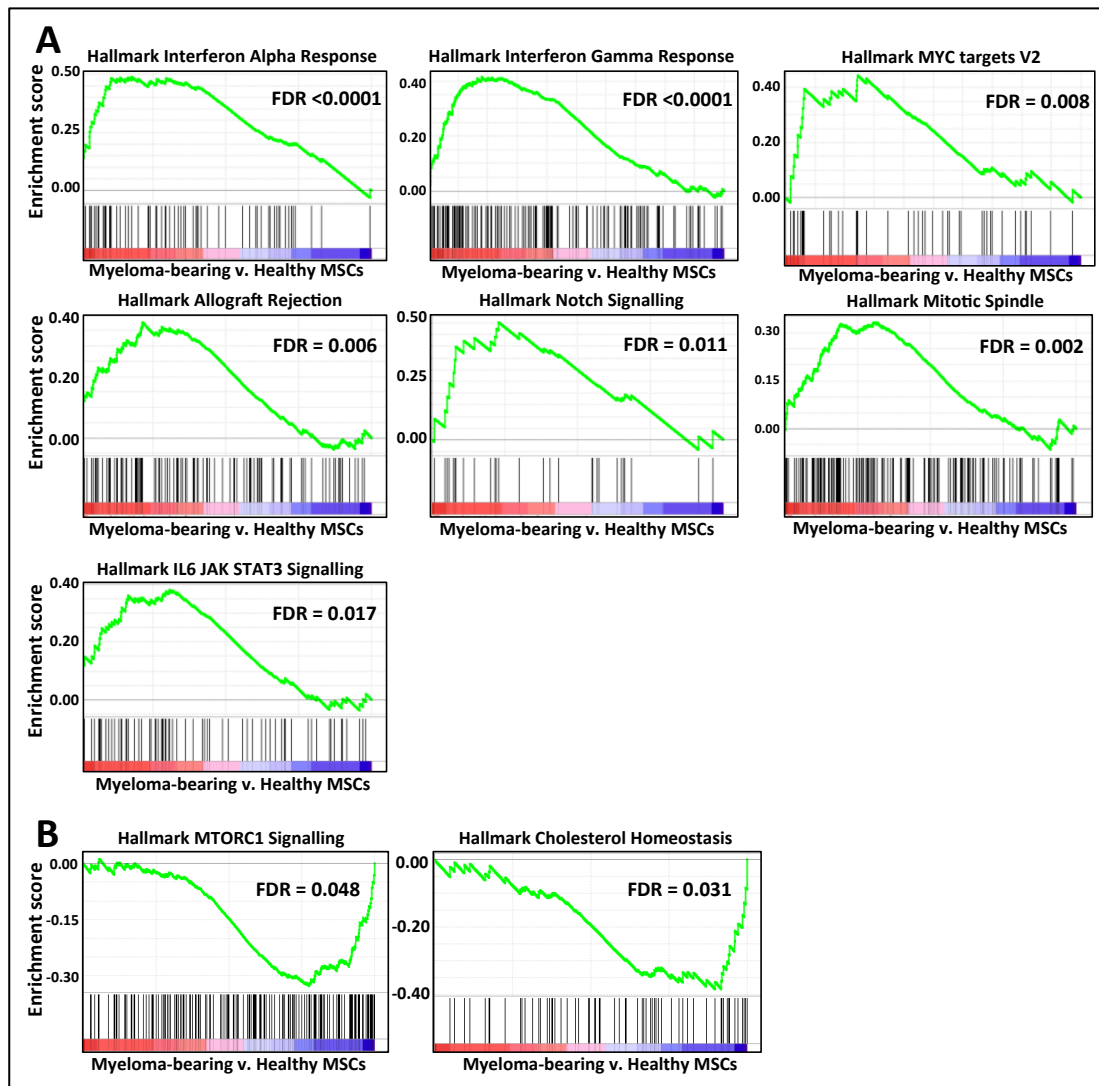


Figure 3.4: Enriched (FDR<0.05) MSigDB Hallmark gene sets in MSCs from 5TGM1-GFP myeloma-bearing and healthy KaLwRij mice.

A: Positively enriched Hallmark gene sets in myeloma-bearing mice.

B: Positively enriched Hallmark gene sets in healthy mice.

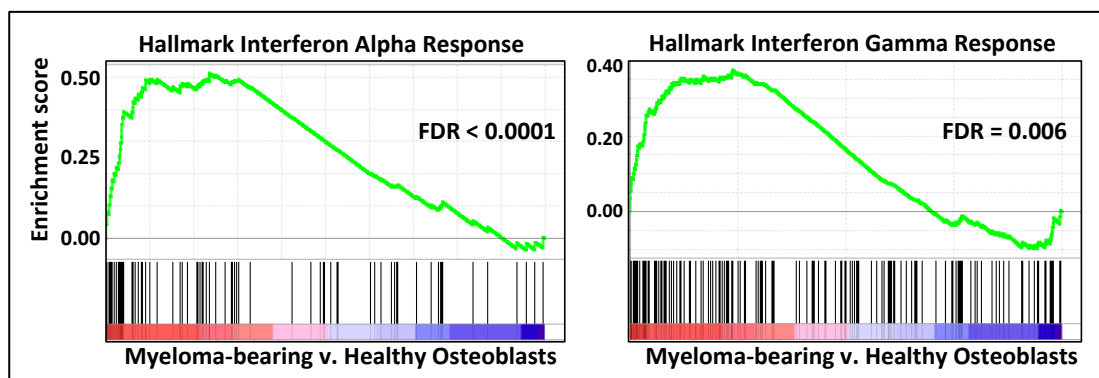


Figure 3.5: Positively enriched (FDR<0.05) MSigDB Hallmark gene sets in osteoprogenitor-osteoblast cells from 5TGM1-GFP myeloma-bearing KaLwRij mice.

There were no MSigDB Hallmark gene sets positively enriched in these cells from healthy mice with FDR<0.05.

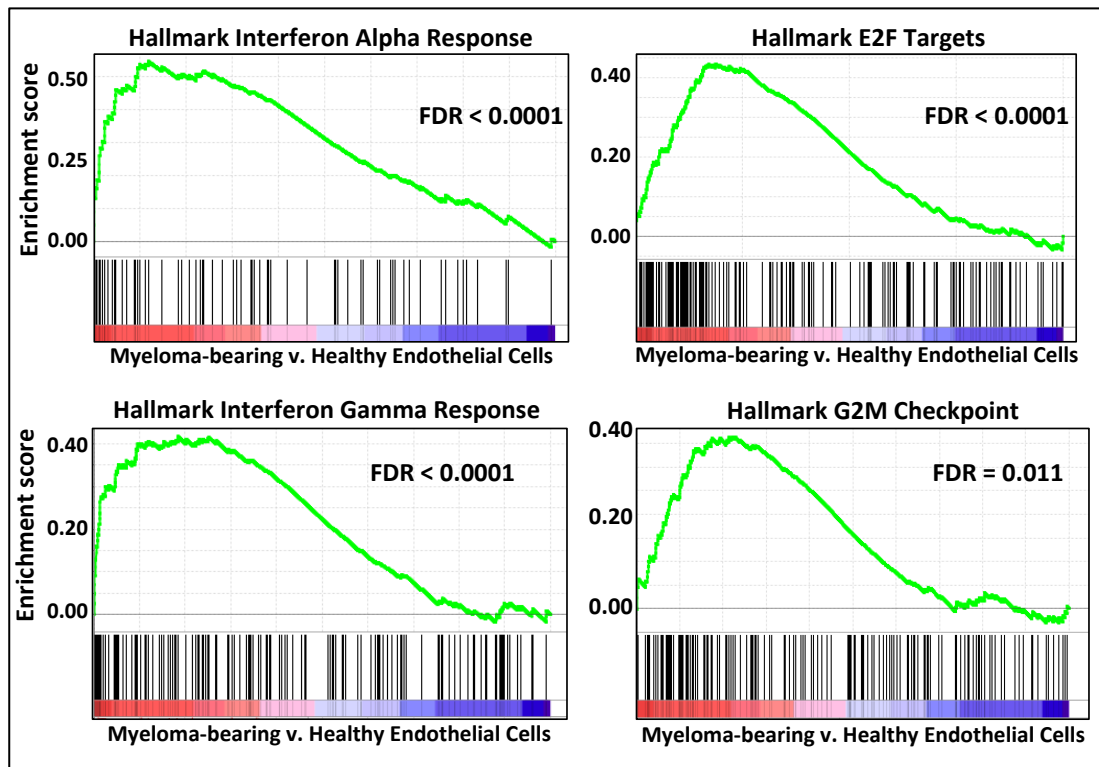


Figure 3.6: Positively enriched (FDR<0.05) MSigDB Hallmark gene sets in endothelial cells from STGM1-GFP myeloma-bearing KaLwRij mice.

There were no MSigDB Hallmark gene sets positively enriched in these cells from healthy mice with FDR<0.05.

Using the Hallmark gene sets gives a ‘broad brush’ overview of some of the cellular processes that may change in niche cells when myeloma invades. However many individual genes in each cell type, not necessarily included in any gene set tested, singly demonstrated a significant fold change in expression between the healthy and myeloma-bearing states. There will inevitably exist in RNASeq datasets considerable ‘noise’ (random variation) between gene expression in individual samples, and the presence of significantly differentially expressed genes that are as yet not curated and poorly understood. Due to the high level of noise and different methods of trying to overcome this, different methods used to identify significance often produce lists of genes without complete overlap. For this reason, as discussed in methods section 3.2.4, two different methods were used here to generate lists of differentially expressed genes, and their results compared. The first method was voom-limma, using significance defined as adjusted p-value (FDR)<0.1 and

$\text{abs}(\log_2\text{FC}) > 1$. This generated only 6 differentially expressed genes between myeloma cells from the two niches (bone lining vs. central bone marrow), and 251 differed between endothelial cells from myeloma-bearing and healthy mice. There were greater transcriptome differences in MSC (82 genes, Fig. 3.7, Supplementary Table 2) than osteoblast (28 genes) subsets. As my hypotheses focused on changes that might mediate bone damage, MSCs had demonstrated the greatest number of significantly enriched Hallmark sets, and MSCs had many more differentially expressed genes than osteoblasts, I decided to take this cell type forwards for further analysis. The second method was GSEA ranking, taking the top 100 genes ranked for differential expression using the signal2noise metric (Supplementary Figure 4). Lists were generated using these two methods of identifying genes differentially expressed between myeloma-bearing and healthy MSCs (Fig. 3.7, Supplementary Table 2 and Supplementary Fig. 4).

In an attempt to further visualise an overview of the differentially expressed genes that already had some level of known function, I decided also to look at individual genes that comprised the leading edges of all the enriched Hallmark gene sets. This ensured that individual genes picked out were not only relatively well curated, but also part of an enriched set representing a biological process, therefore increasing the likelihood that the gene's individual enrichment was biologically meaningful. For this combined leading edge analysis all enriched sets were included, not just those with $\text{FDR} < 0.05$ (Supplementary Figure 5).

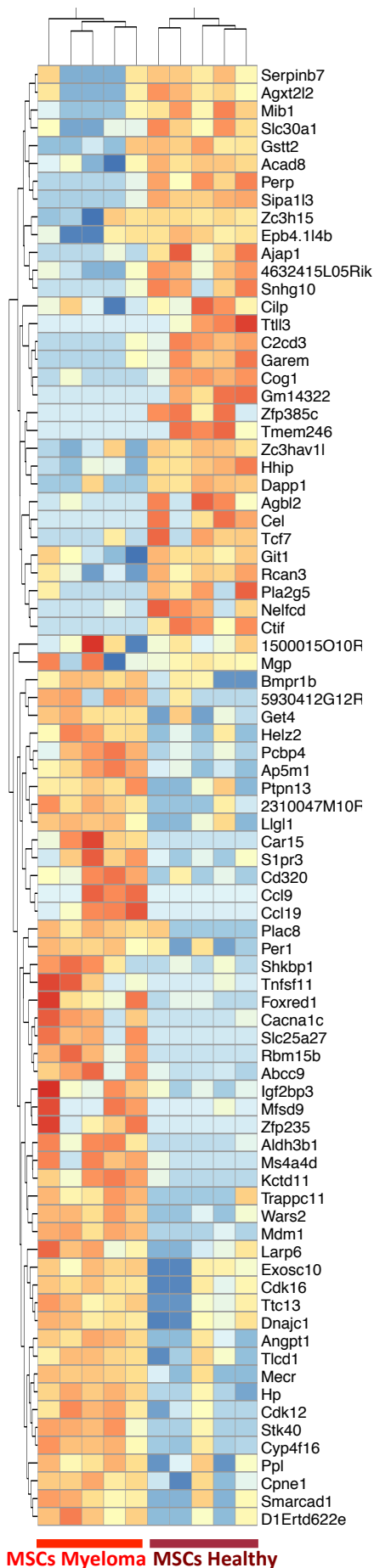


Figure 3.7: Transcriptome profiling of MSCs from bone-lining cells of myeloma-bearing and healthy mice identifies significantly differentially expressed genes between the two states.

Heatmap of MSC gene expression, showing all genes differentially expressed with adj. p-value<0.1 and abs(log2FC)>1 (n = 82).

Each column represents data from a single 100-cell MSC sample from one mouse.

Each row represents relative expression of the gene as labelled.

The orange/blue scale is derived from normalised scores (z-scores) of the rpkm.

One process that is not examined by the Hallmark gene sets is osteogenesis, and few genes of specific relevance to osteogenesis appeared in the Hallmark sets. Looking at the lists of individual differentially expressed genes in MSCs however, there is evidence of gene upregulation that suggests increased vascular remodelling (e.g. *Angpt1*, *Angpt2*, *Kcnj8*, *Postn*, *Wars2*, *Col4a1*) and adipogenesis (e.g. *Adipoq*, *Col4a1*, *Sparcl1*, *Angpt1*, *Coq5*, *Itsn1*, *Fabp4*, *Angptl4*, *Mgll*, *Bcl6*, *Dgat1*, *Apoe*, *Sdhc*) (Fig. 3.7 and Supplementary Figures 4-5). The GSEA top 100-ranked genes (Supplementary Figure 4) tantalisingly reveals cell surface receptors *Smo* and *Bmpr1b* in the top 25 most unregulated genes in MSCs from myeloma-bearing mice, both required for osteogenesis, and *Tgfb2*, overexpression of which is associated with bone loss. Interrogating the data for evidence of the widely reported osteogenic differentiation block in myeloma MSCs required further investigation.

3.3.3 GSEA of MSCs from the bone-lining niche demonstrates alterations in bone homeostasis gene sets in myeloma-bearing mice

To investigate changes in bone homeostasis and remodelling in myeloma driven by gene expression in MSCs, new gene sets were designed, as described in section 3.2.5 above. The three novel gene sets, 'bone formation', 'bone loss' and 'bone remodelling', were interrogated concurrently for enrichment using GSEA (Fig.3.8). Although the 'bone remodelling' set (FDR 0.066) did not reach significance, the 'bone loss' and 'bone formation' gene sets were significantly enriched in bone-lining MSCs from myeloma-bearing mice compared to controls.

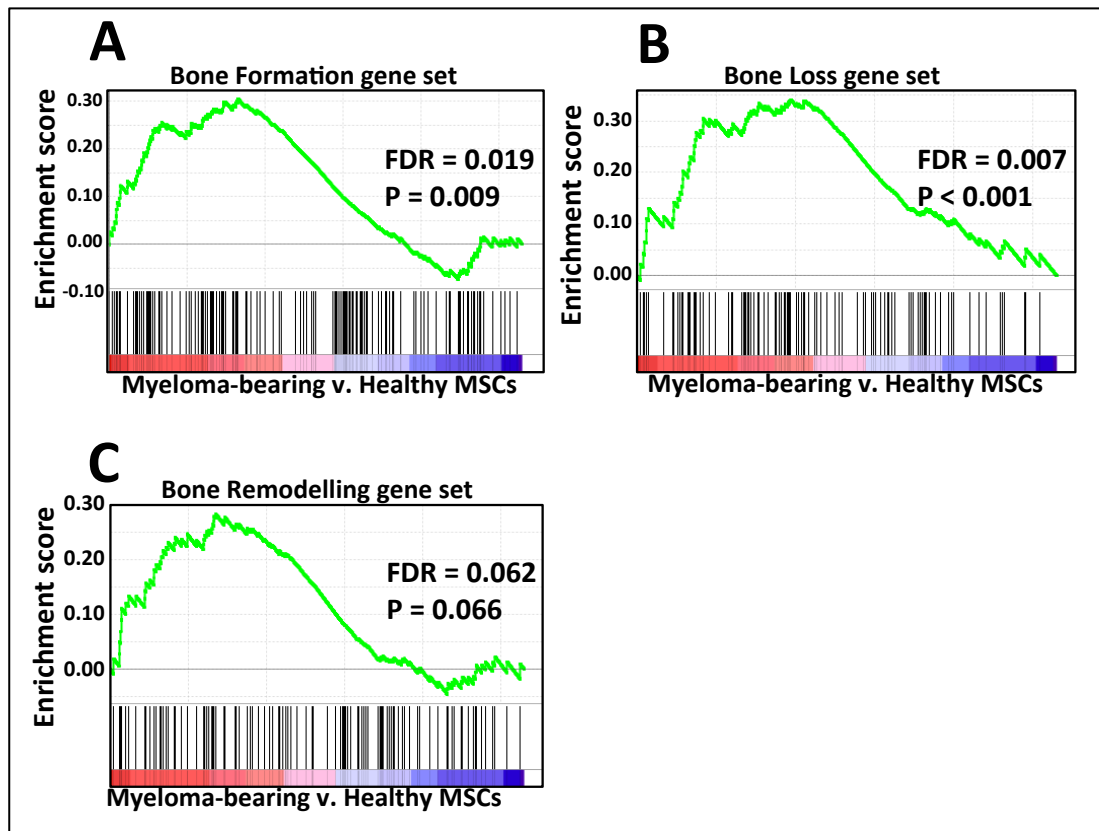


Figure 3.8: Bone homeostasis gene sets show enrichment in MSCs from 5TGM1-GFP myeloma-bearing *KalwRij* mice, in comparison with MSCs from healthy mice.

Only bone formation and bone loss are significant with $FDR < 0.05$.

A: Bone formation gene set

B: Bone loss gene set

C: Bone remodelling gene set

Bone remodelling is a tightly controlled process where bone formation and loss must be balanced in order to maintain bone mass. Even in the early disease model investigated here we noted that, as would be predicted in established myeloma, the enrichment was higher in the bone loss than in bone formation set. However bone formation was also significantly enriched in myeloma-bearing mice, suggesting a complicated and mixed response to the invasion of tumour. The clustering of the hits in the bone formation set (Fig 3.8 A) shows there are also a group of individual genes from this set enriched in healthy mice, also the case with the bone remodelling set, probably the cause of its lack of statistical significance overall (Fig 3.8 A). Biologically, neither of these observations is surprising: particular bone homeostatic pathways and interactions are known to be specifically inhibited (e.g. Wnt

signalling) or upregulated (e.g. RANKL) in myeloma MSCs, whereas other pathways may be unaffected or even have an opposing regulation change as a feedback mechanism.

To identify novel pathways involved in the deregulated bone homeostatic response to tumour invasion, I looked for genes with a potential role in bone homeostasis robustly differentially regulated enough to appear in the lists of significant genes generated by both methods used (Supplementary Table 2 and Supplementary Figure 4). To do this they were combined in a Venn diagram, and the overlapping genes researched for evidence of involvement in bone homeostasis (Fig. 3.9).

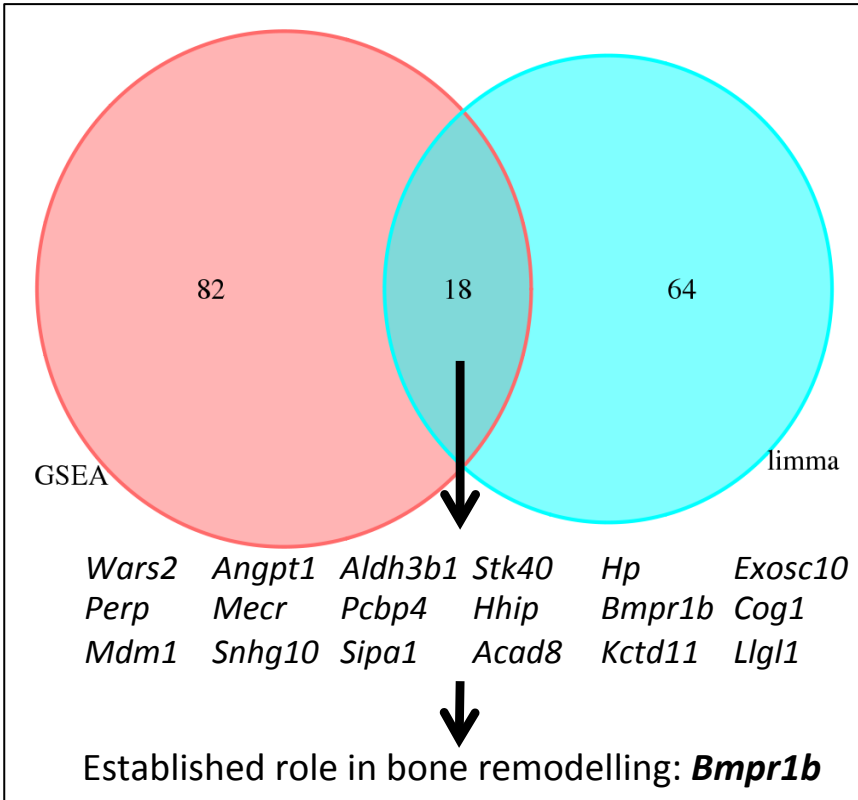


Figure 3.9: Of the genes that overlap between limma-generated differentially expressed genes (adj.p-value<0.1 and abs(log2FC)>1, n = 82) and GSEA-ranked top 100 genes, only *Bmpr1b*/ *Alk6* has an established role in in bone remodelling.

The 18 genes listed were identified by both methods of establishing differential gene expression.

18 genes were found to be shared between both gene lists. *Bmpr1b* (*Alk6*) was the only one with an established role in bone remodelling, although review of the literature suggested some of the other genes had potential for a role: *Angpt1* (angiopoietin 1, shown to mediate

coupling of angiogenesis and osteoblastogenesis (Suzuki *et al*, 2007)), *Sipa1* (Rap-GTPase activating protein; Rap in turn activates Notch signalling (Doi *et al*, 2015)), *Stk40* (deletion enhances adipogenesis in stromal cells (Yu, Hongyao *et al*, 2015)), *Hhip* (hedgehog interacting protein, a hedgehog signalling inhibitor; *Hhip1* mutant mice have delayed skeletal mineralisation (Chuang *et al*, 2003)), *Kctd11* (a ubiquitin ligase component causing inactivation of hedgehog effector Gli (Canettieri *et al*, 2010)) and *Lgl1* (a cell polarity regulator which internalises N-cadherin (Jossin *et al*, 2017)). However, as the evidence of a potential mediating role in osteoblast development for *Bmpr1b* was compelling (Lin, Shuxian *et al*, 2016), and unlike the Notch and Hedgehog pathways its involvement in myeloma bone disease was hitherto undescribed, further analysis of whether the BMP signalling pathway was otherwise dysregulated was performed.

3.3.4 BMP pathway genes are shown to be upregulated in MSCs from the bone-lining niche on invasion by 5TGM1-GFP myeloma

As discussed in Chapter 1, activity of the BMP pathway is known to be required for bone formation and remodelling. However we could find no published evidence regarding the role of this pathway in myeloma bone disease, despite there being some evidence that deregulated TGF β (Matsumoto & Abe, 2011) and Activin (Chantry *et al*, 2010; Vallet *et al*, 2010) signalling enhance myeloma bone disease, members of the same superfamily which all require Smad4 for intracellular signalling and whose receptors are increasingly shown to be able to bind family ligands widely, even if signal transduction has little or no efficiency (Aykul & Martinez-Hackert, 2016) (see Chapter 1).

Examination of the leading edges of the bone homeostasis gene sets was performed, to see if other BMP pathway members were contributing to their enrichment. BMP signalling has

roles in both bone formation and resorption (Sánchez-Duffhues *et al*, 2015), but the gene set combining these processes, bone remodelling, was found to contain only 3 BMP pathway genes, *Acvr1*, *Bmp4* and *Grem1* (Supplementary Table 1). *Acvr1* was nonetheless in the leading edge of the set. Having identified *Bmpr1b* to be differentially expressed, I hypothesized that other members of the BMP pathway may also be differentially expressed. I therefore designed a BMP signalling pathway gene set (Supplementary Table 1), which I found to be significantly enriched in MSCs from myeloma-bearing mice (Fig. 3.10 A). To assess if this enrichment was equivalent in magnitude to that of my other bone homeostasis sets, the bone-remodelling gene set was expanded to include the BMP pathway gene set members (BMP family members added $n = 41$, prior gene set size $n = 151$, BMP pathway members 23% of total). This resulted in a rise in the enrichment score and reduction in the FDR from 0.066 (Fig. 3.8 C) to 0.007 (Fig 3.10 B). The leading edge ($n = 41$) of the expanded gene set was comprised 32% (13/41 genes) of BMP pathway members, targets or interactors, alongside known mediators of myeloma-induced osteolysis such as RANKL (*Tnfsf11*), supporting a role for the BMP pathway in myeloma bone disease (Figure 3.11).

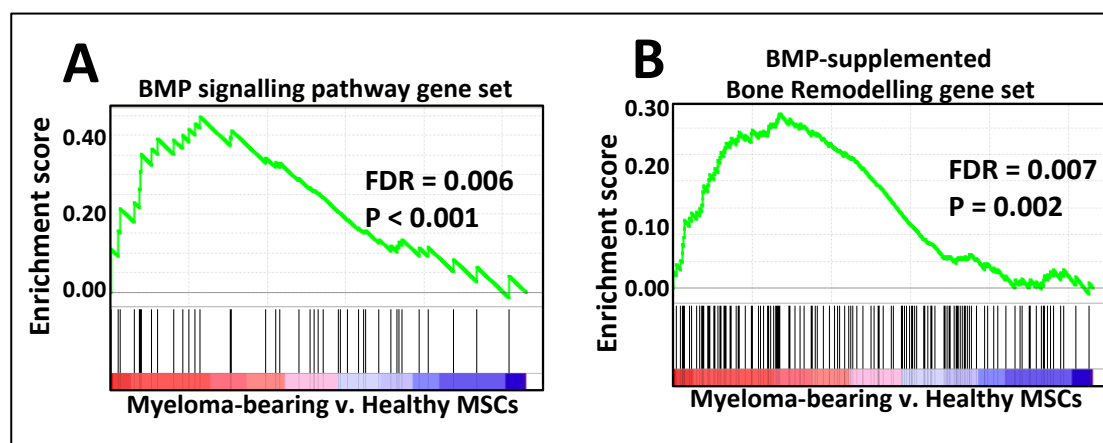


Figure 3.10: The BMP signalling pathway is significantly enriched in MSCs from myeloma bearing mice, and the bone remodelling gene set enrichment is strengthened by the addition of BMP pathway members to the gene set.

A: Enrichment plot of BMP pathway gene set

B: Enrichment plot of bone remodelling gene set (note original in Fig. 3.8 C) with addition of 44 members of the BMP pathway.

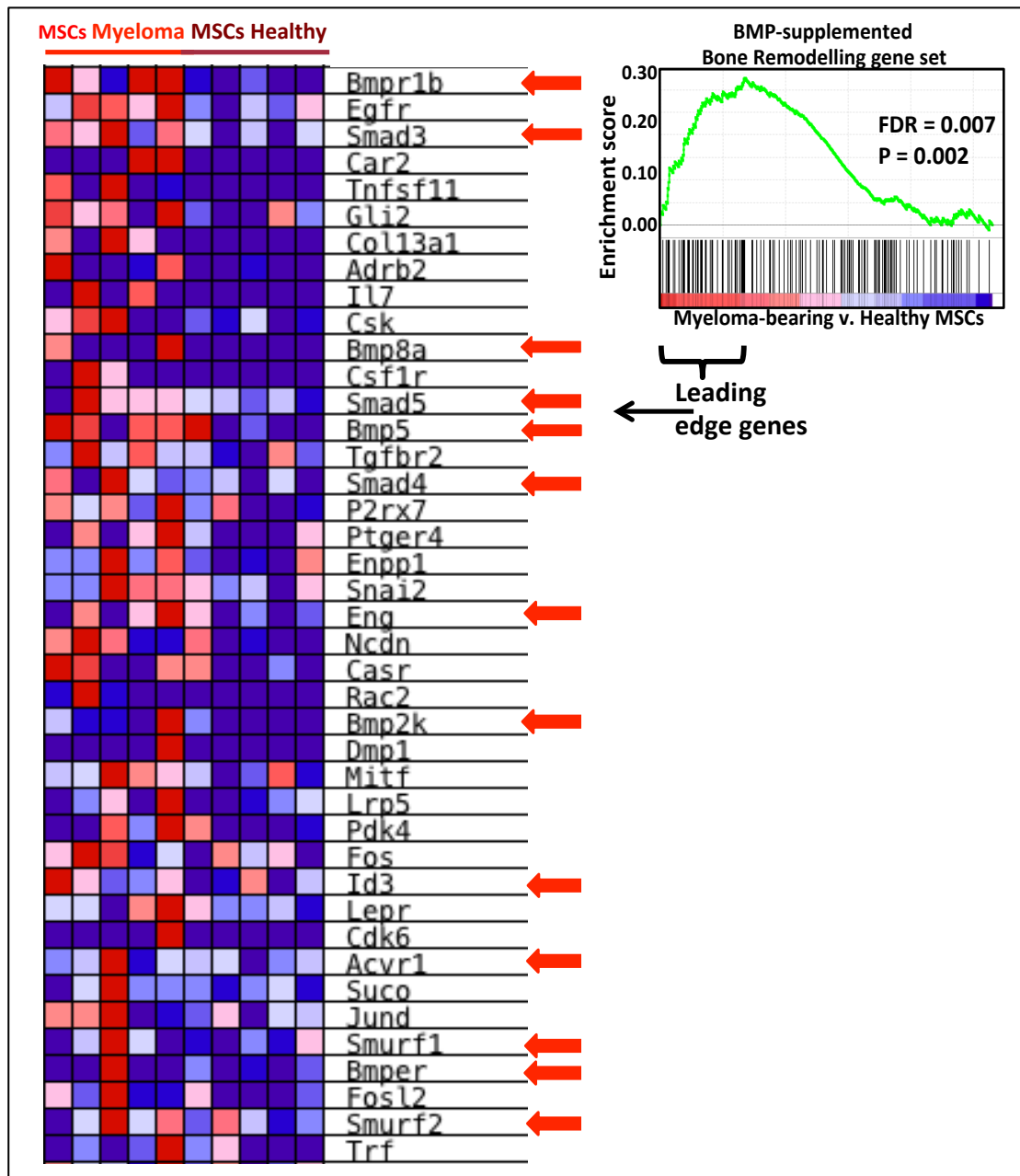


Figure 3.11: The BMP pathway members are overrepresented in the leading edge of the BMP-supplemented bone remodelling gene set

The leading edge (genes which contribute most highly to the enrichment score) comprises 32% BMP pathway members or interactors, as indicated by red arrows. BMP members and interactors make up 23% of the total gene set (n = 192).

3.4 Discussion

This chapter began with the observation that unsupervised profiling of the niche transcriptome changes that occur *in vivo* on myeloma invasion of the bone lining had never been reported. I hypothesized that, in view of the known disruption to bone homeostasis that myeloma causes in this niche, significant transcriptional changes would occur in certain pathways in certain cell types. I further hypothesized that the information yielded on which pathways are up or down-regulated would give novel information about the mechanisms by which bone repair capacity is lost, and therefore new therapeutic targets would be revealed.

Using protocols developed for the investigation of haematopoietic niche maintenance (Nakamura *et al*, 2010), I sorted three endosteal niche cell types and tumour from two locations (bone lining and central marrow) to investigate transcriptional differences between myeloma-bearing and healthy mice, and tumour from different niches. On MDS plotting these populations separated by cell type (Fig. 3.2), and marker gene expression confirmed their identities were as expected (Fig 3.3). Nakamura *et al* demonstrated that Alcam-Sca1⁺ MSCs had the potential to differentiate down osteogenic or adipogenic pathways, although my gene expression analysis confirmed some evidence of early osteogenic differentiation in this subset (*Sox9*, *Col1a1* and *Runx2*) and it is likely to contain a range of early commitment stages. The Alcam⁺Sca1⁻ fraction however reportedly retained poor ability to pursue adipogenic differentiation (Nakamura *et al*, 2010) and our analysis confirmed a range of osteoblastic markers uniquely present or enriched in this population (*Bglap*, *Col1a1*, *Sox9*, *Cdh2*, *Bmp7*, *Sp7*).

There are many developmental steps from skeletal stem cell to matrix-secreting osteoblast or osteocyte, and it was not possible in this dataset, without single cell sequencing and

many more markers, to assess gene expression changes in more defined developmental subsets, although that would be of great interest in early myeloma bone disease and has not previously been done. Since the initiation of this work, high throughout single cell sequencing has become more feasible and accessible; with these technological developments, more information could be gained by sequencing high numbers of unsorted single cells from the bone lining niche, and allowing high-dimensional data plotting (e.g. t-SNE) and clustering to determine sub-phenotypes of niche cells. Thus the magnitude and nature of changes between myeloma-bearing and healthy mice could be compared from one osteoblast developmental stage to the next, so enabling a focus on stages of most difference in the search for the 'point of differentiation block'. Even more excitingly, the lack of a need of cell surface markers to sort cell types pre single cell analysis means that for the first time, these techniques could be applied to human trephine specimens; if single bone lining cells could be obtained from such specimens by digestion, their transcriptomes could be used to define MSC to osteoblast or stromal developmental stage, and how each stage may be altered in number and behaviour between healthy individuals and those with MGUS and myeloma. Obtaining trephines from healthy individuals remains a logistical challenge to this type of experiment however.

Only 6 genes were significantly different between the myeloma bone-lining and central marrow compartments, and the preparation technique had introduced bias, so these differences were not investigated further. 251 genes were differentially expressed between endothelial cells, 82 between MSCs and 82 between osteoblasts from myeloma-bearing and healthy mice. With only 5 transcriptomes per condition, high noise-to-signal is likely to mean this is under representative, but also that validation of findings is essential.

Datasets of entire transcriptomes yield far more information than can be thoroughly investigated in the course of one research project. Despite the fact that many of the differences revealed between myeloma-bearing and healthy mice may have represented relevant and novel biological information, it was necessary to decide which cell type to focus on, then which gene or pathway expression differences to take forward as the focus of the research project. Gene set enrichment analysis (GSEA) using a screening panel of gene sets such as the Hallmark sets is a helpful technique that can give an overview of changes in non-specific biological processes. Unsupervised analysis using the Hallmark sets initially revealed common upregulation of interferon alpha and gamma response genes in all bone lining niche cell types, Notch signalling in MSCs and cell cycle-related sets in MSCs and endothelial cells. That MSCs and endothelial cells proliferate in response to myeloma is already well reported (Noll *et al*, 2014b; Ribatti *et al*, 2014), so upregulation of gene sets consistent with this occurring (mitotic spindle and Myc response in MSCs, E2F and G2M checkpoint in endothelial cells) is not necessarily novel information. Upregulation of Notch signalling in bone and stromal cells on contact with myeloma is also previously reported (Delgado-Calle *et al*, 2016a; Nefedova *et al*, 2004). Enrichment of the interferon and allograft rejection response sets are consistent with immune recognition of, and response to, the invading tumour, and a T cell infiltration of the myeloma environment. This finding is interesting, firstly because IFN- α has historically been used as a treatment in myeloma due to its immunomodulatory and anti-tumour effects (Parker *et al*, 2016), but also because IFN- γ secreting Th1 cells are reported to be lower than normal in human myeloma, with a skew instead to IL6 and IL17-secreting T cells, driving osteoclastogenesis and part of the picture of T cell anergy and abnormal tumour tolerance (Li *et al*, 2014; Noonan *et al*, 2010). However, the IL6 response was also enriched in MSCs. The KalwRIJ 5TGM1 myeloma model, where a very aggressive myeloma arrives suddenly in a previously immunologically healthy marrow may not in this regard be fully representative of human myeloma, which develops slowly

over time, progressing from MGUS, with gradual development of immune tolerance. For this reason we concluded that this model was not ideal to recapitulate the immune microenvironment of myeloma development, and instead pursued gene sets that might represent transcriptomic changes related to the onset of bone disease. This resulted in a decision not to take forward further investigations using endothelial cell data, but instead to focus on the transcriptome changes in non-fate committed MSCs, which were greater than those in cells already committed to an osteoblast fate.

The exact stage(s) of osteogenic differentiation blocked by myeloma are unknown. Downregulation of master transcriptional regulator *Runx2* is a feature (Roodman, 2011), but this is a relatively late marker of preosteoblast commitment (Rutkovskiy *et al*, 2016). It is likely that genes upstream of the point of differentiation block could be upregulated in response to downstream differentiation block. Alternatively, in the early disease stage analysed here, with likely patchy myeloma-bone lining contact, mixed features of blocked and activated osteoblast differentiation may be present. I sorted bone-lining cells from mice with relatively early myeloma infiltration (central marrow myeloma cell infiltration ranged from 1.7 to 7.5%, mean 3.24%, of total live mononuclear cells sorted) to ensure adequate cells could be collected; in later disease this population becomes extremely scarce. Pre-designed bone formation, bone loss and bone remodelling gene sets were tested for enrichment as a means of assessing whether changes in bone lining MSCs from myeloma-bearing mice demonstrated the expected deregulated bone homeostasis. However, in addition to 'bone loss' gene set enrichment (FDR 0.007), we also observed 'bone formation' gene set enrichment (FDR 0.019). In early stages of myeloma bone disease, osteoblasts are reportedly increased, in an attempt to maintain normal coupling between osteoclasts and osteoblasts in the face of increased osteoclastic activity (Bataille *et al*, 1991). Therefore, it is not surprising that mixed features of blocked and activated osteoblast differentiation were

seen. The bone remodelling gene set did not reach significant enrichment (FDR 0.066). As a combination of the other two sets, the bone remodelling set contained elements of each, which may have led to no overall enrichment in one direction or the other.

As the bone homeostasis gene set enrichments suggested that the MSC transcriptome data was capable of showing myeloma-induced perturbations to bone homeostasis, I proceeded to use different methods of assessing differential gene expression (limma and GSEA ranking analyses) and took forward overlap genes only to consider for further investigation. Of the 18 genes that featured in this overlap (Fig. 3.9), *Bmpr1b* stood out as having well-established evidence of a role in bone homeostasis. This led me to examine changes in the BMP pathway specifically, and relative to a broad set of bone homeostasis genes. A BMP signalling gene set was significantly enriched in myeloma-bearing MSCs (FDR 0.006, Fig. 3.10). As BMP signalling in bone lining cells reportedly has roles in stimulating both osteoclasts and osteoblasts, I used the BMP set to supplement the bone remodelling set, increasing its size by 23% with BMP pathway members, with the purpose of comparing the magnitude of BMP pathway enrichment to that of other bone homeostasis genes. This resulted in significant enrichment of the bone remodelling set, with FDR dropping from 0.066 to 0.007. Its new leading edge was comprised 32% BMP pathway members.

The enrichment of BMP pathway members relative to bone homeostasis genes as a whole in MSCs from myeloma-bearing mice led me to hypothesize that either this represented a feedback effect of downstream osteogenesis block, or that BMP signalling had a direct role in the osteoblastic differentiation block occurring in bone lining cells in myeloma bone disease. If the former hypothesis were correct, inhibiting BMP signalling would not improve bone disease, as the downstream block would still be present. However, if the latter hypothesis were correct, inhibiting BMP signalling would modify the differentiation block

and osteoblastogenesis and bone formation in myeloma would improve. The next stage of this project was planned to test the experimental question generated by these hypotheses. The effect of the disruption of BMP signalling on myeloma-bone interactions *in vivo* was investigated, and this work is presented in chapters 4 and 5.

Chapter 4 Disruption of BMP signalling in myeloma-bone interactions

4.1 Introduction

In chapter 3, the transcriptome profile differences between bone-lining niche cells of healthy and 5TGM1 myeloma-bearing KaLwRij mice were discussed. As my aim was to discover bone therapeutic targets, and the transcriptome differences were greater in Sca1+ALCAM⁻ cells (MSCs / osteoblast progenitors) than Sca1-ALCAM⁺ cells (committed osteoblasts), differentially expressed genes in MSCs were taken forwards for further investigation. Differential gene expression and GSEA enrichment score rankings were combined to identify a small number of robustly differentially regulated genes, which included *Bmpr1b* among 18 genes differentially expressed by both methods. BMP pathway signalling is highly relevant in bone cell fate commitment and differentiation (Salazar *et al*, 2016), so this pathway was then specifically interrogated for differential expression in MSCs from healthy vs. myeloma-bearing mice. Differential expression of genes involved in BMP signalling was significantly enriched in these cells in myeloma-bearing mice. It was also found that by supplementing my previously-designed non-significantly enriched bone-remodelling gene set with BMP pathway members, this set became highly significantly enriched, with BMP pathway members forming 32% of the leading edge genes. This led me to hypothesize that BMP signalling is deregulated in osteoblast progenitors of the myeloma bone lining niche, and therefore could represent a therapeutic target in myeloma bone disease.

4.1.1 BMPs in myeloma

BMP signalling has been previously investigated in myeloma, although not in relation to bone homeostasis in the tumour niche. Kawamura *et al* first showed direct application of BMP2 to kill myeloma cell lines and primary human samples *in vitro* by induction of apoptosis (Kawamura *et al*, 2000). They went on to hypothesize that as BMP2 had a known

role in inducing bone formation, it may have a role in myeloma both due to its anti-tumour effect and the potential to 're-build' lost bone. Others have repeated this work with a wider range of BMP ligands (Hjertner *et al*, 2001; Olsen *et al*, 2014; Ro *et al*, 2004). Holien *et al* went on to demonstrate that *in vitro* BMP-induced myeloma cell apoptosis was mediated by downregulation of *MYC* expression by intracellular SMAD signalling, and that some patients with constitutive *MYC* activation were resistant to BMP-mediated apoptosis (Holien *et al*, 2012). Critically, all of this work was performed *in vitro* with isolated myeloma cell lines or patient samples, and these experiments were not replicated *in vivo* or using *in vitro* tumour microenvironment models.

BMP ligand expression across a range of human myeloma samples has been analysed by those looking for disease outcome biomarkers. Two groups have shown that BMP6 is the main BMP expressed by primary myeloma cells (Grcevic *et al*, 2010; Seckinger *et al*, 2009). Seckinger *et al* found BMP6 expression to be a marker of superior patient survival. Grčević *et al* showed that BMP6, BMP4 and their receptors and targets were overexpressed in primary myeloma samples, and in potentially contradictory findings to the above data, showed that exogenous BMPs protected myeloma cell lines from bortezomib-induced apoptosis, instead inducing pro-survival proteins ID1, ID2 and IL6, while downregulating pro-apoptotic CDKN1A and BAX.

Finally, it has been shown that excess circulating BMPs may mediate the inflammatory anaemia commonly observed in myeloma and linked to hepcidin overexpression (Sharma *et al*, 2008). BMP2 was found to be elevated in myeloma patient serum, and addition of neutralising anti-BMP2/4 significantly abrogated the stimulatory effect of myeloma patient serum on hepcidin promoter activity in HuH7 hepatocellular carcinoma cells, whereas anti-IL6, anti-BMP6 and anti-BMP9 did not (Maes *et al*, 2010). I concluded that the effects of

BMP signalling perturbation on myeloma *in vivo* had not been adequately assessed, either on the tumour itself or its accompanying pathological effects. This led me to investigate methods by which this could be achieved.

4.1.2 LDN193189 (LDN) is a small molecule inhibitor of Type 1 BMP receptor signalling

BMP signalling has previously been experimentally inhibited *in vivo*, particularly in the investigation of ameliorating the inherited bone-forming disease Fibrodysplasia Ossificans Progressiva (FOP) where a mutation in the ACVR1 Type 1 BMP receptor enables BMP signalling to be activated by Activin A rather than only BMP ligands, leading to a phenotype of heterotopic ossification of skeletal muscle at points of inflammation (Hatsell *et al*, 2015). LDN193189 is a small molecule inhibitor of SMAD phosphorylation; it targets the kinase activity of the Type 1 BMP receptor intracellular kinase domain by reversibly competing for the ATP binding pocket. It was developed as a specific and potent inhibitor of ALK2, ALK3 and ALK6 Type 1 BMP receptors (Figure 4.1) (Cuny *et al*, 2008) for the purpose of *in vivo* experimental use, and inhibition of BMP signalling *in vivo* with this small molecule antagonist was shown to prevent ossification in mouse models of FOP (Yu *et al*, 2008). It has been shown to be safe and has Type 1 BMP receptor antagonistic activity in a range of murine disease models (Balboni *et al*, 2013; Steinbicker *et al*, 2011b; Whissell *et al*, 2014), including dosing for up to 20 weeks (Derwall *et al*, 2012). Of note, I wished to generally inhibit Type 1 BMP signalling as both *Bmpr1b* (Alk6) and *Acvr1* (Alk2) were significantly enriched on GSEA analysis. Although I considered ways to agonise BMP signalling for comparison, there is no Type 1 BMP receptor signalling agonist currently feasible for prolonged *in vivo* use, as recombinant BMPs themselves have a short half-life. BMP pathway upregulation has been described *in vivo* using Anti-Gremlin1 antibody (Ciucan *et al*, 2013)

but this reagent could not be obtained. Another method described is upregulation of BMP signalling by inhibiting

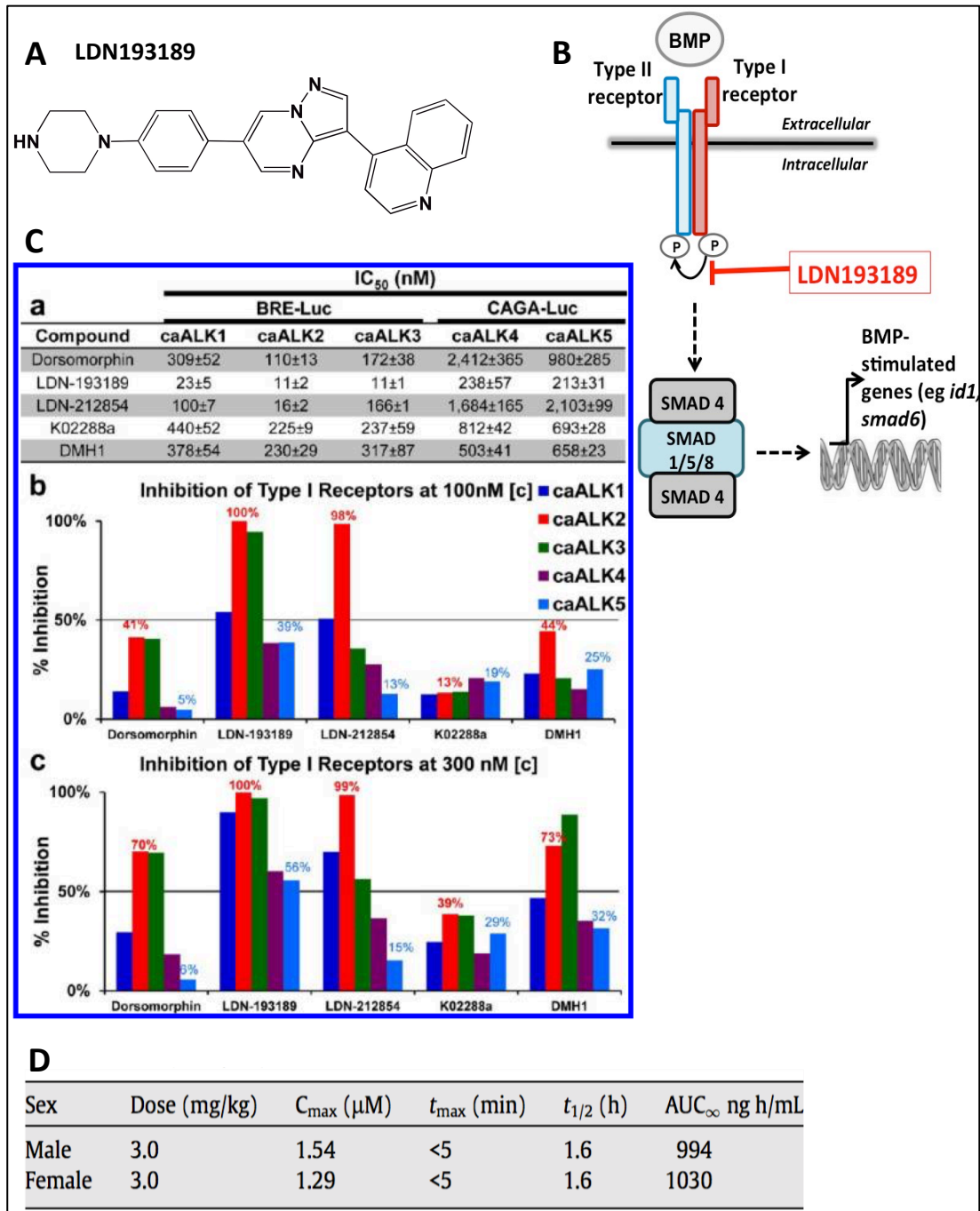


Figure 4.1: Structure and activity of Type 1 BMP signalling small molecule inhibitor LDN193189 (reproduced with permission as below).

A: Structure of LDN193189

B: Diagram of mechanism by which LDN193189 inhibits SMAD phosphorylation by BMPRI intracellular kinase domain, so abrogating BMP pathway signalling.

C: Potency and selectivity of BMP inhibitors based on BMP (BRE-Luc) and TGF-β (CAGA-Luc) transcriptional activity mediated by constitutively active ALK1-5. (a) In vitro cell-based assay IC₅₀ measurements against constitutively active BMP (caALK1, 2, and 3) and TGF-β (ALK4 and 5) type I receptors. (b,c) Inhibition of caALK1-5 by BMP inhibitors at various concentrations. At 100nM LDN193189 equally inhibits ALK2 and ALK3 at near 100% potency. Data shown are calculated on the

basis of IC₅₀ generated from at least 3 independent experiments, with data plotted as mean ± SEM. Reprinted with permission from Mohedas, AH, Xing, X, Armstrong, KA, Bullock, AN, Cuny, GD, Yu, PB (2013) Development of an ALK2-biased BMP type I receptor kinase inhibitor. ACS chemical biology, 8, 1291-1302. Copyright 2013 American Chemical Society (Mohedas *et al*, 2013).

D: Pharmacokinetic analysis of LDN193189 in plasma following bolus intraperitoneal administration in mice (N = 3/sex). Reprinted with permission from Elsevier from Cuny, GD, Yu, PB, Laha, JK, Xing, X, Liu, JF, Lai, CS *et al* (2008) Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. Bioorg Med Chem Lett, 18, 4388-4392 (Cuny *et al*, 2008).

E3 ubiquitin ligase Smurf1- mediated Smad 1/5 degradation (Cao *et al*, 2014). However, the small molecule inhibitors described have not to our knowledge ever been used *in vivo*.

I decided to use LDN193189 for inhibition of Type 1 BMP signalling in a murine myeloma model, to assess the effect on tumour burden, bone destruction and anaemia.

4.1.3 Type 1 BMP receptors in bone homeostasis

Despite sharing many ligands and the same intracellular signalling machinery, type 1 BMP receptors Acvr1 (Alk2), Bmpr1a (Alk3) and Bmpr1b (Alk6) have distinct roles in osteogenesis, which can also differ between developmental and adult bone homeostasis. As described above, when Acvr1 signalling is aberrantly activated outside bone, heterotopic ossification occurs. However, bone-conditional Acvr1 knock out in embryonic or newborn mice leads to increased bone mass (Kamiya *et al*, 2011). In 2T3 pre-osteoblastic cells *in vitro*, Bmpr1b truncation blocks osteogenic mineralisation and overexpression enhances it (Chen *et al*, 1998). Conditional Bmpr1b knockdown under Col1-Cre causes severely reduced bone density; however Bmpr1b null mice had only mild ossification defects. Bmpr1a knockout has a varied effect depending on the promoter site used and age at which knockout occurs; in embryonic mice, when Bmpr1a knockout used mature osteoblast gene Og2-Cre, reduced bone mass was seen. However in older mice (10 months), or mice of any age using Col1-Cre (expressed earlier in osteoblastogenesis), increased bone mass is always seen (Lin, S. *et al*, 2016). Enhanced bone mass following Acvr1 or Bmpr1a KO has since been shown to be

mediated by reduced levels of downstream Wnt inhibitors Dkk1 and Sost (Kamiya *et al*, 2011; Kamiya *et al*, 2010), and also a reduced RANKL/OPG ratio leading to reduced osteoclastogenesis, as RANKL and OPG are also downstream targets of both Bmpr1a and Wnt signalling in osteoblasts (Baron & Kneissel, 2013; Kamiya, Nobuhiro *et al*, 2008).

4.1.4 Hypotheses of this chapter

To summarise, BMPs are previously reported to induce the apoptosis of myeloma cells *in vitro*, and BMP signalling induces hepcidin expression in the liver; hepcidin mediates inflammatory anaemia. Knockdown of type 1 BMP receptors under bone-specific promoters has lead to increased bone mass phenotypes in mice.

As reported in this thesis, transcriptomic profiling has showed BMP signalling to be upregulated in the mesenchymal progenitor cells of the bone-lining niche in multiple myeloma.

Based on these observations:

Hypothesis 1: *In vivo* a BMP signalling antagonist will increase tumour burden.

Hypothesis 2: Inhibition of Type 1 BMP signalling *in vivo* will reduce bone destruction in multiple myeloma.

Hypothesis 3: Inhibition of Type 1 BMP signalling *in vivo* will improve inflammatory anaemia in multiple myeloma.

4.2 Experimental design and specific methods

Experiments to assess the effect of inhibiting BMP signalling *in vivo* in myeloma were preceded by *in vitro* experiments. The purpose of these experiments was to extend published observations of the effects of BMPs on myeloma tumour cells *in vitro*, by incorporating elements of the bone marrow microenvironment, and BMP inhibition by LDN193189. The methodology for *in vitro* cell culture experiments is described in chapter 2, with specific details added below. LDN193189 was then tested *in vivo* using the KaLwRij/5TGM1 murine myeloma model, also as described in chapter 2. The experimental design is described below, with further details of analysis methods in chapter 2 as specified. This 30-mouse experiment was repeated twice, although certain analyses (bone histomorphometry and hepcidin analyses) were only performed on the repeat experiment, meaning n = 60 for some indices but n = 30 for others (as indicated in appropriate figure legends). Finally, in a repeat experiment of that described in chapter 3, bone lining niche sorting and RNASeq analysis of bone lining cells from myeloma-bearing mice treated with LDN193189 or vehicle was performed. The methodology for this experiment is as that described in section 3.2 and below.

4.2.1 *In vitro* experiments to determine the effects of BMPs and LDN193189 on myeloma cells in culture

Only specific details pertaining to individual experimental conditions are reported here.

BMP gene expression by myeloma cells

Bmp2, *Bmp4* and *Bmp6* gene expression (Fig. 4.4 A) was measured using RNA harvested from 5TGM1 myeloma cells (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control) after 24h incubation either alone or across a transwell from murine 2T3 cells that had undergone a 10-day mineralisation protocol. Replicate wells were shown to have mineralised with Alizarin Red staining.

Viability and proliferation assays

AlamarBlue® cell viability assays were performed with the addition of either Human BMP6 (R&D systems) and/or LDN193189 (Sigma), dosed as indicated at time of cell plating (Fig. 4.4 B&C). After 72 hours incubation, 10% AlamarBlue® (10µl) was added and fluorescence measured after 3 hours. For fluorescence expressing cell contact experiment (Fig. 4.4 D), HS5 cells (to recapitulate stromal support) were seeded for culture and allowed to adhere for 4 hours. Control wells contained no HS5 cells. Media was then replaced with fresh media containing suspended 5TGM1-GFP cells, with/without additional experimental treatments as specified. GFP fluorescence was read at 72 hours.

Gene expression in stromal and hepatocellular carcinoma cell lines

Confluent 2T3 cells were treated with 100nM LDN193189 and/or 200ng/ml hBMP6 and harvested after duration indicated (Fig. 4.5 A-C). Nuclear extracts were prepared and phosphorylated-SMAD1/5/8 relative to total SMAD1 protein quantified by western blot. *Id1* gene expression was measured on RNA harvested after 24h incubation with 100nM LDN193189 or control media (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control).

Confluent human hepatocarcinoma HuH7 cells were treated with 100nM LDN193189 or control media for 24h (Fig. 4.5 D); harvested RNA was used to measure *HAMP* gene expression (qRT-PCR, $2^{-\Delta CT}$ method using *GAPDH* as endogenous control).

Stromal cell culture from myeloma patient bone marrow samples

Mononuclear cells were isolated from fresh bone marrow aspirates donated by myeloma patients (Supplementary Table 3). Adherent cells (BMSCs) were grown to confluence, reseeded and treated for 24 h with LDN 100nM or ALK3-FC 10µg/ml, before RNA extraction and qRT-PCR, $2^{-\Delta CT}$ method using *GAPDH* as endogenous control (Fig. 4.5 E).

Osteoclast differentiation experiments

For osteoclast differentiation experiments, LDN 100nM was added to osteoclastogenic media from the day after monocyte seeding until mature osteoclast formation was observed in control wells. Wells were then fixed, stained and multinucleated cells enumerated (Fig. 4.10).

Gene expression in UMR106.01 rat osteosarcoma cell line

Confluent UMR106.01 cells were treated with 100nM LDN193189 or control media for 24h, followed by RNA extraction and qRT-PCR ($2^{-\Delta CT}$ method using *Hprt* as endogenous control) (Fig. 4.15 C).

4.2.2 *In vivo* experiments to test the effects of LDN193189 on 5TGM1 murine myeloma, bone disease and anaemia

On day 0, mice were weighed and inoculated IV with 2×10^6 5TGM1 GFP+ cells. From day 5 LDN193189 (3mg/kg, Sigma) or vehicle (20% (2-hydroxypropyl)-beta- cyclodextrin) was injected IP daily. Mice were assessed daily and weighed weekly.

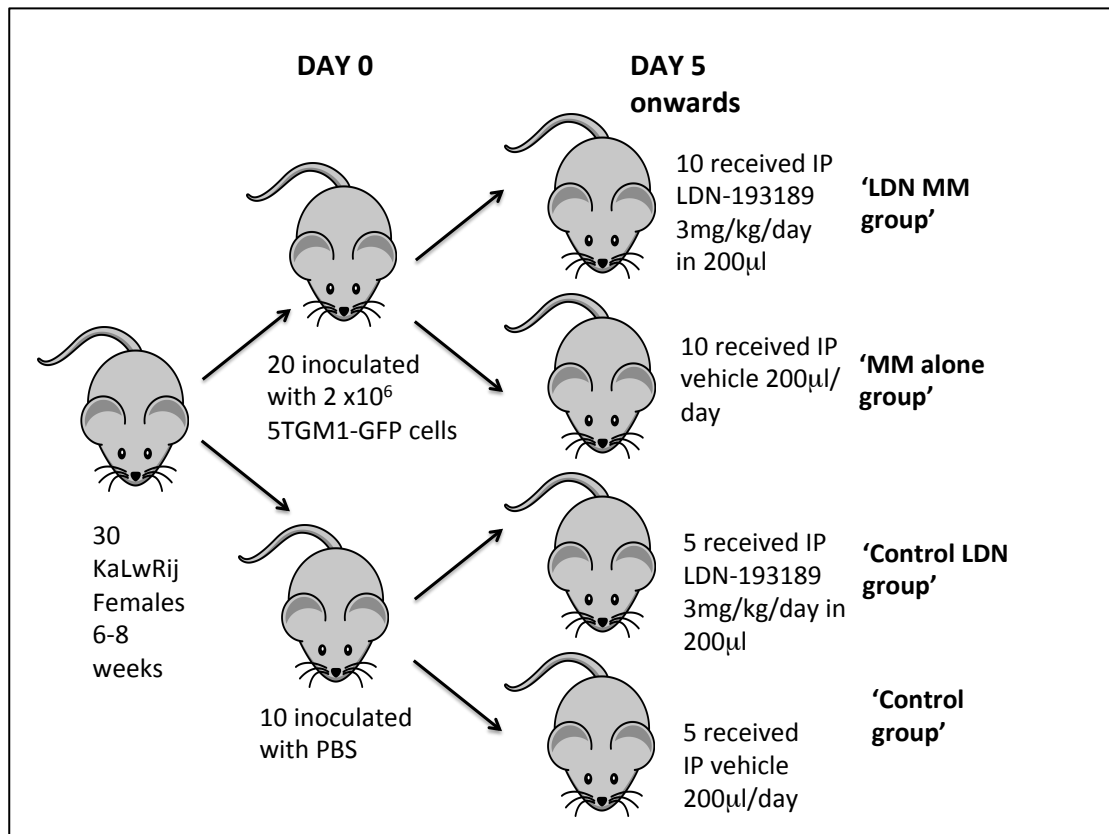


Figure 4.2: Experimental design for LDN193189 or vehicle treatment of healthy and 5TGM1-GFP myeloma-bearing KaLwRij mice.

Mice were culled when the first mouse displayed sign of hind limb weakness, which occurred on day 27 for the first replicate of this experiment, and day 25 for the second replicate. On one experimental replicate, 200 μ l 2mg/ml calcein solution was injected IP to every mouse on day 22 and day 25 in order to facilitate dynamic bone formation histomorphometry assays. After cull, relevant samples were collected, processed and the following analyses performed all as described in chapter 2:

- Tibial MicroCT- generated trabecular and cortical bone density indices (BV/TV)
- Histomorphometry of decalcified, formalin fixed, paraffin embedded femur sections stained with haematoxylin and for TRAP activity, analysed with Osteomeasure software
- Histomorphometry of methyl/butyl methacrylate plastic embedded non-decalcified lumbar vertebral sections, calcein incorporation analysed with Osteomeasure software.

- FACS measurement of GFP-positive 5TGM1 cell proportion of total spleen and bone marrow cell suspensions to assess tumour burden
- Serum IgG2b ELISA to assess global 5TGM1 tumour burden
- Serum TRAP5b activity to assess global osteoclast activity
- Bone marrow plasma Dkk1 and Sclerostin quantification
- Liver *Hamp1* gene expression (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control)
- Serum Hepcidin ELISA

4.2.3 RNASeq of sorted bone lining niche components of myeloma-bearing mice treated with LDN193189 or vehicle

16 10-week old mice, 8 males and 8 females, were inoculated IV with 1.5×10^6 5TGM1 GFP+ cells (Figure 4.3). From day 5 LDN193189 (3mg/kg, Sigma) or vehicle (20% (2-hydroxypropyl)-beta-cyclodextrin) was injected IP daily. Mice were sex and weight matched between LDN193189 and vehicle-treated groups. They were assessed daily and weighed weekly. As described in chapter 3, only 6 bone lining cell samples were sorted per day, meaning that Day 0 IV infusion of myeloma cells or PBS, and then Day 23 cull, were staggered over three consecutive days. Bone lining niche cell FACS-assisted sorting and cDNA library preparation using the SmartSeq2 protocol were performed exactly as described in chapters 3 and 2 respectively, so are not repeated here. However in contrast to the sequencing described in chapter 3, 75bp paired-end sequencing of cDNA libraries using Illumina HiSeq4000 was performed, as a result of facilities upgrade at the Wellcome Trust Centre for Human Genetics Sequencing Facility, University of Oxford, between the two experiments. As the results of these two RNASeq experiments were not intended to be compared to each other, we concluded that a different sequencing depth was an acceptable

variation between them. Not all samples amplified or yielded adequate quality cDNA libraries, so sequencing was performed on 10 endothelial samples, 11 osteoblast samples, 13 MSC samples, 13 central marrow myeloma samples and 11 bone lining myeloma samples. Data analysis was performed as previously described in section 3.2.3, with two differences: firstly, paired-end sequencing data where reads were identified as PCR duplicates using Samtools (Li *et al*, 2009) were discarded. Secondly, read counts were used for the identification of global differential gene expression between specified populations using the edgeR package alone (Robinson *et al*, 2010). Genes were considered differentially expressed between populations if they had an adjusted *p*-value (FDR) of less than 0.05. Supplementary data for this RNASeq dataset is in supplementary figures 6 (quality assessment of mapping), 7 (cell type phenotype heatmap) and 8 (MDS plot).

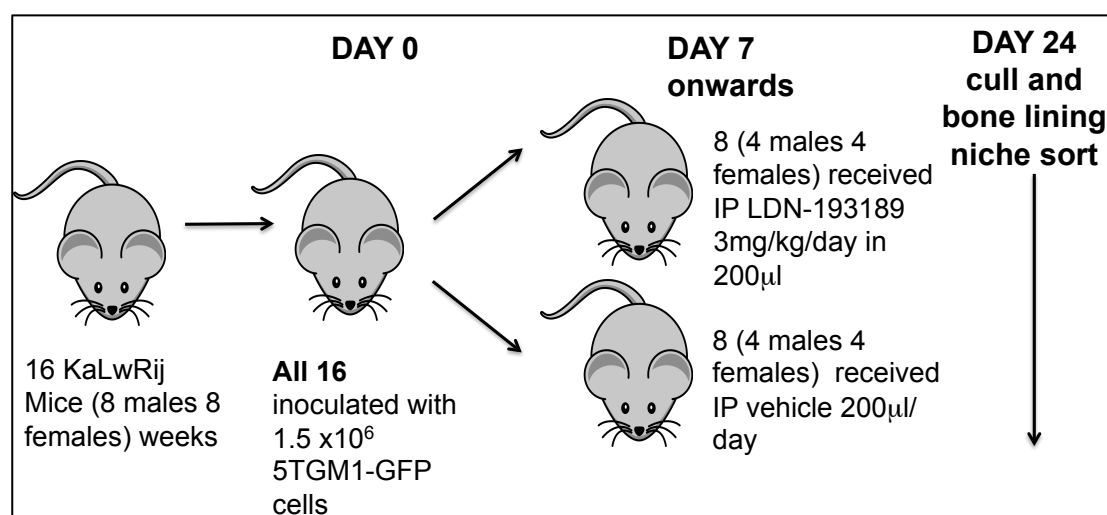


Figure 4.3: Experimental design for LDN193189 or vehicle treatment of KaLwRij mice with 5TGM1 myeloma prior to bone lining niche sorting for RNASeq.

4.3 Results

4.3.1 BMP6 reduces the viability of myeloma cell lines *in vitro* but LDN193189 and stromal contact abrogate this effect

As discussed, BMP6 expression has been reported to be associated with superior survival in patients. I first assessed BMP ligand gene expression in 5TGM1 murine myeloma, the cell line used in our *in vivo* model. This showed BMP6 to be expressed (Fig. 4.4 A), but not BMP2 or BMP4 (no expression detected, data not shown). Because BMP signalling was found to be enriched in bone lining cells on myeloma invasion (chapter 3), the effect on myeloma BMP6 expression of co-culture with mineralised murine osteoprogenitor 2T3 cells was performed, the mineralised layer modelling the endosteal niche surface. This showed that myeloma BMP6 expression was increased on co-culture. Exposure to increasing dose of recombinant human BMP6 reduced the viability of human myeloma cell line JJN3 and murine myeloma line 5TGM1 in culture, but not MM1-S (Fig. 4.4 B). The two sensitive cell lines were therefore tested again with increasing doses of LDN added concurrent to the BMP6.

LDN193189 almost completely reversed the BMP6 response in both cell lines (Fig. 4.4 C) although at 400nM LDN193189, the viability of JJN3 cells began to fall. At none of the doses tested did it enhance viability above baseline. Having shown that LDN193189 protected myeloma cells from BMP-induced cell death, I investigated what the effect of the myeloma microenvironment was on BMP-induced cell death. Stromal adhesion is reported to induce chemoresistance in myeloma cells (Bjorklund *et al*, 2014), but had not to our knowledge been assessed in relation to BMP-induced toxicity. I therefore used a cell fluorescence model to monitor 5TGM1-GFP myeloma cell growth while attached to stromal cells, as this was the myeloma cell line be used in the *in vivo* model. Using GFP fluorescence intensity as a proxy for cell growth, I found that only when 5TGM1 cells were cultured alone did BMP6

reduce growth; either stromal adhesion or LDN193189, or both, maintained cell growth not significantly different from control condition (Fig. 4.4 D).

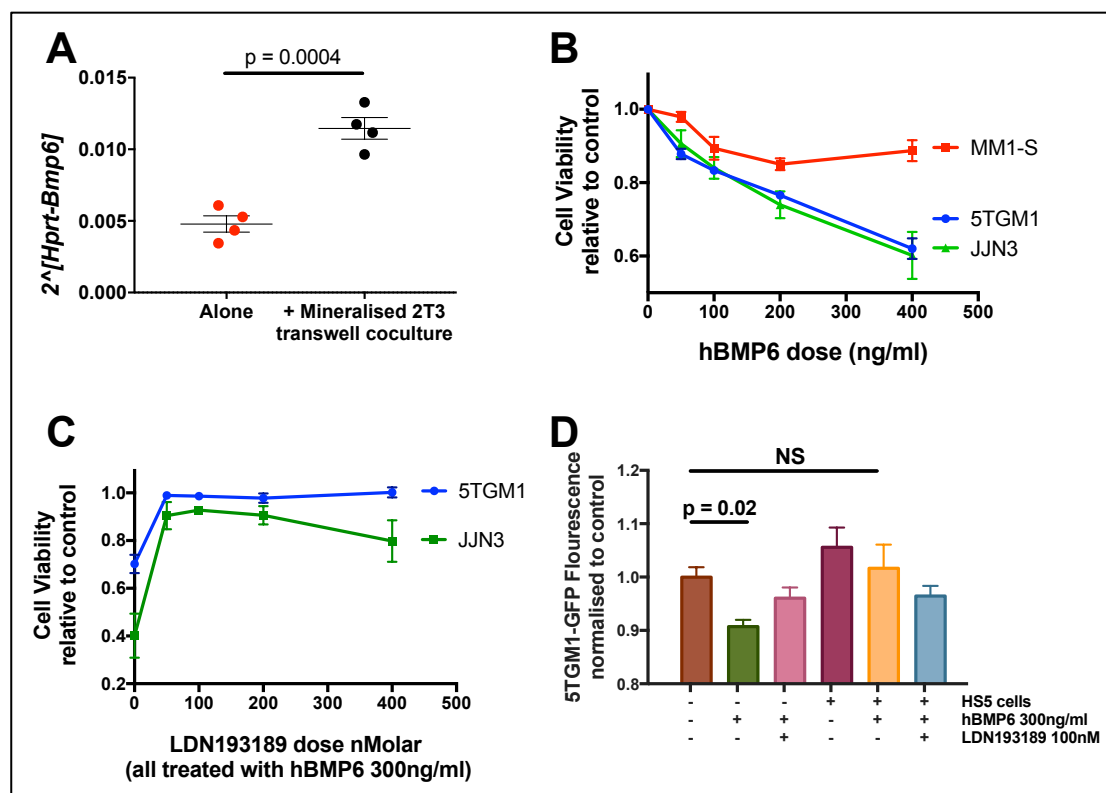


Figure 4.4: BMP6 reduces the viability of myeloma cell lines *in vitro* but LDN193189 and stromal contact abrogate this effect.

A: Gene expression of BMP6 by 5TGM1 murine myeloma cells alone and on 24h transwell co-culture mineralized 2T3 cells. $2^{-\Delta CT}$ method using *Hprt* as endogenous control (n=4 replicates, mean+SEM, unpaired T test)

B: Effect of BMP6 on JJN3, MM1-S (human) and 5TGM1 (mouse) myeloma cell line viability at 72h. Viability expressed relative to viability in control conditions (n=4 replicates, mean+SEM)

C: Dose effect of LDN193189 on BMP6-induced myeloma growth inhibition at 72h. Viability expressed relative to viability in control (non BMP6 exposed) condition (n=3 replicates, mean+SEM)

D: Effect of hBMP6, LDN193189 and contact co-culture with stromal line HS5 on proliferation of 5TGM1-GFP cells at 72h (GFP fluorescence intensity as proxy for growth) (n=4 replicates, mean+SEM, 1 way ANOVA, Sidak's multiple comparisons test)

4.3.2 LDN193189 inhibits BMP pathway activity in osteoblast progenitor and hepatoma cell lines and *ex vivo* human stromal cells

My second and third hypotheses concerned the effects of LDN193189 on bone lining niche cells and hepatocytes (the main source of endogenous hepcidin). I therefore sort to test the effect of LDN193189 on cell lines best approximating these cells *in vitro*, prior to *in vivo* use (Fig. 4.5). These experiments confirmed significant inhibition of BMP pathway activity by

LDN193189, as measured by phosphorylated SMAD1/5/9 western blotting at 30 minutes and 1-hour incubation, and expression of target genes *Id1* in 2T3 cells, and *HAMP*

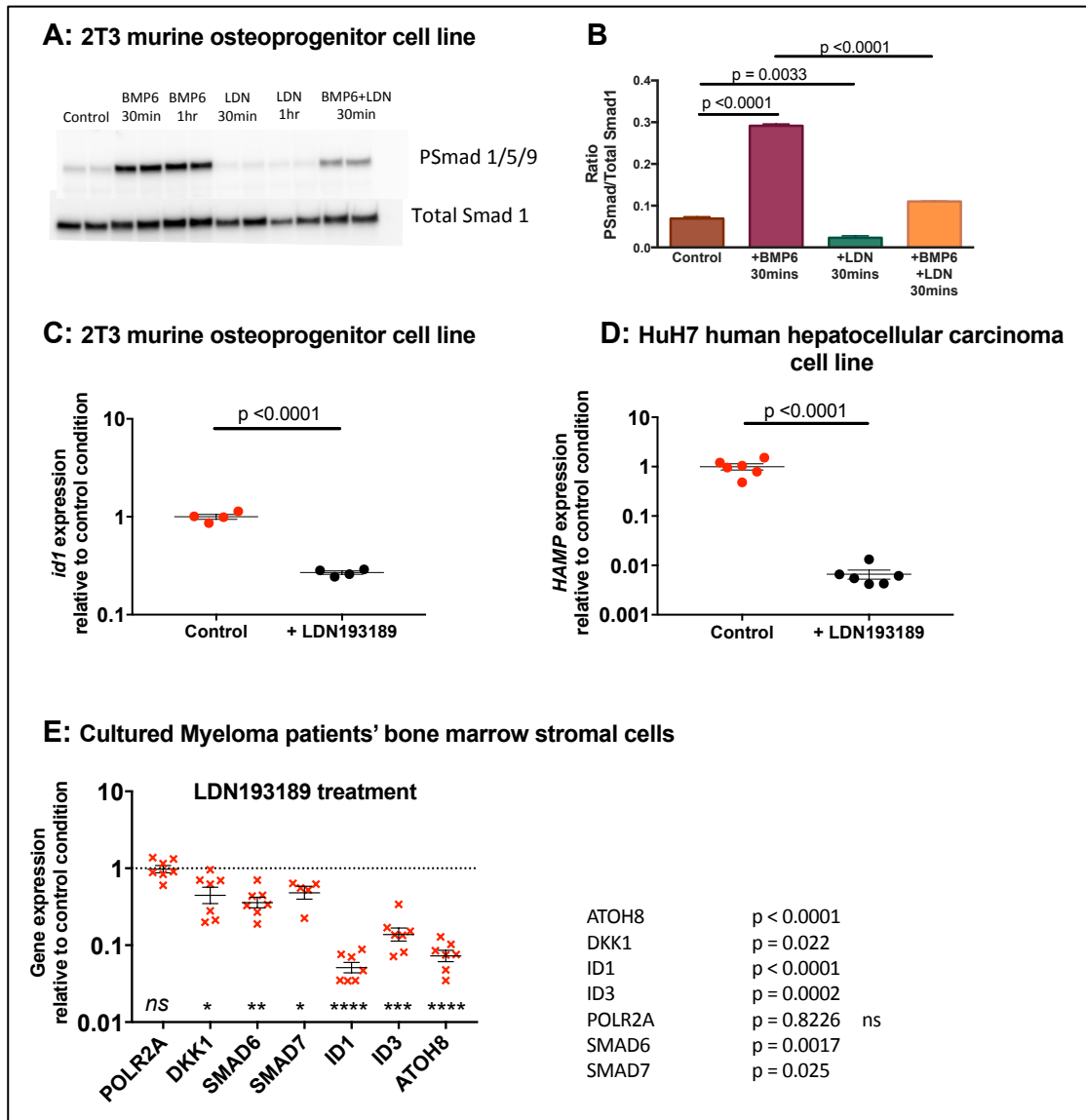


Figure 4.5: LDN193189 inhibits BMP signalling in 2T3 murine osteoprogenitor, HuH7 human hepatocellular carcinoma cell lines and ex vivo human stromal cells.

A: Western blot of Phosphorylated Smad1/5/9 complex and Total Smad 1 in 2T3 cells treated with human BMP6 (200ng/ml) and/or LDN (100nM) for duration as marked.

B: Quantitative densitometry of western blot in (A), (1 way ANOVA, Sidak's multiple comparisons test)

C: Gene expression of *Id1* by 2T3 cells cultured alone and in presence of 100nM LDN193189. $2^{-\Delta CT}$ method using *Hprt* as endogenous control (24h incubation, n = 4 replicates) (unpaired T test).

D: Gene expression of *HAMP* by HuH7 cells; all treated with 200ng/ml BMP6, with or without 100nM LDN193189. $2^{-\Delta CT}$ method using *GAPDH* as endogenous control (18h incubation, n=6 replicates) (unpaired T test).

E: Expression of *POLR2A* (control) and BMP-response genes *SMAD7*, *SMAD6*, *ID1*, *ID3*, *ATOH8*, and *DKK1* by patients' bone marrow stromal cells after 24h incubation with 100nM LDN193189. $2^{-\Delta CT}$ method using *GAPDH* as endogenous control (n=7 samples, mean+SEM, p values generated with paired ratio t-tests as each sample was individually normalised to control due to baseline variation between patient samples, with correction for false discovery rate by Benjamini & Hochberg method.)

in HuH7 cells. Finally, to assess mechanistic relevance to human tissue, I cultured bone marrow stromal cells obtained from fresh bone marrow aspirates from myeloma patients and tested the effect of 24 hours treatment with LDN193189 on gene expression (Fig. 4.5 E), which confirmed suppressed expression of BMP target genes *SMAD7*, *SMAD6*, *ID1*, *ID3*, *ATOH8*, and *DKK1*.

4.3.3 LDN193189 has no effect on 5TGM1-GFP myeloma disease burden in

KaLwRij mice

Healthy and myeloma-bearing mice were treated with 3mg/kg LDN193189 or vehicle control once daily, from day 5 after 5TGM1-GFP tumour inoculation until cull. Tumour burden was quantified by serum IgG2b quantification, measuring the monoclonal antibody produced by the malignant plasma cells as a surrogate of tumour burden, and by percentage of GFP-expressing cell content of the bone marrow and spleen. This demonstrated no difference in tumour burden, by either method, between control and LDN193189-treated animals (Fig. 4.6 A-C). Weight gain over experiment course was also unaffected by LDN193189 treatment (Fig. 4.6 D). However when bone-lining cells were sorted for RNASeq, it was noted that the proportion of myeloma cells adherent to the bone lining (i.e. requiring dissociation with collagenase rather than simple flushing), was increased in LDN193189-treated mice relative to total myeloma fraction (Fig. 4.6 E).

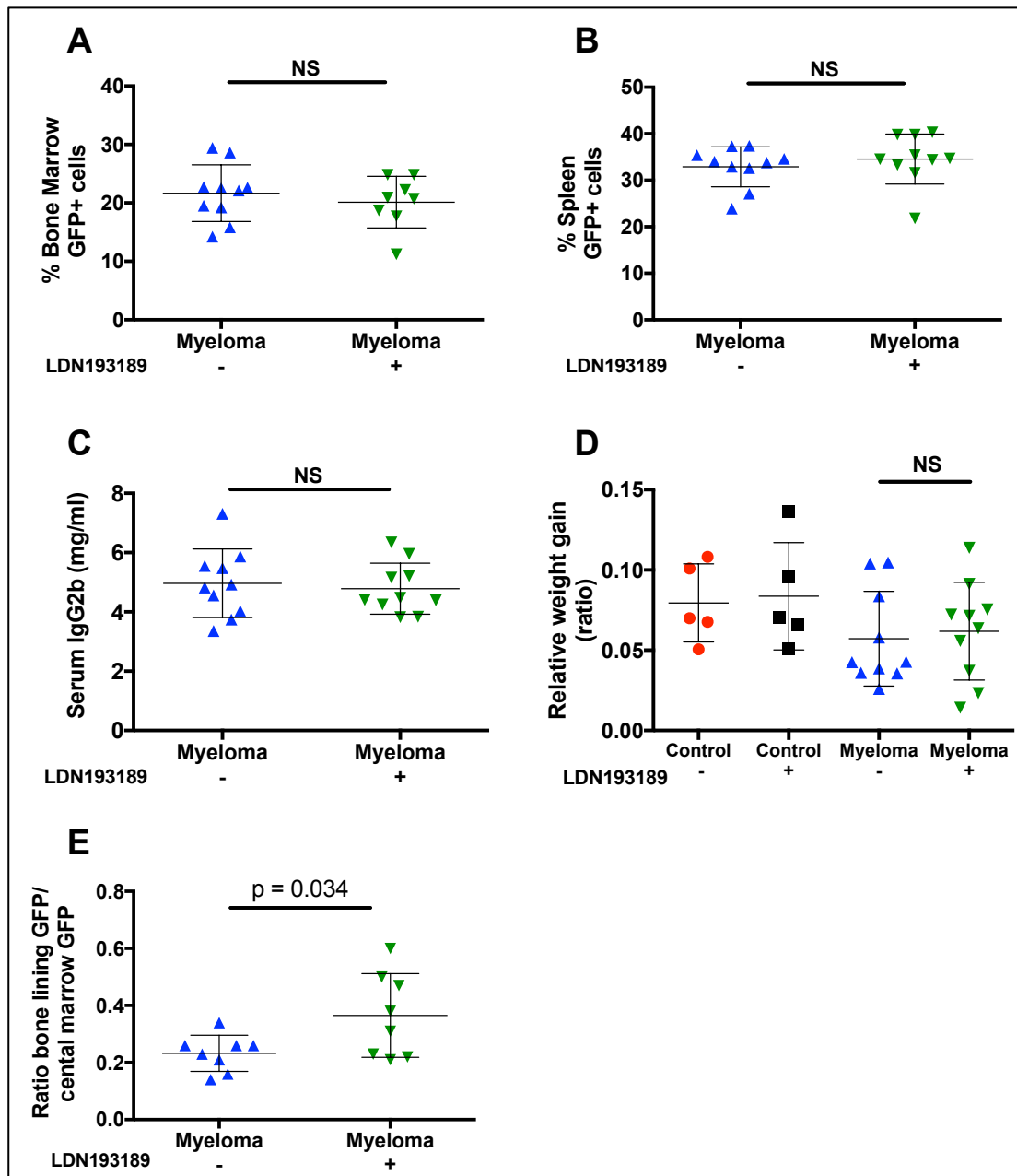


Figure 4.6: Tumour burden is no different between vehicle- and LDN193189-treated myeloma-bearing mice but an increased proportion of myeloma is adherent to the bone lining with LDN193189 treatment.

Data from a single $n = 30$ experiment shown, as is representative of both replicates.

A: Percentage of bone marrow mononuclear cells expressing myeloma-specific GFP

(Note two data points missing from LDN193189-treated group due to bleach contamination of sample before analysis.)

B: Percentage of spleen mononuclear cells expressing myeloma-specific GFP

C: Serum IgG2b level (mg/ml)

D: Relative weight gains over course of experiment (total weight change/starting weight)

E: Proportion of total GFP+ cells which are adherent to bone lining cells (i.e. found in the enzyme-digested fraction rather than the bulk fraction).

All p values generated using unpaired T tests.

4.3.4 LDN193189 improves bone mass in 5TGM1-GFP-bearing KaLwRij mice

Total metaphyseal bone density (measured as bone volume / total volume, BV/TV), cortical thickness, trabecular bone density (BV/TV) and derived trabecular thickness were quantified in right tibiae from both experimental replicates using microCT images (see methods), which was also used to count discrete cortical surface lesions on reconstructed images. All these indices were significantly improved in disease-bearing animals treated with LDN193189 (Fig. 4.7 A – D, F, Figure 4.8). Histomorphometric assessment of BV/TV, performed on formalin-fixed paraffin embedded right femoral sections stained with haematoxylin and TRAP (Fig. 4.7 E), also showed significantly higher BV/TV in LDN193189-treated disease-bearing animals.

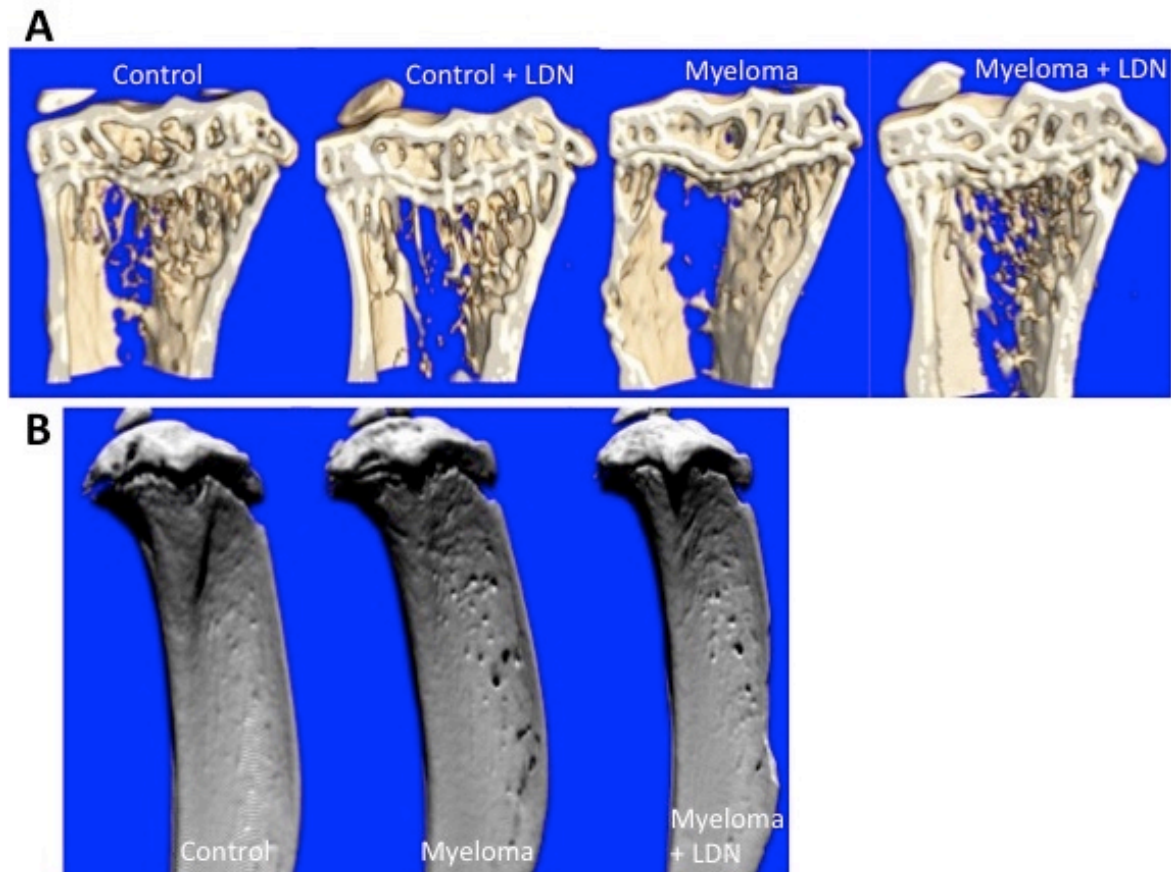


Figure 4.8: Inhibition of BMP signaling with LDN193189 reduces myeloma bone disease *in vivo*. Reconstructed microCT images of (A) tibial sagittal sections showing trabecular architecture and (B) surface cortical lesions on tibial surface.

4.3.5 Osteoclast numbers and activity are reduced in LDN193189-treated myeloma bearing mice, and LDN193189 reduces osteoclast formation *in vitro*.

Having observed the increased bone mass and reduced cortical lesion numbers in myeloma-bearing mice treated with BMP inhibitor LDN193189, despite no change in tumour burden, I wished to determine the mechanism by which this occurred. As discussed in the introduction, the cause of increased bone destruction in myeloma is increased bone surface osteoclast formation and bone lysing activity, so I initially assessed the effect of LDN193189 treatment on osteoclast numbers at the bone surface (Fig. 4.9 A-C).

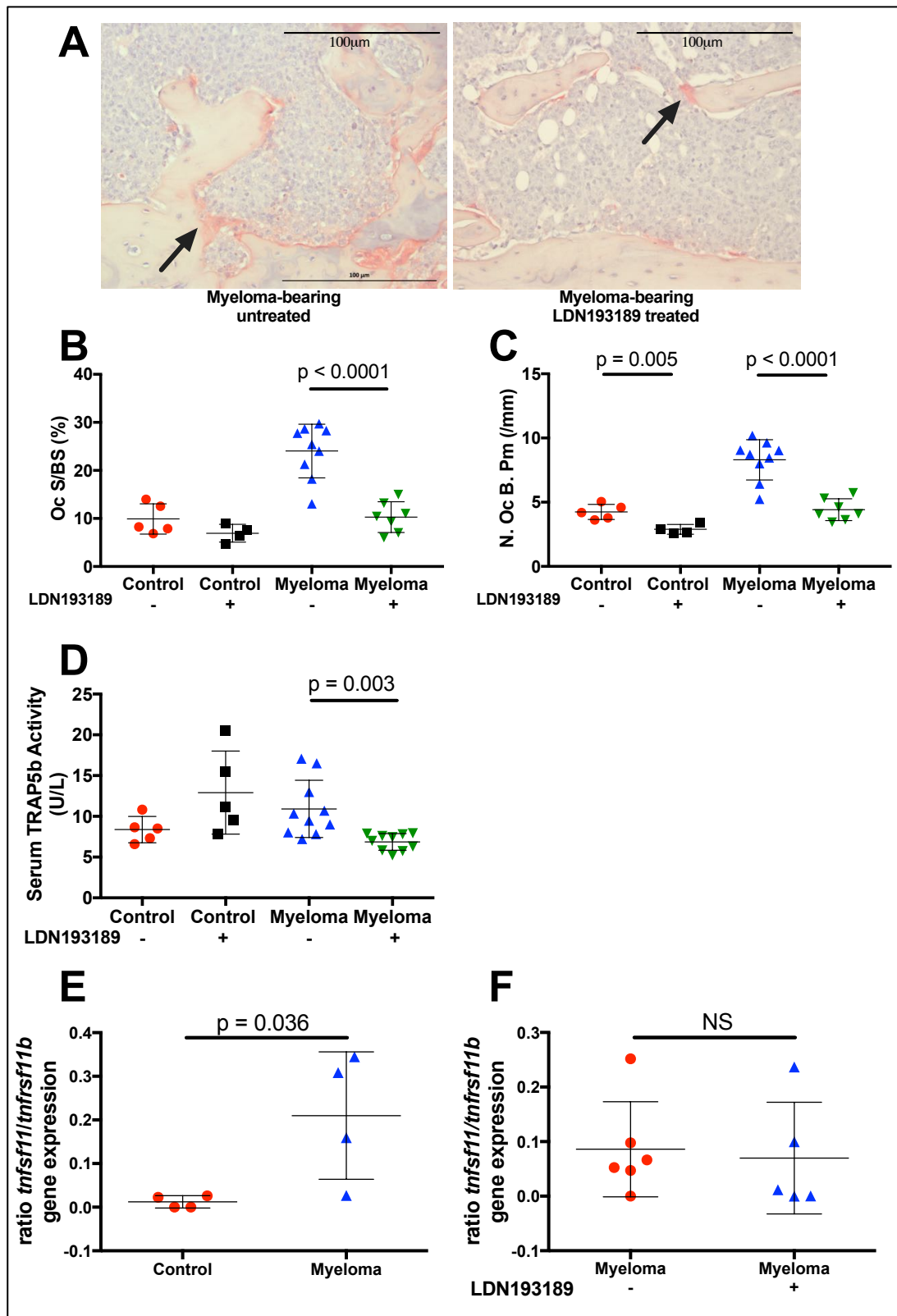


Figure 4.9: Inhibition of BMP signaling reduces osteoclast number *in vivo* by a non-RANKL-mediated mechanism

A: Osteoclasts (indicated with arrows) from myeloma-bearing mice: vehicle vs. LDN193189-treated (TRAP and haematoxylin stained).

B-C: Histomorphometric analysis of TRAP-stained osteoclasts in control and LDN193189-treated healthy and myeloma-bearing mice:

B: Osteoclast surface/ trabecular surface ratio (OcS/BS)

C: Osteoclast bone surface density (N.Oc.B.Pm)
 D: Serum TRAP5b concentration, indicating global osteoclast burden, in vehicle vs. LDN193189-treated healthy and myeloma-bearing mice.
 E-F: RNASeq-generated *in vivo* *Tnfsf11:Tnfrsf11b* (RANKL:OPG) expression (RPKM) in osteoblasts.
 E: Gene expression ratio in healthy vs. myeloma-bearing mice
 F: Gene expression ratio in vehicle vs. LDN193189-treated myeloma-bearing mice
 All p values generated using unpaired T tests.

Using histomorphometry to count osteoclast numbers, and to determine the ratio of osteoclast-occupied trabecular surface to unoccupied trabecular surface, I found LDN193189-treated myeloma-bearing mice had fewer osteoclasts, occupying less bone surface than vehicle-treated myeloma-bearing mice (Fig. 4.9 A-C). Although these experiments were not powered to see if this was also the case in healthy mice, I also noted a reduction in osteoclast numbers in healthy LDN193189-treated animals. Merely counting osteoclasts does not however indicate their effective function; I therefore measured serum tartrate-resistant acid phosphatase (TRAP) 5b, an enzyme secreted into the circulation by osteoclasts during bone resorption and used as a marker of active bone resorption (Halleen *et al*, 2006). This did not significantly vary in healthy animals, but in myeloma-bearing mice, there was a significant drop in TRAP5b levels with LDN193189 treatment, indicating that not only were osteoclast numbers reduced, but also bone resorption activity (Fig. 4.9 D).

Osteoclast formation is enhanced by a range of inflammatory and pro-bone remodeling cytokines, but RANKL (gene *Tnfsf11*) is the key osteoclast-inducing cytokine in myeloma-bone disease. The ratio of RANKL to its soluble decoy receptor, OPG (gene *Tnfrsf11b*), can be used as an indicator of RANKL osteoclast-recruiting activity and has been shown to be elevated in myeloma (Roodman & Dougall, 2008). I used RNASeq-generated data to observe changes in this ratio in ALCAM+Sca1- osteoblasts at the transcription level (ratio RPKM values, see chapter 2 RNASeq methods), which had the highest expression of these genes of the cell types I analysed. This ratio was calculated in both RNASeq experiments, firstly in osteoblasts from myeloma-bearing vs control mice, and secondly in osteoblasts from vehicle vs LDN193189-treated myeloma-bearing mice. Although numbers were small there was a

significant increase in the *Tnfsf11/Tnfrsf11b* ratio in myeloma-bearing mice, indicating a relative increase in RANKL levels as would be expected (Fig. 4.9 E). There was however no change in the *Tnfsf11/Tnfrsf11b* ratio in LDN193189-treated versus vehicle-treated myeloma-bearing mice (Fig. 4.9 F), suggesting that change in the transcription level of these genes in bone lining osteoblasts is not the mechanism by which LDN193189 suppresses osteoclast formation.

If the suppression of osteoclast formation by LDN193189 were due to alteration of RANKL or other cytokine signaling from the microenvironment, it would only occur in the presence of microenvironment cells. We therefore decided to test the effect of LDN193189 on osteoclast differentiation from isolated monocytes *in vitro*, using human CD14⁺ monocytes. Only if the mechanism of LDN193189 were direct on osteoclast precursors themselves would this suppress osteoclast formation. These experiments showed a direct suppression of osteoclast formation by LDN193189 similar to the suppression seen with positive control OPG (Fig. 4.10), indicating that BMP signaling inhibition with LDN193189 directly suppresses osteoclast formation.

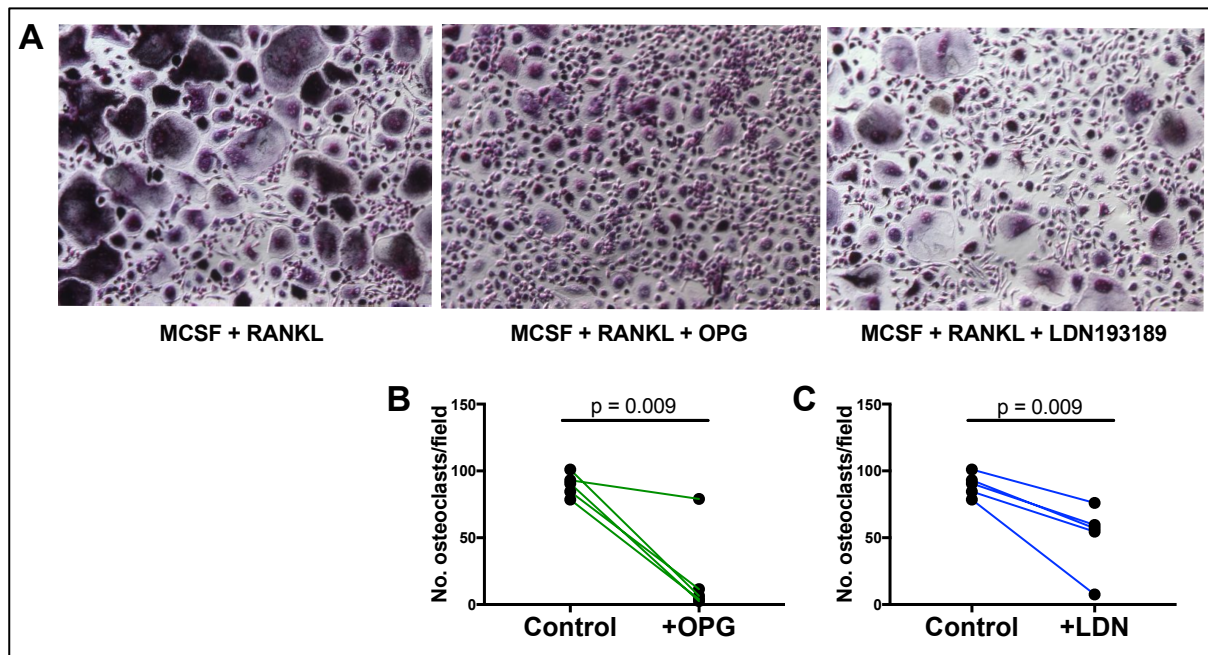


Figure 4.10: Inhibition of BMP signaling with LDN193189 directly reduces osteoclast formation *in vitro*

A: Pooled human CD14⁺ PBMC osteoclast differentiation, with treatments as indicated commenced 24h after seeding. TRAP stained at D10. Images taken at 70x magnification.

B-C: Quantified osteoclasts (≥ 3 nuclei) when CD14⁺ PBMCs treated as (A) were enumerated. Results from same experimental replicate are paired to account for inter-replicate variation in baseline (n=5 replicates). P values generated using paired T tests.

B: Control vs OPG treatment

C: Control vs LDN193189 treatment (see chapter 2 for methodology)

4.3.6 Bone lining cell number and thickness, and bone formation, are increased in LDN193189-treated myeloma bearing mice

In order to optimally reverse the bone homeostasis pathology of myeloma, not only must the enhanced osteoclast formation and activity be halted, but osteoblast suppression must also be reversed. Therefore I next investigated whether BMP inhibition with LDN193189 improved osteoblast numbers and new bone formation in myeloma-bearing mice.

Histomorphometric analysis demonstrated significantly more bone lining cells following treatment of myeloma-bearing mice with LDN (Fig. 4.11 A). Blinded histological measurement of the bone lining cell layer in healthy mice (the layer was too damaged in myeloma-bearing mice for accurate measurement) revealed that treatment with

LDN193189 resulted in a thicker layer (Fig. 4.11 B-C), a morphological characteristic related

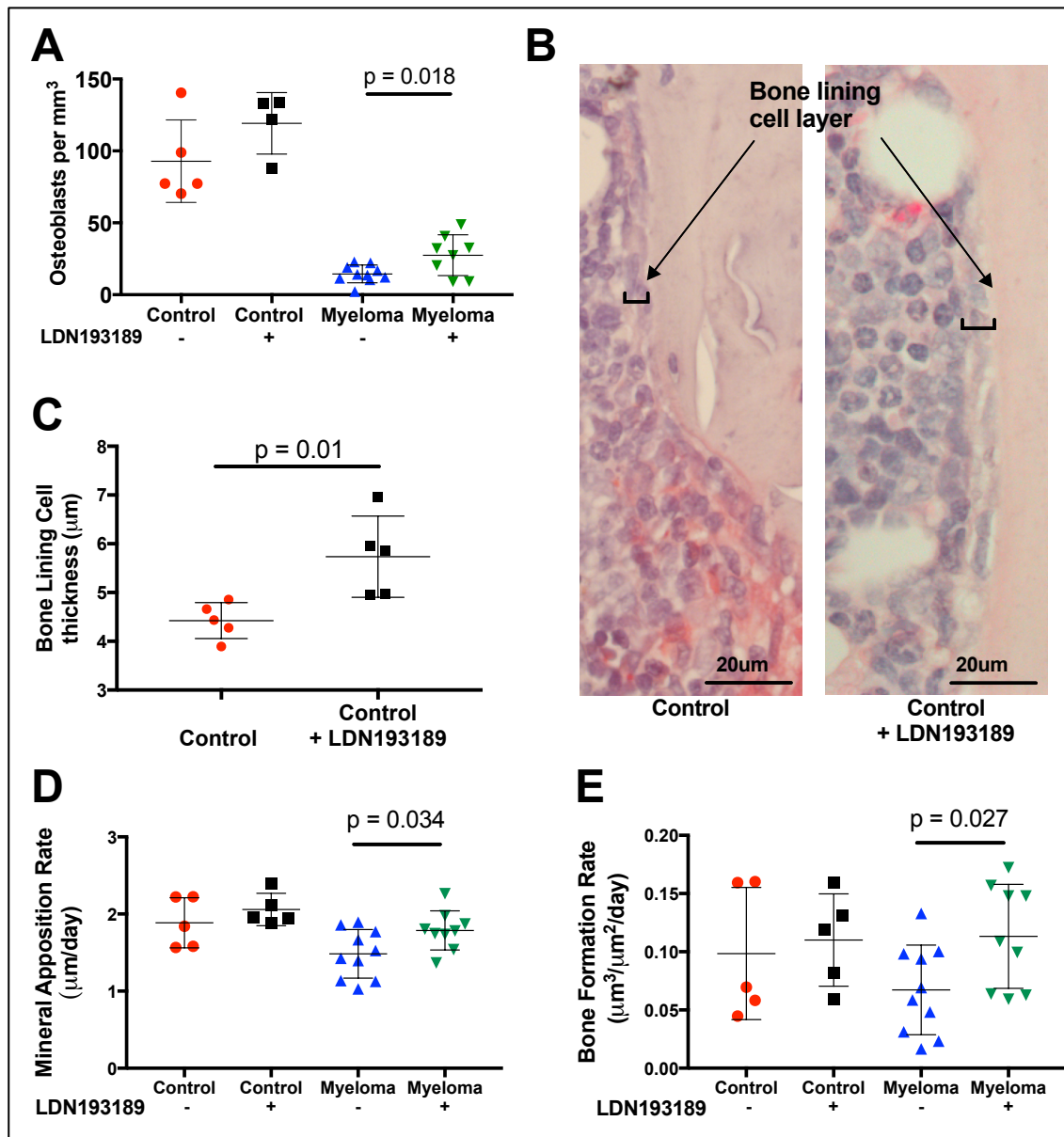


Figure 4.11: Inhibition of BMP signaling with LDN193189 increases bone lining cell number and thickness, and bone formation rates.

A: Osteoblasts per mm³ (Bone lining cell density, N.Ob/T.Ar) in healthy and myeloma-bearing mice treated with vehicle or LDN193189

B: Images of bone lining cell layer (haematoxylin and TRAP stained femur sections) in healthy mice treated with vehicle or LDN193189

C: Bone lining cell layer thickness (µm) in healthy mice treated with vehicle or LDN193189

D: Mineral apposition rate (µm/day)

E: Bone formation rate (µm³/µm²/day)

All p values generated using unpaired T tests.

to the conversion of quiescent bone lining cells to active matrix-secreting osteoblasts and recently associated with inhibition of sclerostin (Kim *et al*, 2017). Using quantitation of bone formation with calcein double labelling, myeloma-bearing mice were found to increase

mineral apposition and bone formation rates following LDN193189-treatment (Fig. 4.11 D-E).

4.3.7 RNASeq analysis of MSCs from myeloma-bearing mice shows enrichment of gene sets compatible with osteoblast differentiation with LDN193189 treatment.

In order to investigate a mechanistic basis for the increase in bone lining cell number and thickness and bone formation observed, RNASeq transcriptome analysis of MSCs from myeloma-bearing mice treated with LDN193189 or vehicle was performed. As it was the MSC (Sca1+ALCAM-) compartment that had previously shown significant BMP pathway enrichment in myeloma-bearing mice, I focused only on these cells when assessing the effect of LDN193189 on transcriptome changes. That pharmacological BMP signalling inhibition was occurring was evidenced by the relative downregulation of BMP target genes *Id1*, *Id2* and *Tnfrsf11b* in MSCs of LDN193189-treated mice. Of note, *Bmpr1b*, the significant upregulation of which in MSCs from myeloma-bearing mice first highlighted the BMP pathway in these cells, was also downregulated by LDN193189 treatment (Supplementary Figure 10).

86 genes were differentially expressed with FDR <0.05 in MSCs from myeloma-bearing mice treated with LDN193189 vs vehicle (Supplementary Table 4). Some genes noted to be associated with bone matrix formation were significantly upregulated (highlighted yellow in table; *Xpr1*, *Vdr*, *Tram2*, *Col9a1*, *Lrg1*) but the bone formation gene set described in chapter 3 was not. GSEA was performed to search for enriched gene sets. There were no enriched sets with an FDR <0.05 from the Hallmark collection (Liberzon *et al*, 2015), so the GSEA-recommended, less stringent FDR of <0.25 was also used to assess enrichment. This identified 6 Hallmark gene sets enriched with FDR < 0.25 (Fig. 4.12).

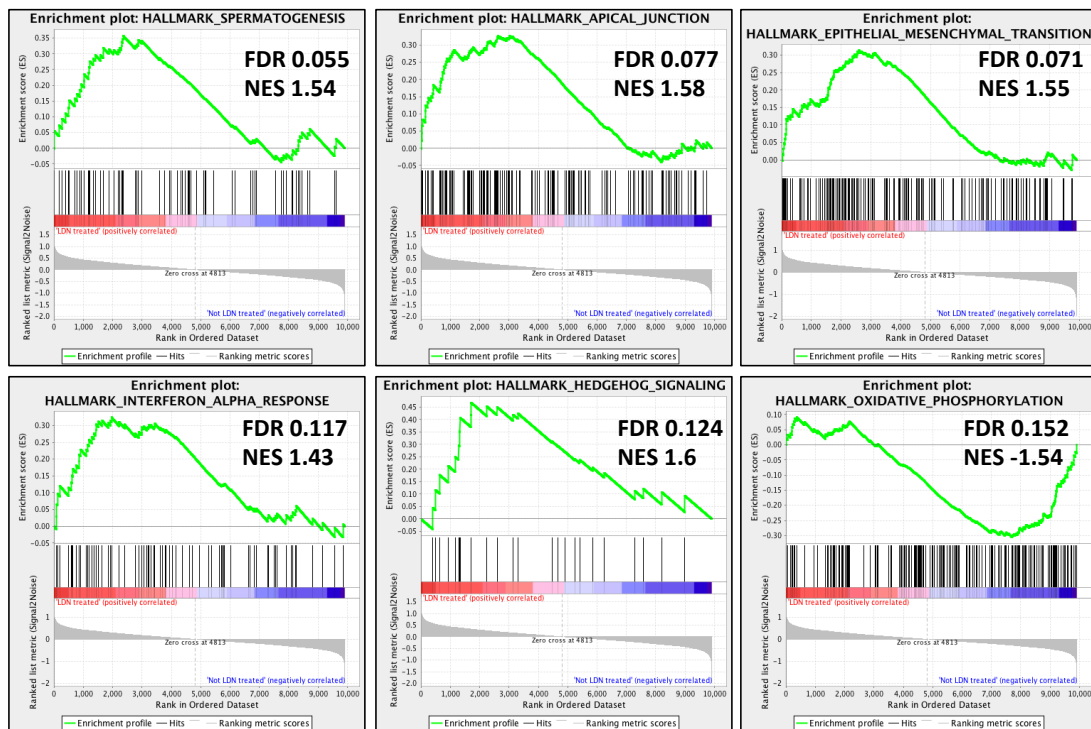


Figure 4.12: Hallmark gene sets enriched with FDR <0.25 in MSCs from LDN193189-treated vs vehicle treated myeloma-bearing mice

FDR = false discovery rate; NES = normalised enrichment score (explanation of GSEA plots, Supplementary Figure 3)

Of these gene sets, only ‘Hedgehog Signalling’ is specifically associated with bone formation, but the parallels of cell fate determination, differentiation and adhesion (particularly with ‘apical junction formation’ and ‘EMT’) can be seen in these gene sets in relation to mature osteoblast formation from a precursor. To investigate these parallels, I compared the leading edges of the above gene sets to the ‘bone remodelling’ and ‘bone formation’ gene set leading edges (which, although were not significantly enriched overall in LDN193189-treated animals, did have significantly upregulated genes within them). Certain collagens and integrins, appearing in one of the enriched Hallmark sets and also the bone gene sets, were upregulated (Fig 4.13). However, these Hallmark gene sets lacked specificity to my experimental hypothesis. I therefore next used the much larger Molecular Signatures Database curated gene set collection (MSigDB C2 collection, n = 3334 gene

sets) (Subramanian *et al*, 2005), to search for more specific molecular associations of the increased bone mass observed.

Four gene sets, all from the Reactome Database (Fabregat *et al*, 2016), met pre-set enrichment criteria of FDR < 0.25 and n > 25 (Fig 4.14, full gene sets listed in Supplementary Table 5). These were 'Nuclear Receptor Transcription', in which the most highly enriched gene was *Vdr*, Vitamin D Receptor (noted above to be a differentially expressed gene), and which includes other nuclear receptors associated with promotion of cell maturation and differentiation, such as sex hormone and retinoic acid receptors; 'Collagen formation', 'Extracellular matrix organisation' and 'NCAM1 interactions'. The enrichment of the latter three gene sets was driven by overlapping content of high numbers of collagen genes associated with matrix formation and cell-matrix adhesion. The transition from early osteoblast through to osteocyte is marked by the progressive encasement of the osteoblast cell at the bone surface by the matrix being secreted, so is essentially driven by matrix formation. The enrichment of these collagen-formation associated gene sets (discussed further in following discussion section) was therefore highly suggestive of differentiation towards a more mature osteoblast phenotype and increasing bone formation.

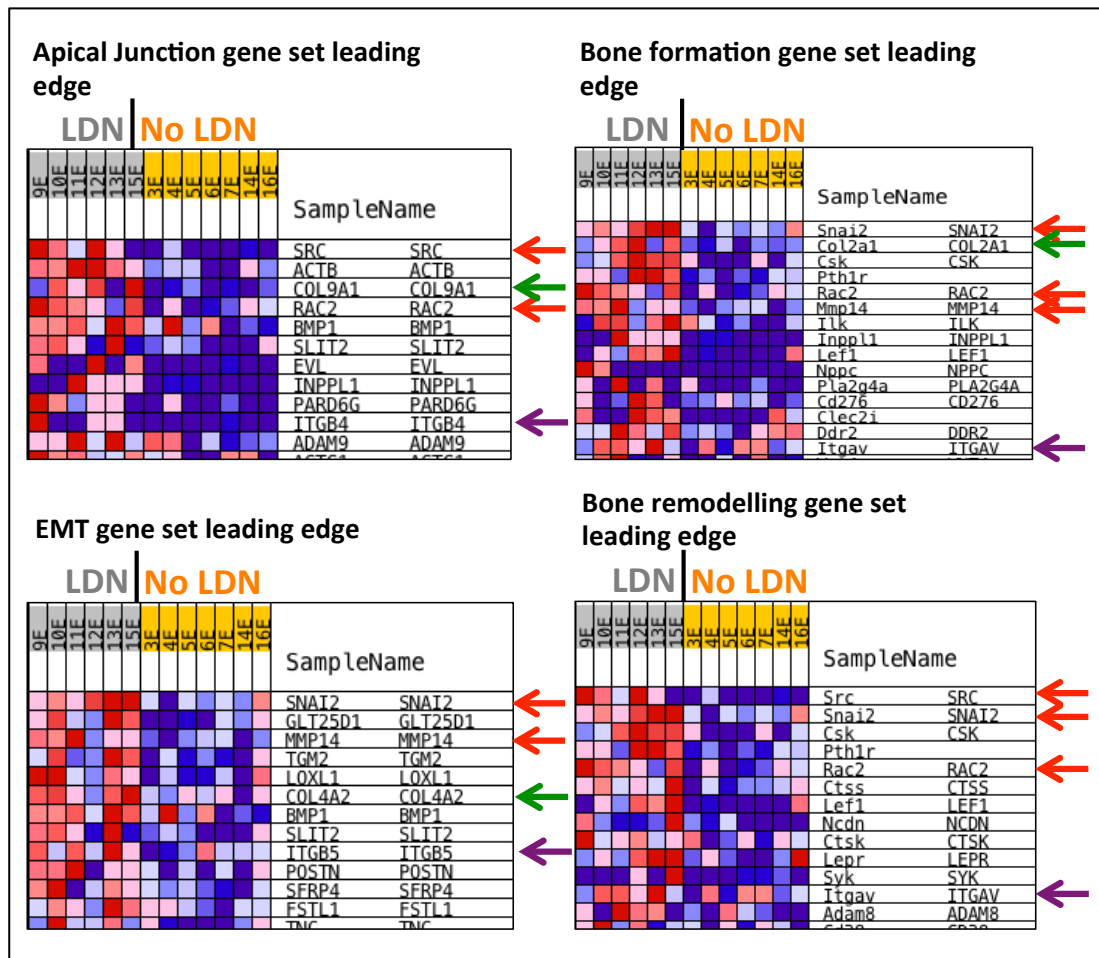


Figure 4.13: Leading edge genes from Hallmark gene sets 'Apical junction' and 'EMT' show overlap with leading edges of previously described 'Bone Formation' and 'Bone Remodelling' gene sets, in MSCs from LDN193189-treated vs vehicle treated myeloma-bearing mice.

Leading edge genes overlapping between a bone set and a Hallmark set are marked with red arrows. Collagen and Integrin family genes, noted to appear frequently, are marked with green and purple arrows respectively.

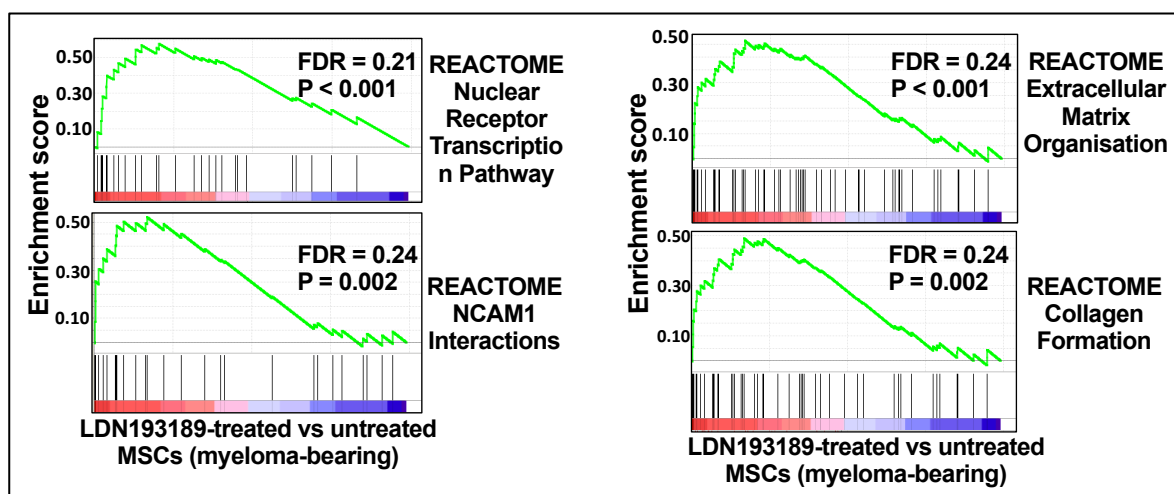


Figure 4.14: Four REACTOME gene sets were enriched with FDR < 0.25, n > 25 genes.

GSEA interrogation of the MSigDB C2 collection in MSCs from LDN193189 vs vehicle treated myeloma-bearing mice.

4.3.8 LDN193189 does not appear to reduce Dkk1 or Sclerostin in myeloma-bearing mice.

As discussed in chapter 1, the control of Dkk1 and Sclerostin expression by BMP type 1 signalling has been previously reported in mice with conditional inducible knockout of Alk2 or Alk3 receptors (Kamiya *et al*, 2011; Kamiya *et al*, 2010; Kamiya, N. *et al*, 2008), both of which are inhibited by LDN193189. This is proposed to be the mechanism by which these mice exhibit a phenotype of higher bone mass. Having shown that LDN193189 increased bone mass and promoted osteoblast development in myeloma-bearing mice, I hypothesized that this was mediated by environmental reduction of either or both of these Wnt inhibitors. As the main source of both inhibitors are osteocytes, which are technically difficult to extract, I had not performed RNASeq analysis on them. I therefore measured the bone marrow plasma level of these proteins using a Luminex panel, but neither were significantly reduced in the bone marrow plasma with LDN193189 treatment in myeloma-bearing mice (Fig. 4.15 A-B). Of note, bone marrow stromal cells cultured from myeloma patients had shown a significant reduction in Dkk1 expression with LDN193189 treatment *in vitro* (Fig. 4.5 E), but again, these cells do not express sclerostin. I therefore used rat osteosarcoma line UMR106.01 to assess LDN193189 effect on sclerostin expression *in vitro*. This cell line was used as a model of osteocytes due to its reliable expression of sclerostin which is only expressed by mature osteocytes, and amenability for knockdown experiments (Sedaghati *et al*, 2016) (see chapter 5); however, I found that sclerostin expression in this cell line was not reduced by treatment with LDN193189 (Fig. 4.15 C).

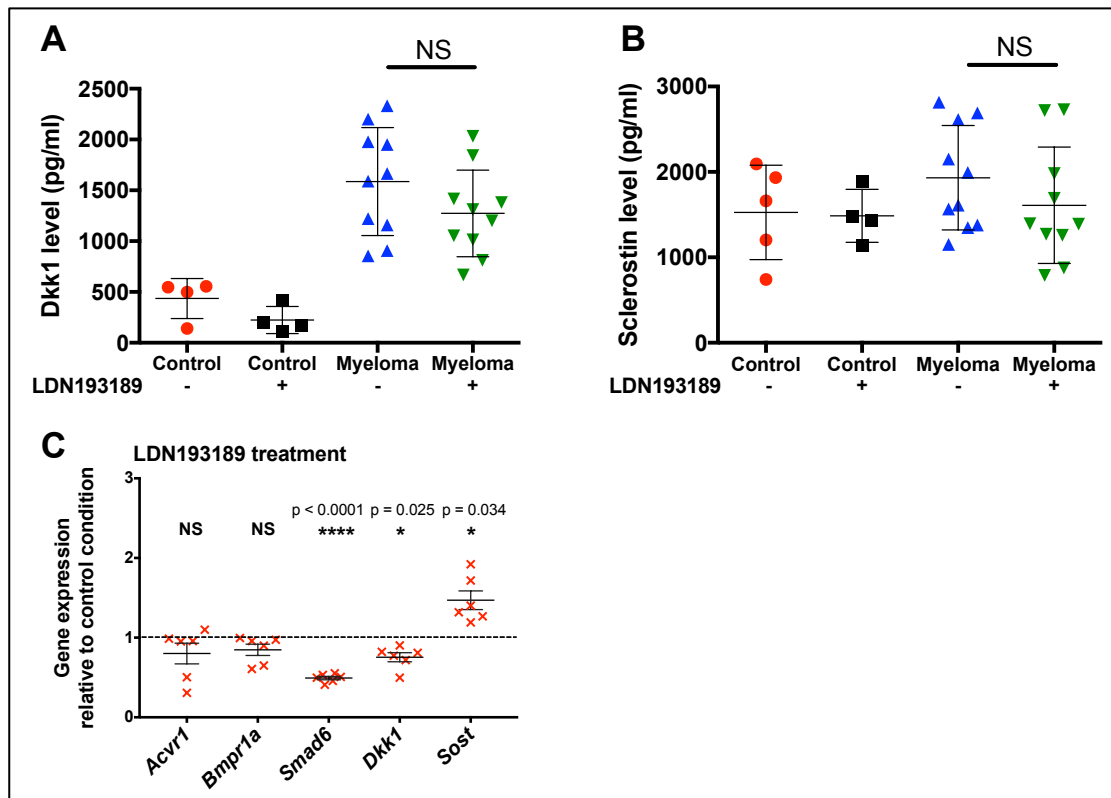


Figure 4.15: LDN193189 treatment does not reduce Dkk1 or Sclerostin levels in myeloma-bearing mice, but does reduce Dkk1 gene expression in osteosarcoma cell line UMR106.01.

A: Bone marrow plasma Dkk1 level (pg/ml)

B: Bone marrow plasma sclerostin level (pg/ml)

A-B: p values generated using unpaired T tests.

C: Effect of incubation with LDN193189 (100nM, 24h) on expression of *Acvr1*, *Bmpr1a*, *Smad6*, *Dkk1* and *Sost* (Sclerostin) genes in rat osteosarcoma line UMR-106.01. P values generated using one-way ANOVA with multiple comparisons to control condition using Dunnett's multiple comparisons test.

4.3.9 LDN193189 reduces Hepcidin gene expression but not serum protein level in myeloma-bearing mice.

LDN193189 has already been shown to reduce *HAMP* gene expression in HuH7 cells (Fig 4.5

D). *In vivo*, we found LDN193189 reduced hepatic *Hamp1* (Hepcidin) gene expression in

myeloma-bearing mice, but decreased serum hepcidin only in non-tumour mice (Fig. 4.16).

Technical sample collection problems rendered samples unsuitable for haemoglobin

measurement, so whether myeloma-bearing mice were anaemic, and if so, whether this was

ameliorated by LDN193189, could not be assessed.

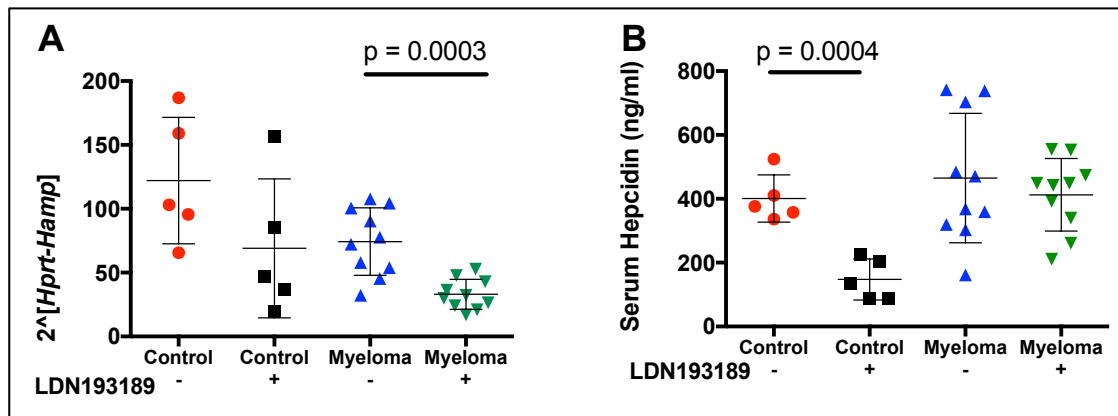


Figure 4.16: LDN193189 reduced hepatic Heparin gene expression in myeloma-bearing mice, but only reduced serum Heparin level in healthy mice.

A: *Hamp1* gene expression in liver tissue samples from healthy and myeloma-bearing LDN193189 or vehicle treated mice. $2^{-\Delta CT}$ method using *Hprt* as endogenous control.

B: Serum hepcidin level (ng/ml) in healthy and myeloma-bearing LDN193189 or vehicle treated mice. P values generated using unpaired T tests.

4.4 Discussion

I initially hypothesized that *in vivo*, a BMP signalling antagonist may increase tumour but would also reduce bone destruction and improve inflammatory anaemia in multiple myeloma. I found that tumour burden was unaffected other than an apparent redistribution of tumour to adherent sites at the bone lining; however, bone mass was increased following LDN193189 treatment, and lesions were decreased, with direct effects demonstrated on osteoclasts and osteoblasts. Although I was unable to assess anaemia in these experiments, I found Heparin gene expression to be reduced in the hepatic tissue of LDN193189-treated myeloma-bearing mice. My anaemia hypothesis therefore remains incompletely tested by these experiments, and further work with improved assays of anaemia indices is required to determine if BMP signalling inhibition can mitigate the anaemia of inflammation in myeloma.

Reports, and my own data, that BMPs are toxic to myeloma cell lines and patient samples *in vitro* prompted me to hypothesize that BMP inhibition would enhance tumour growth *in vivo*, not a feature that would be compatible with its clinical use.

However on the cell lines I tested, although LDN193189 did abrogate the BMP-induced toxicity, it did not enhance growth above baseline in BMP-expressing cell lines. I then found that stromal contact, clearly highly relevant to the *in vivo* setting, also protected myeloma cells from BMP-induced toxicity. These two findings allowed me to review this hypothesis, which was then confirmed when total tumour burden was unchanged with LDN193189 treatment.

Although novel for BMPs, the finding that stromal contact protects tumour cells from drug-induced cell death is not unexpected, and provides compelling reasons why novel therapeutic strategies should not be tested on tumour cells in isolation *in vitro*. Although this concept of cell adhesion-mediated drug resistance (CAM-DR) was first described in myeloma cells (Damiano *et al*, 1999), it has since been shown to be relevant in a range of malignancies (Meads *et al*, 2008). Mechanisms include cytokine-induced pro-survival signals (for example IL6) and contact-mediated protection, where hyaluronan-binding protein CD44 (Bjorklund *et al*, 2014), Notch signalling (Nefedova *et al*, 2004) and integrin-mediated interactions have all been shown to enhance survival. CD44 expression, allowing matrix adhesion, is in many cancers a marker of stemness (Chanmee *et al*, 2015), whereas anti-Jagged1 (Notch ligand) has recently been shown to reverse osteoblast-induced chemoresistance in breast cancer bone metastases (Zheng *et al*). Exosome-mediated transfer of pro-survival factors from stromal to myeloma cells has also been reported (Wang, 2014). Often inseparable from drug resistance is quiescence; the ability of quiescent cells to re-enter cell cycle and propagate tumour after therapy survival means that the adherent, quiescent, drug-resistant population are sometimes considered to be 'cancer

stem cells', although in this context, the cell of propagation and cell of origin should not be confused.

I found that the bone lining-adherent tumour fraction was increased on treatment with LDN193189. In myeloma, specific adhesion to the bone lining has been shown to induce quiescence, drug resistance and tumour-propagating features (Lawson *et al*, 2015a).

Therefore the increase of tumour cells in this site in our model may indicate an increase in quiescent cells. In active myeloma this could risk an increased residual disease fraction after therapy, known to be associated with poorer survival (Munshi *et al*, 2017). However in premalignant disease (MGUS) or myeloma in remission, it could potentially delay time to progression, and this would be interesting to explore in future *in vivo* studies.

LDN193189 improved cortical and trabecular thickness, bone matrix formation and osteoblast numbers, and also reduced bone lesions and osteoclast numbers. Using isolated CD14+ osteoclast precursors *in vitro*, the osteoclast-inhibitory effect was shown to be direct, and not mediated via either RANKL or other microenvironmental interactions. Reduction of osteoclasts *in vivo* results in preservation of bone matrix and therefore of osteoblasts (Croucher *et al*, 2003), so it was initially possible that the observation of higher osteoblast numbers was indirect, due to reduced remodelling. However, on unbiased GSEA of over 3000 gene sets, MSCs from myeloma-bearing LDN193189-treated mice showed significant enrichment in three gene sets, 'collagen formation', 'extracellular matrix formation' and 'NCAM1 interactions' (Fig. 4.14), all driven by enhanced expression of over 15 different collagen genes associated with matrix formation and cell-matrix adhesion, suggesting development of the osteoblast phenotype. Alk3/BMPR1a inhibition has been previously noted to improve collagen structure and trabecular strength (Zhang *et al*, 2016). The additionally enriched 'Nuclear Receptor Transcription' set represents a set of steroid hormone receptors and their associated nuclear binding apparatus, including the vitamin D

and retinoid X receptors. I found the *Vdr* gene was highly enriched in MSCs from LDN-treated myeloma-bearing mice; retinoid X receptors alpha (*Rxra*) and beta (*Rxrb*) were also enriched members of the gene set (Supplementary Table 5). *Vdr* is a marker of developing osteoblasts (Wang, Yongji *et al*, 2014) and is reported to have a role in ensuring osteogenic rather than adipogenic differentiation, interestingly by suppressing *Dkk1* expression (Cianferotti & Demay, 2007). Later, as osteoblasts differentiate to osteocytes, the vitamin D and retinoid X receptors heterodimerize and by their DNA binding initiate the transcriptomic changes that lead to osteoblast to osteocyte transition (St. John *et al*, 2014). In summary, the RNASeq data I generated from LDN193189-treated myeloma mice lends gene expression support to my findings that BMP inhibition promotes osteoblast differentiation and bone matrix formation *in vivo*, as well as reducing osteoclasts and bone resorption.

Dysregulation of various signalling pathways have been implicated in the maturation block of osteoblasts in myeloma, but not previously the BMP pathway. BMP signalling is best known for its osteoinductive properties, so the use of BMP inhibition to promote bone mass is initially counterintuitive. However as discussed above, despite lack of previous work on this pathway in myeloma bone homeostasis, the control of expression of Wnt inhibitors *Dkk1* and *Sclerostin* by BMP type 1 signalling has been previously reported in adult mice with conditional inducible knockout of *Alk2* or *Alk3* receptors (Kamiya *et al*, 2011; Kamiya *et al*, 2010; Kamiya, N. *et al*, 2008), both of which are inhibited by LDN193189. As *Dkk1* and *Sclerostin* are also heavily implicated in the maturation block of osteoblasts in myeloma (Delgado-Calle *et al*, 2017; Heath *et al*, 2009; McDonald *et al*, 2017), it was tempting to hypothesize that the mechanism of increased osteoblast differentiation we observed with LDN193189 treatment was mediated by the reduction in these Wnt inhibitors. *In vitro* experiments indicated that this hypothesis may be correct at least for *Dkk1*, as both cultured stromal cells from myeloma patients' bone marrow samples, and rat

osteosarcoma line UMR106.01 showed a significant fall in Dkk1 expression on LDN193189 treatment. The bone lining cells also exhibited a change to a thicker morphology similar to that recently described on treatment with anti-Sclerostin antibodies (Kim *et al*, 2017). However, when sclerostin and Dkk1 levels in the bone marrow plasma were measured directly, no difference was detected between LDN193189 and vehicle treated mice (Fig. 4.15). This single method of measurement may have been flawed; it relied upon accurate dilution of the bone marrow plasma with exactly 2ml PBS (a much higher volume than the total volume of original plasma in the crushed bones) in every case as the bone marrow was crushed out of the bones; if this volume altered at all the dilution of the proteins contained would have been inaccurate.

To pursue further evidence of whether sclerostin and/or Dkk1 levels were reduced by BMP inhibition in myeloma-bearing mice, I considered either measuring them by different methods on our samples from these experiments (for example, quantitative fluorescence microscopy, (Lee & Iimura, 2017)), or considering alternative methods of inhibiting BMP signalling that might have a more complete inhibitory effect. One of my concerns with LDN193189 was its short half-life, 1.6 hours (Fig. 4.1); as I had only dosed the mice once daily, so BMP inhibition would not have been continuous. I tried repeating the experiment with a twice daily IP dosing of LDN193189 (data not shown), but under the severity permissions of Prof. Edwards' UK Home Office Project Licence (see methods) the maximum we could perform this for was 14 days and unfortunately this led to the experiment needing to be terminated early before bone disease had developed. In addition, LDN193189 is unlikely to have a safety profile compatible with consideration for use in humans. I therefore decided to progress to alternative methods of the inhibition of BMP signalling, that firstly would give continuous inhibition between doses, and secondly might be compatible with use as a future human therapeutic. Data produced with this intention is presented and discussed in Chapter 5.

Chapter 5 Inhibiting Type 1 BMP Signalling: Molecules with Clinical Potential in Myeloma

5.1 Introduction

It has been shown in chapter 3 that a gene set comprising ligands, receptors, antagonists, targets and intracellular components of the BMP pathway is upregulated in MSCs of the myeloma bone-lining niche. In chapter 4, inhibition of type 1 BMP signalling was shown to halt cancer-associated bone destruction in the KaLwRij mouse myeloma model. Evidence was presented that the small molecule Type 1 BMP receptor inhibitor LDN193189 led to osteoclast suppression and osteoblast differentiation *in vivo*, and *in vitro* could directly suppress human osteoclast differentiation. In addition, LDN193189 suppressed liver *Hamp1* gene expression *in vivo*, but this did not extend to a fall in circulating hepcidin protein levels. It is unclear whether LDN193189 may ever be suitable for use as a human therapeutic; as described in chapter 1, type 1 BMP signalling has diverse and critical roles. Although LDN193189 has been under development as a possible therapy in FOP and inflammatory anaemia [<https://ncats.nih.gov/bridgs/projects/active/protein-inhibitors-blood-bone-disorders>], a pan-inhibitor of type 1 BMP signalling may produce unacceptable side effects, or off-target kinase interactions (Mohedas *et al*, 2013). LDN193189 also has a short half-life (Fig. 4.1), meaning that even once-daily dosing such as that used in chapter 4 may not provide consistent receptor blockade. It is therefore possible that a greater benefit to bone mass could be provided by a more pharmacokinetically stable therapeutic.

I next sought to recapitulate the beneficial *in vivo* effects of LDN193189 in the KaLwRij myeloma model using molecules specifically targeted to the BMP pathway, but which had been designed as human therapeutics. There are no reports of these molecules being previously used in the cancer: bone niche setting.

5.1.1 Anti-BMP6

As discussed in section 4.1.1, BMP6 is the main BMP reported to be expressed by human myeloma cells (Grcevic *et al*, 2010; Seckinger *et al*, 2009), and was also expressed in 5TGM1 murine myeloma cells (Fig. 4.4). Anti-BMP6 human IgG4 antibody was obtained under MTA from Kymab Ltd (protein batch KYAB1246). This is a fully human antibody derived from their 'Kymouse' antibody generation technology. In brief, transgenic mice containing the entire human immunoglobulin variable gene repertoire, but murine constant regions, are exposed to the human antigen of interest (i.e. in this case BMP6). Their immune response to this antigen exposure produces high-affinity antibodies with long human-like complementarity-determining region 3 (CDR3H), broad epitope coverage and strong signatures of somatic hypermutation (Lee *et al*, 2014). The molecule is active (cross-reactive) against human and mouse BMP6 with similar potency. It neutralises BMP6 activity *in vitro* as measured by the neutralisation of a hepcidin reporter gene assay in HeopG2 cells. It has high specificity for BMP6 and does not cross react with other BMPs, for example BMP5, BMP7 or BMP2/4. Affinity for BMP6 (KD) is in the nM range (all unpublished data supplied by Kymab, summarized at <http://www.google.ch/patents/US8318167>).

5.1.2 Alk3-Fc solubilised ligand trap

Solubilised murine Alk3-Fc (RAP661) was obtained under MTA from Acceleron Pharma. This compound will bind and therefore neutralise ligands that would normally bind to the Alk3 (BMPR1a) receptor (Fig. 5.1), and has been previously shown to increase bone volume in an ovariectomised mouse model of osteoporosis (Baud'huin *et al*, 2012). Of note, although *in vitro* the membrane-bound Alk3 receptor can bind BMPs 2, 4, 5, 6, 7 and 8 with high affinity (Yadin *et al*, 2016), *in vivo* the preference for removal of any particular ligand by soluble Alk3-Fc will depend upon its relative abundance in any local environment, presence

of co-receptors that might preferentially facilitate binding either to the soluble trap or to the competing membrane-bound native Alk3, and the abundance of competing receptors and inhibitors (see chapter 1).

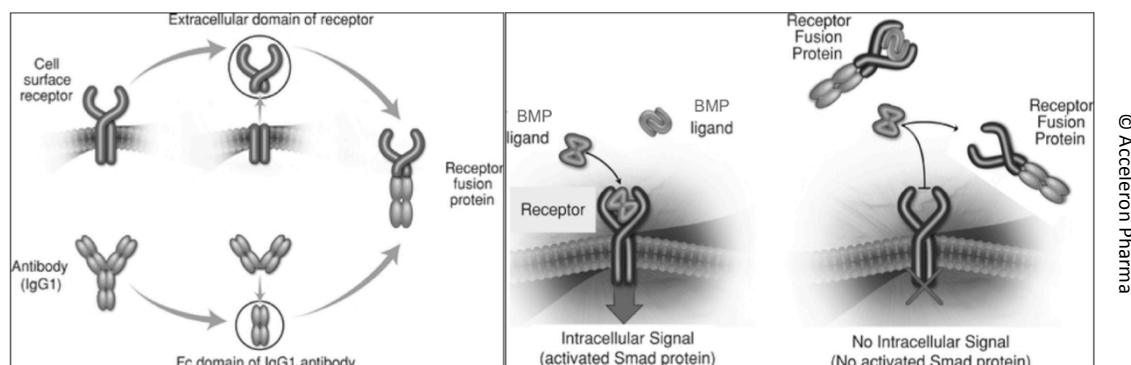


Figure 5.1: Ligand trap design of RAP661.

The ligand-binding component of the Alk3 cell surface receptor is joined to Fc domain of IgG1. This solubilises the receptor so that it may act in a similar ligand trapping / removing fashion to natural BMP inhibitors such as noggin. Reproduced with permission from Acceleron Pharma.

5.1.3 Hypotheses of this chapter

In chapter 4, LDN193189 was shown to increase bone mass in myeloma bearing mice, and reduce liver hepcidin expression but only in control mice. BMP6 is the main BMP produced by myeloma cells (note that this is associated with good prognosis) (Grcevic *et al*, 2010; Seckinger *et al*, 2009). BMP6 is also the main BMP which signals to promote hepcidin transcription in states of functional iron restriction (Ganz, 2011). Alk3/ Bmpr1a receptor signalling has been shown to mediate the hepatic BMP-hepcidin axis (Steinbicker *et al*, 2011a), and osteocyte sclerostin secretion (Kamiya *et al*, 2016). Alk3-Fc has previously been shown to improve bone mass in osteoporosis models (Baud'huin *et al*, 2012). I therefore hypothesized:

Hypothesis 1. Anti-BMP6 treatment will improve bone mass in myeloma bone disease and suppress hepcidin expression, which may or may not improve haemoglobin. It will not reduce tumour burden, and could potentially increase it.

Hypothesis 2: Alk3-Fc treatment will improve bone mass in myeloma bone disease, increase osteoblastogenesis and decrease osteoclastogenesis. It may suppress sclerostin levels. It will suppress hepcidin expression, which may or may not improve haemoglobin. It will not reduce tumour burden.

5.2 Experimental design

For analysis of both these drugs, the KaLwRij 5TGM1 myeloma model was used as described in chapter 2.

5.2.1 Specific details of *in vivo* experiments to determine the effects of anti-BMP6 and Alk3-FC

Anti-BMP6 experiment

On day 0, 21 mice were weighed and inoculated IV with 2×10^6 5TGM1 GFP+ cells. On day 5, IP anti-BMP6 or isotype control antibody treatment was commenced at 10mg/kg/dose, three times per week. Mice were assessed daily and weighed weekly.

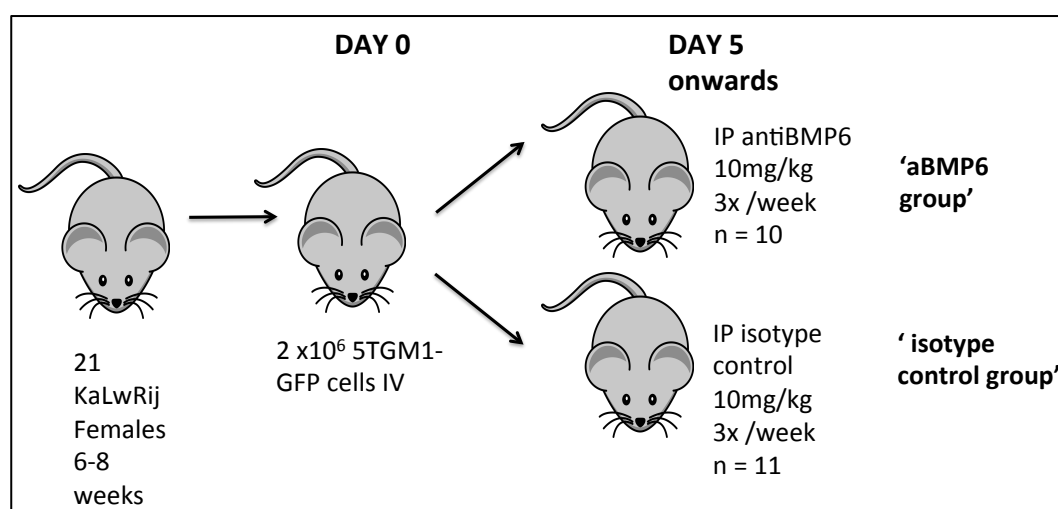


Figure 5.2: Experimental design of anti-BMP6 or isotype control use in KaLwRij mice with 5TGM1 myeloma.

All mice were culled when the first mouse displayed sign of hind limb weakness, which occurred on day 22. Tissues were processed and analysed as described in chapter 2, to provide the following data:

- Tibial MicroCT- generated trabecular and cortical bone density indices (BV/TV)
- FACS measurement of GFP positive proportion of spleen and bone marrow cell suspensions to assess tumour burden
- Serum IgG2b ELISA to assess global 5TGM1 tumour burden
- Liver *Hamp1* gene expression (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control)
- Serum Hepcidin ELISA
- Serum iron
- Serum transferrin (human transferrin assay)

Of note, this transferrin test (described in chapter 2) has not been validated for use in the measurement of murine transferrin. However on testing, the positive correlation of its quantitation with hepcidin, and negative correlation with serum iron suggest with high probability that it measures transferrin level to some extent (Figs. 5.5 and 5.6). Serum transferrin data presented here is therefore viewed as pilot, non-validated data that is relative within the experiment but not an absolute measurement.

Alk3-Fc Ligand Trap experiment

On day 0, 30 mice were weighed and inoculated IV with 2×10^6 5TGM1 GFP+ cells, or PBS control. On day 5, IP Alk-Fc or vehicle control treatment was commenced at 10mg/kg/dose, twice per week (Fig. 5.3). Mice were assessed daily and weighed weekly.

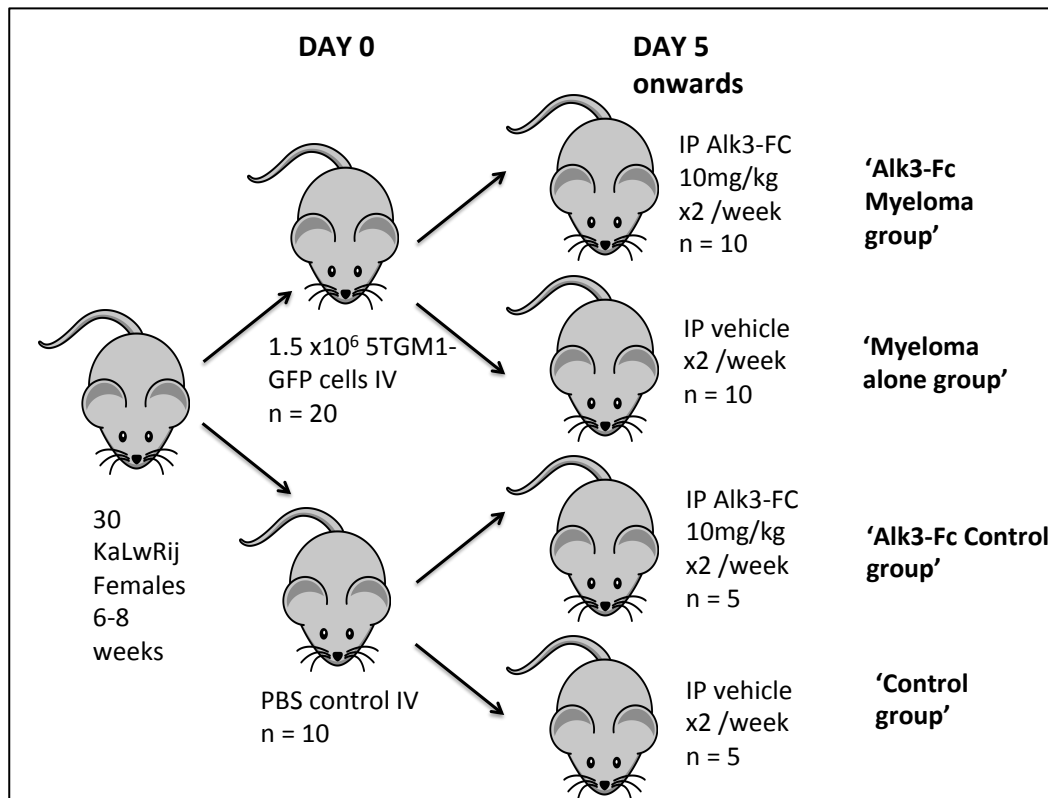


Figure 5.3: Experimental design of Alk3-Fc or vehicle control use in KaLwRij mice with 5TGM1 myeloma.

All mice were weighed then culled when the first mouse displayed sign of hind limb weakness, which occurred on day 20. Again, tissues were processed and analysed as described in chapter 2, to provide the following data:

- Tibial MicroCT- generated trabecular and cortical bone density indices (BV/TV)
- Histomorphometry of decalcified, formalin fixed, paraffin embedded femur sections stained with haematoxylin and for TRAP activity, analysed with Osteomeasure software
- FACS measurement of GFP positive proportion of spleen and bone marrow cell suspensions to assess tumour burden
- Serum IgG2b ELISA to assess 5TGM1 tumour burden
- Serum TRAP5b ELISA to assess global osteoclast activity
- Bone marrow plasma Dkk1 and Sclerostin quantification

- Liver *Hamp1* gene expression (qRT-PCR, $2^{-\Delta CT}$ method using *Hprt* as endogenous control)
- Serum Hepcidin ELISA

5.2.2 Specific details of *in vitro* experiments to determine the effects of Alk3-FC

In vitro experimental techniques are discussed in chapter 2, so only specific details pertaining to individual experimental conditions are reported here.

HAMP gene expression experiments

Confluent human hepatocarcinoma HuH7 cells were treated with human BMP6 0.2µg/ml, LDN193189 100nM, ALK3-FC at either 10, 1, 0.5 or 0.1µg/ml, or control media for 24h (Fig. 5.15); harvested RNA was used to measure *HAMP* gene expression (qRT-qPCR, $2^{-\Delta CT}$ method using *GAPDH* as endogenous control). Of note, n = 4 replicates were performed of this experiment, 2 using human ALK3-FC, 2 using murine Alk3-FC.

Osteoclast differentiation experiments

Osteoclast differentiation was performed as described in chapter 2. Alk3-FC 10 µg/ml was added to osteoclastogenic media from day after seeding until mature osteoclast formation in control wells, when wells were fixed, stained and multinucleated cells enumerated (Fig. 5.12).

siRNA BMP receptor knockdown experiments in UMR106.01 rat osteosarcoma cell line

Rat UMR-106.01 cells were transfected with siRNAs targeting either rat *Alk2*, *Alk3* or both. The total concentration of siRNA added was 100nM (50nM per target when both targeted together). Cells were harvested after a further 48 hours. 100nM LDN193189 was added to

equivalent wells 24 hours before harvest for comparison. RNA extraction and qRT-PCR were performed using the $2^{-\Delta CT}$ method with *Hprt* as endogenous control (Fig. 5.14).

Stromal cell culture from myeloma patient bone marrow samples

Mononuclear cells were isolated from fresh bone marrow aspirates donated by myeloma patients. Adherent cells (BMSCs) were grown to confluence, reseeded and treated for 24 h with LDN 100nM or ALK3-FC 10µg/ml, before RNA extraction and qRT-PCR, $2^{-\Delta CT}$ method using *GAPDH* as endogenous control (Fig. 5.14).

5.3 Results

5.3.1 Anti-BMP6 improves anaemia, with no effect on myeloma disease burden or bone disease in 5TGM1 myeloma-bearing mice

KaLwRij mice were inoculated with 5TGM1-GFP myeloma, and from day 5 treated with anti-BMP6 or isotype control antibody three times weekly as described above. Indices of disease burden at cull demonstrated that anti-BMP6 had no effect on accumulation of tumour cells in bone marrow or spleen, global tumour burden as measured by serum IgG2b, or weight loss (Fig. 5.4 A-D).

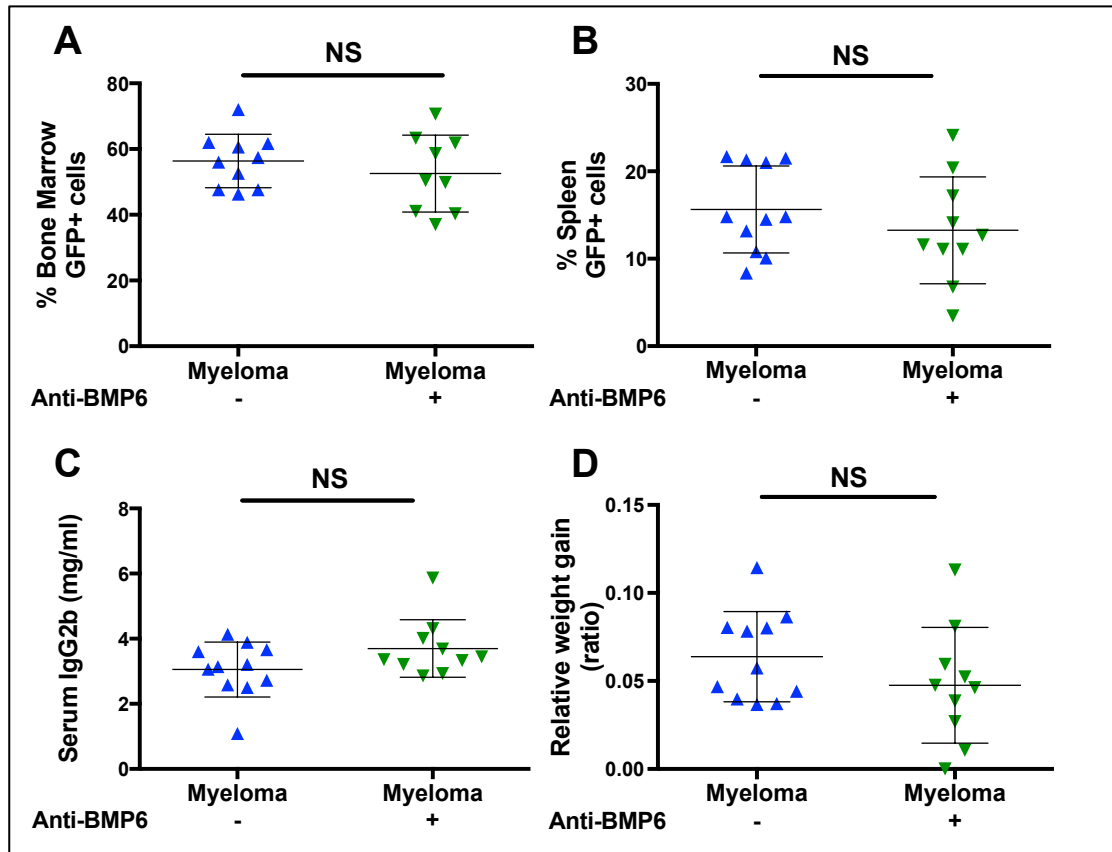


Figure 5.4: Tumour burden is no different between isotype control- and anti-BMP6-treated myeloma-bearing mice.

Indices of disease burden in myeloma-bearing mice treated with anti-BMP6 (n = 10) or isotype control antibody (n = 11). Each point represents one mouse.

A: Percentage of bone marrow mononuclear cells expressing myeloma-specific GFP (1 data point missing from each group due to sample contamination)

B: Percentage of spleen mononuclear cells expressing myeloma-specific GFP

C: Serum IgG2b level (mg/ml)

D: Relative weight gain over course of experiment (total weight change/starting weight)

To investigate the effect of anti-BMP6 on myeloma-induced anaemia, I investigated indices of anaemia and iron metabolism in myeloma-bearing mice (Fig. 5.5 and 5.6). Treatment with anti-BMP6 caused an elevation in haemoglobin from mean 13.5 g/dL to mean 14.2g/dL (Fig. 5.5 A), with associated reduction in liver *Hamp1* (hepcidin) mRNA (Fig. 5.5 B), and serum hepcidin level (Fig. 5.5 C). As might be expected with reduced hepcidin production, serum iron availability significantly increased (Fig. 5.5 E), and serum transferrin level significantly fell (Fig. 5.5 E).

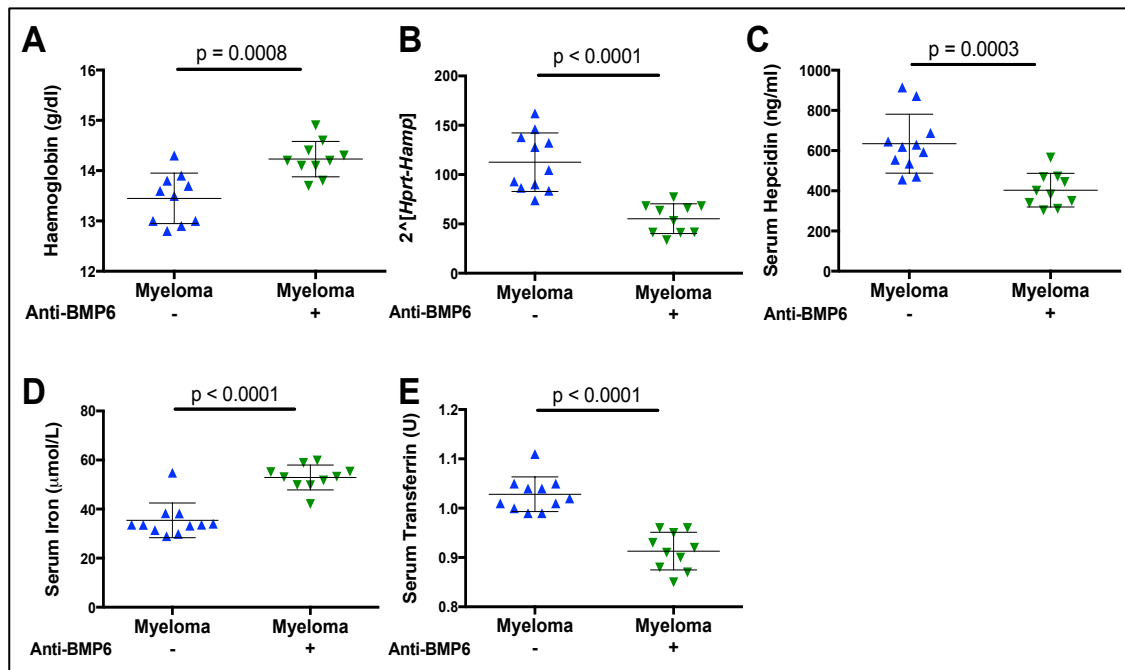


Figure 5.5: Haemoglobin is significantly raised, hepcidin suppressed, and iron availability increased in anti-BMP6-treated myeloma-bearing mice.

Indices of anaemia and iron metabolism in myeloma-bearing mice treated with anti-BMP6 (n = 10) or isotype control antibody (n = 11).

A: Plasma haemoglobin at cull (g/dL)

B: *Hamp1* gene expression in liver tissue samples. $2^{-\Delta CT}$ method using *Hprt* as endogenous control.

C: Serum hepcidin level (ng/ml)

D: Serum iron level ($\mu\text{mol/L}$)

E: Presumed serum transferrin level (U, using human serum transferrin assay)

P values generated using unpaired T tests.

Previous experiments using LDN193189 failed to yield accurate haemoglobin measurements for comparison, but from previous work (Drakesmith lab, data not shown), haemoglobin measurement in healthy age-matched mice from this and the background C57Bl6 strain, taken by the same method and analysed on the same machine, indicate a usual range of 14.5-16 g/dL. These data therefore provide evidence that mildly lowered haemoglobin is found in KaLwRij mice with 5TGM1-GFP myeloma, which is moderately ameliorated by anti-BMP6.

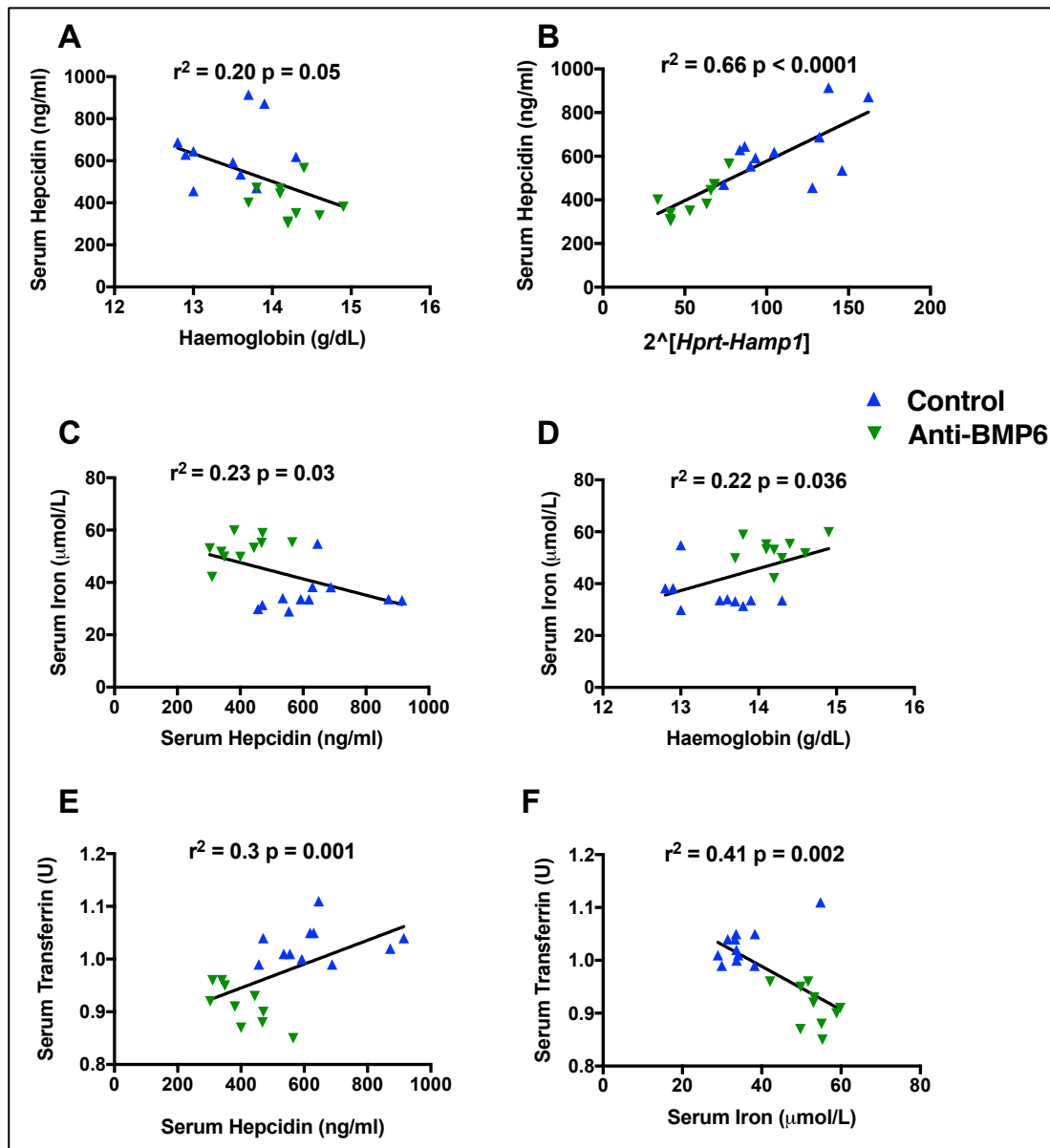


Figure 5.6: Indices of anaemia and iron metabolism correlate in myeloma-bearing mice treated with anti-BMP6 (n = 10) or isotype control antibody (n = 11).

- A: Haemoglobin vs. serum hepcidin
- B: Serum hepcidin vs. liver *Hamp1* (hepcidin) gene expression
- C: Serum hepcidin vs. Serum iron
- D: Serum hepcidin vs. serum transferrin
- E: Haemoglobin vs. serum iron
- F: Serum iron vs. serum transferrin

Unpublished Drakesmith Group data suggests that in female, age matched mice, using the same analyser used in this experiment, the normal range for serum iron is 25-35 $\mu\text{mol/L}$, and transferrin 0.8 to 0.9 U. Of note, in another experiment, heavily anaemic malaria-parasitised female age-matched mice had a raised transferrin of mean 1.2U (data not

shown). In comparison, in this experiment, myeloma-bearing control mice have serum iron in the high-normal range (mean 35.4 $\mu\text{mol/L}$), which rises above normal range with anti-BMP6 (mean 52.9 $\mu\text{mol/L}$). In contrast, the transferrin is slightly high in myeloma-bearing control mice (mean 1.0 U), but falls back to normal range on treatment with anti-BMP6 (mean 0.9 U). *Hamp1* (Hepcidin) mRNA and hepcidin serum levels demonstrate close correlation (Fig. 5.6 B), as should be the case if techniques are accurate, but these techniques do not generate values comparable with other experiments. In summary, on anti-BMP6 treatment, hepcidin gene transcription and serum levels fall, serum iron rises, transferrin falls and haemoglobin corrects. As discussed in detail later in section 5.4, this is consistent with a release of stored iron and resultant increase in erythropoiesis.

To look for changes in bone disease with anti-BMP6 treatment, microCT was performed on each formalin-fixed right femur, and reconstructed images analysed as described. Anti-BMP6 treatment did not elicit significant improvement in trabecular bone volume (BV/TV, Fig. 5.7 A), nor total cortical bone volume (Fig. 5.7 B). As these most sensitive indices of changes to bone microarchitecture demonstrated no differences, further histological analyses were not undertaken.

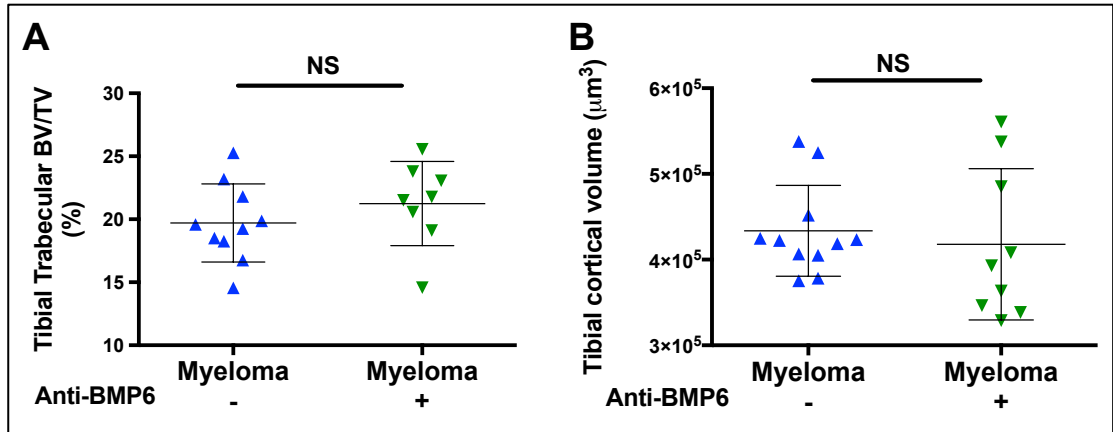


Figure 5.7: Indices of bone volume in myeloma-bearing mice treated with anti-BMP6 (n = 10) or isotype control antibody (n = 11).

Reconstructed tibial metaphyseal images analysed using Bruker CTAn software to generate:

A: Trabecular BV/TV (bone volume / total volume)

B: Cortical volume in μm^3

5.3.2 ALK3-Fc improves bone volume, with no effect on myeloma disease burden or anaemia in 5TGM1 myeloma-bearing mice

Healthy and myeloma-bearing mice were treated with IP ALK3-Fc or vehicle (PBS) twice weekly, from day 5 after 5TGM1-GFP tumour inoculation until cull. Indices of disease burden at cull demonstrated that ALK3-Fc had no effect on the accumulation of tumour cells in bone marrow or spleen, or global tumour burden as measured by serum IgG2b (Fig. 5.8 A-C). However diseased mice treated with ALK3-Fc gained significantly more weight (relative to their starting weight) than untreated disease mice (Fig. 5.8 D).

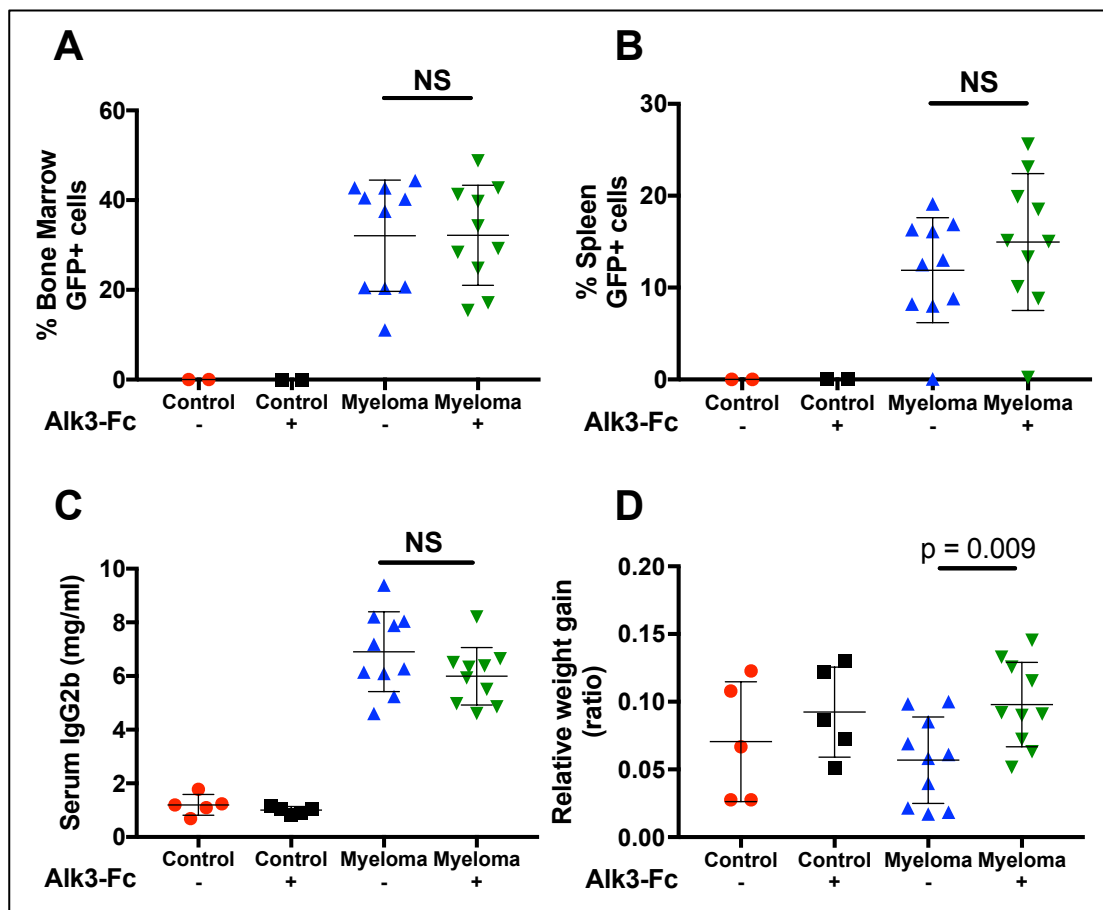


Figure 5.8: Tumour burden is no different between isotype control- and ALK3-Fc-treated myeloma-bearing mice.

Indices of disease burden in healthy (n = 5/group) and myeloma-bearing mice, treated with ALK3-Fc (n = 10) or isotype control antibody (n = 10).

A: Percentage of bone marrow mononuclear cells expressing myeloma-specific GFP

B: Percentage of spleen mononuclear cells expressing myeloma-specific GFP

C: Serum IgG2b level (mg/ml)

D: Relative weight gains over course of experiment (total weight change/starting weight)

All p values generated using unpaired T tests.

On day of cull it was striking that 5 mice (50% of total) in the myeloma group developed hindlimb weakness and loss of tail tone, indicating spinal collapse due to tumour infiltration. However, no mice in the Alk3-Fc treated group showed any similar gait abnormalities, and were normal in behaviour and appearance. Alk3-Fc was shown to have a highly significant effect in augmenting bone volume, both in healthy and myeloma-bearing mice (Fig. 5.9 A-E, Fig. 5.10 A). This was seen in both trabecular and cortical parameters, by both microCT of tibiae and histomorphometry of femora analyses. However, Alk3-Fc did not lead to a reduction in the number of cortical lytic lesions as was seen with LDN193189 (Fig. 5.9 F, Fig. 5.10 B).

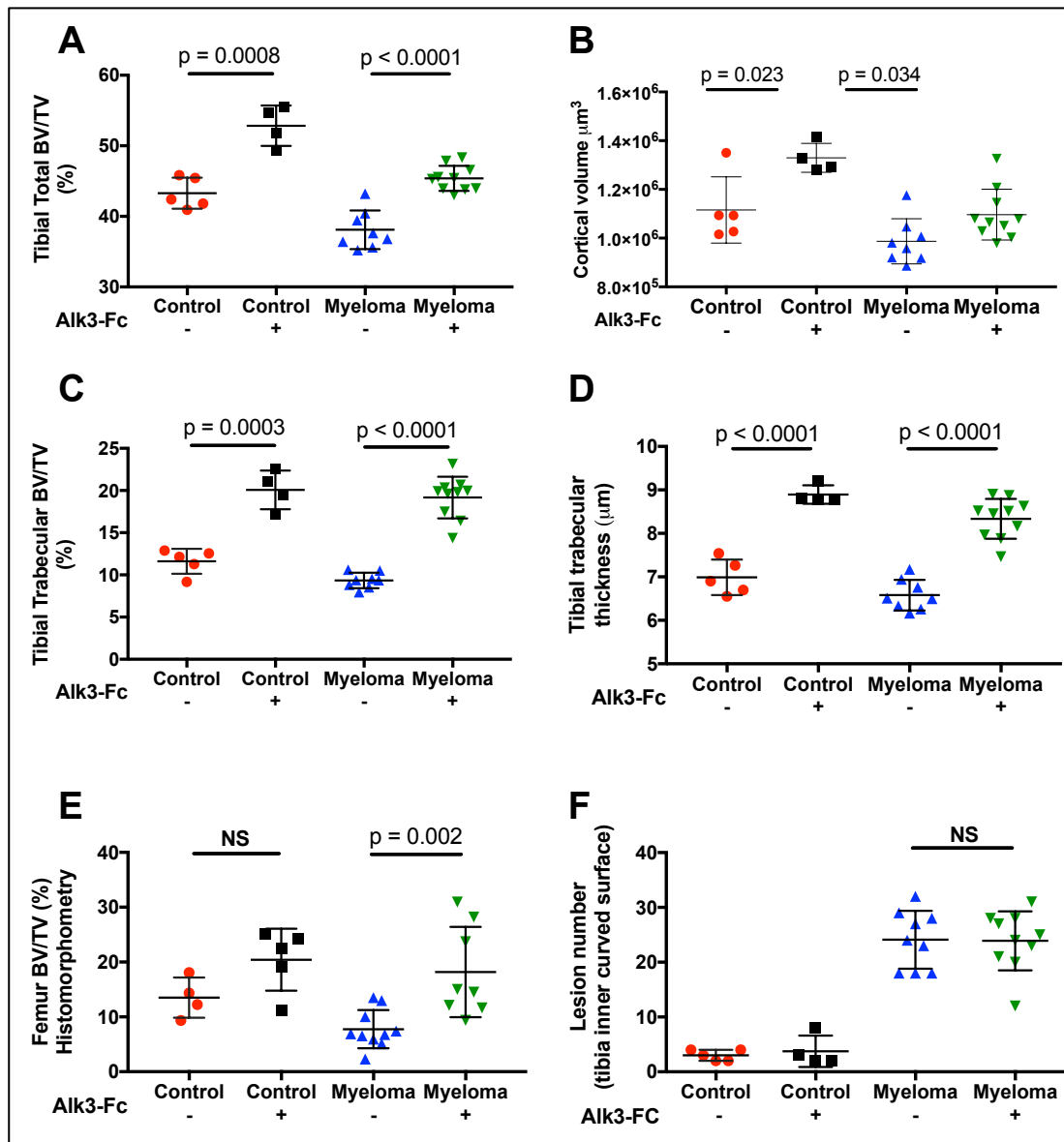


Figure 5.9: Inhibition of BMP signaling with Alk3-Fc reduces myeloma bone disease *in vivo*

Reconstructed tibial metaphyseal images analysed using Bruker CTAn software to generate (A-D & F):

A: Total BV/TV (bone volume / total volume)

B: Cortical volume in μm^3

C: Trabecular BV/TV

D: Trabecular thickness in μm

E: Histomorphometric generated BV/TV (femoral metaphyseal sections).

F: Enumerated cortical lytic lesions on right medial tibial surface

All p values generated using unpaired T tests.

Note: 2 myeloma alone animals had severe bone damage such that tibial metaphyses disintegrated on harvest. CTs were not assessable. One Alk3-Fc-treated control tibia was broken in processing and therefore did not undergo microCT.

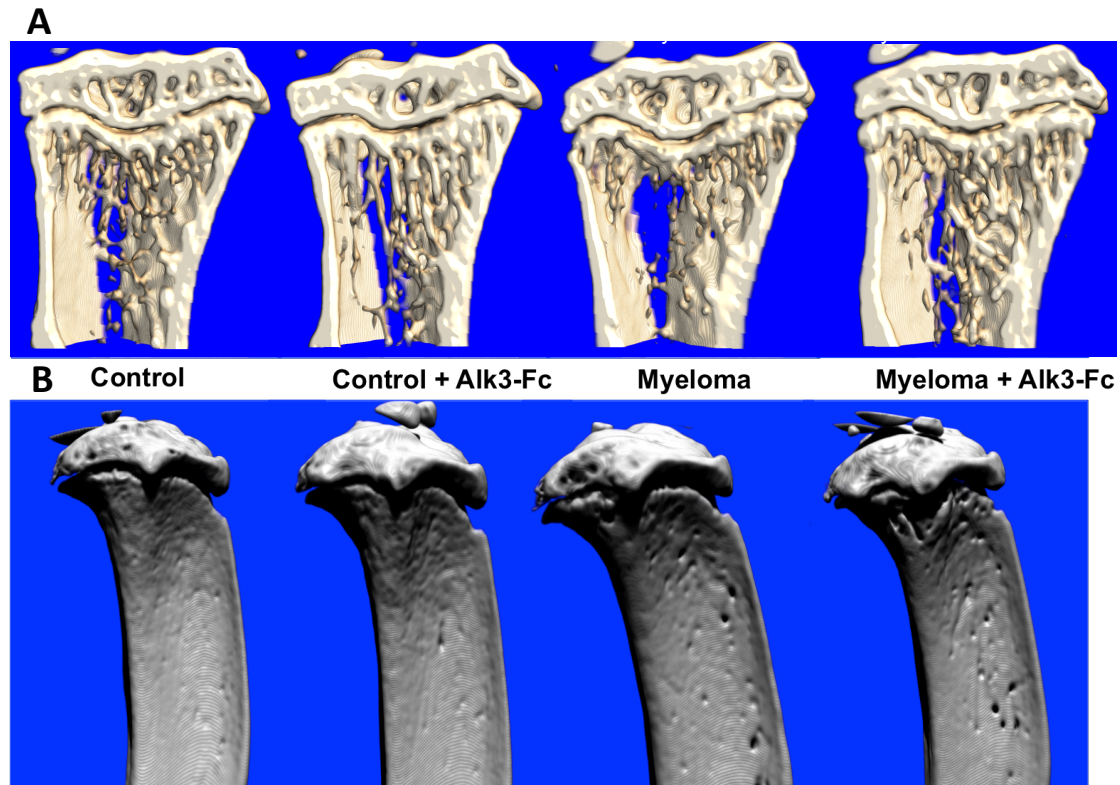


Figure 5.10: Inhibition of BMP signaling with Alk3-Fc reduces myeloma bone disease *in vivo*. Reconstructed microCT images of (A) tibial sagittal sections showing trabecular architecture and (B) surface cortical lesions on tibial surface.

5.3.3 ALK3-FC reduces *in vivo* osteoclasts in myeloma-bearing mice, and *in vitro* osteoclastogenesis

Histomorphometry revealed detailed cellular information regarding treatment with Alk3-Fc. Blinded histomorphometric analysis revealed a reduction in number and density of TRAP-staining cells with Alk3-FC treatment (Fig. 5.11 A-C). To confirm that the observed osteoclast reduction reflected global osteoclast activity, serum TRAP5b was measured by ELISA. This demonstrated a significant drop in serum TRAP5b with Alk3-Fc use in myeloma-bearing and control mice (Fig. 5.11 D), again indicating that Alk3-Fc use reduced osteoclast burden.

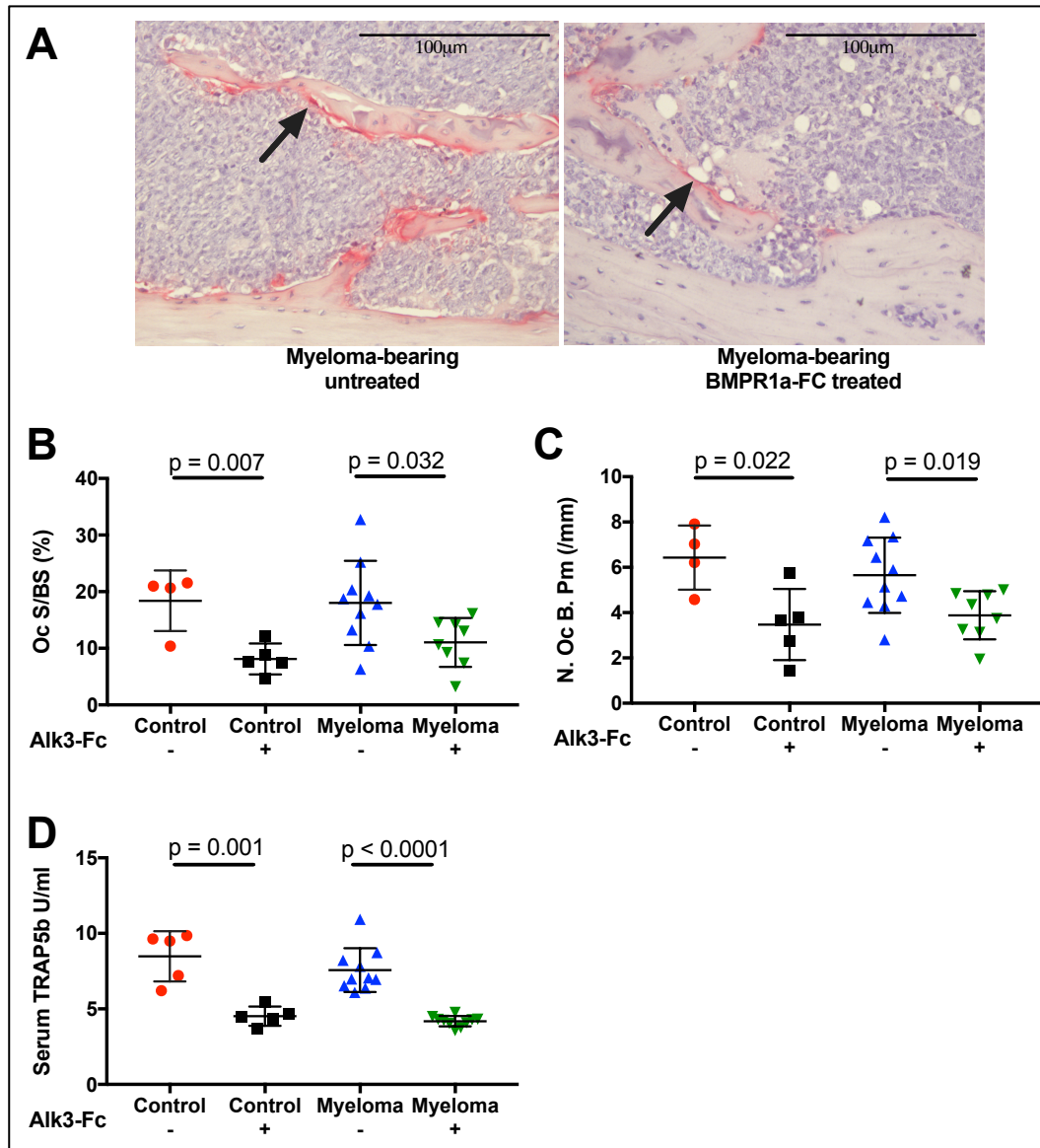


Figure 5.11: Inhibition of BMP signaling with Alk3-Fc reduces osteoclast number *in vivo*

A: Osteoclasts (indicated with arrows) from myeloma-bearing mice: Vehicle vs. Alk3-Fc-treated (TRAP and haematoxylin stained).

B-C: Histomorphometric analysis of TRAP-stained osteoclasts in control and Alk3-Fc-treated healthy and myeloma-bearing mice:

B: Osteoclast surface/ trabecular surface ratio (OcS/BS)

C: Osteoclast bone surface density (N.Oc.B.Pm)

D: Serum TRAP5b concentration, indicating global osteoclast burden, in vehicle vs. Alk3-Fc-treated healthy and myeloma-bearing mice.

All p values generated using unpaired T tests.

In vitro osteoclast differentiation assays were performed using Alk3-Fc to assess whether

this drug had a directly suppressive effect on osteoclast differentiation, or the *in vivo*

observed effect could be explained as indirect (for example via changing expression of

RANKL expression in other bone marrow microenvironment cells, as is the purported case

with anti-sclerostin (Wijenayaka *et al*, 2011)). Alk3-Fc, as LDN-193189 (chapter 4), was shown to significantly reduce osteoclast differentiation in isolated CD14+ PBMCs in the absence of RANKL-secreting cells of the bone marrow microenvironment, suggesting this drug also has a direct effect on osteoclast precursors (Fig 5.12). Of note in this experiment using human PBMCs, human ALK3-Fc was used (see chapter 2) rather than RAP661, a murine version. As an additional comparison, in contrast to ALK3-Fc, an activin ligand trap (ACTR11a-Fc) slightly increase osteoclastogenesis (Fig. 5.12 D), demonstrating BMP and Activin inhibition have non-equivalent effects on myeloma bone disease in this model system.

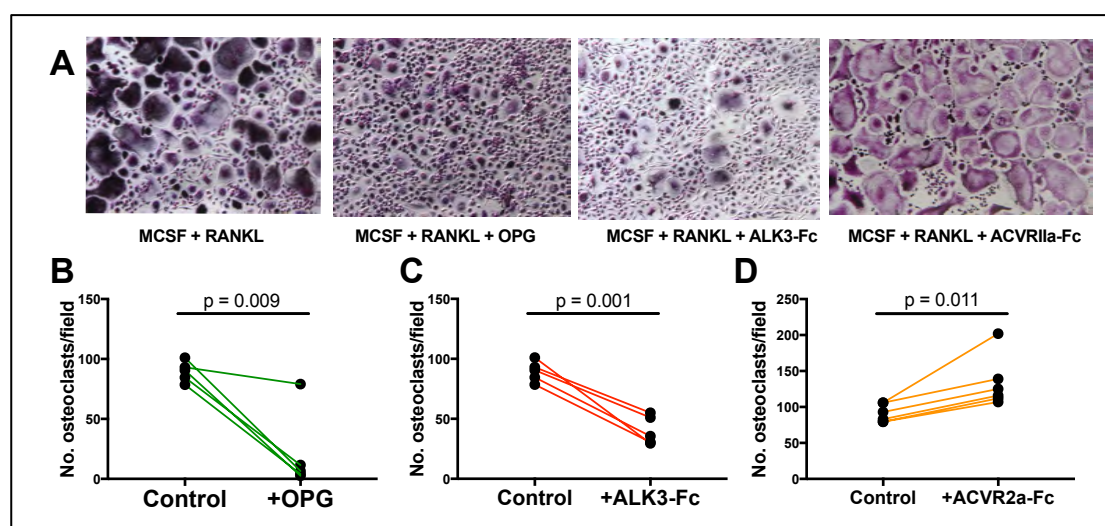


Figure 5.12: Inhibition of BMP signaling with ALK3-Fc directly reduces osteoclast formation *in vitro*

A: Pooled human CD14+ PBMC osteoclast differentiation, with treatments as indicated commenced 24h after seeding. TRAP stained at D10. Images taken at 70x magnification.

B-C: Quantified osteoclasts (≥ 3 nuclei) when CD14+ PBMCs treated as (A) were enumerated. Results from same experimental replicate are paired to account for inter-replicate variation (n=5 replicates). P values generated by paired T test.

B: Control vs OPG treatment

C: Control vs ALK3-Fc treatment (see chapter 2 for methodology)

D: Control vs ACVR2a-Fc treatment

5.3.4 ALK3-FC increases *in vivo* bone lining cells numbers and thickness in myeloma-bearing mice, and reduces bone marrow plasma sclerostin

Using histomorphometry to quantify bone-lining cells, Alk3-Fc treatment was found to increase their numbers (Fig. 5.13 A) while reducing osteoclast numbers, in a manner similar

to, but more dramatic than, LDN193189 treatment (chapter 4). I noted that the rows of bone-lining cells were more easily picked out in the Alk3-Fc treated animals, both in healthy and myeloma-bearing animals (Fig. 5.13 B). A recently published report had noted the same phenomenon in mice treated with anti-sclerostin, and attributed this to reactivation of the bone-lining layer to active osteoblasts (Kim *et al*, 2017).

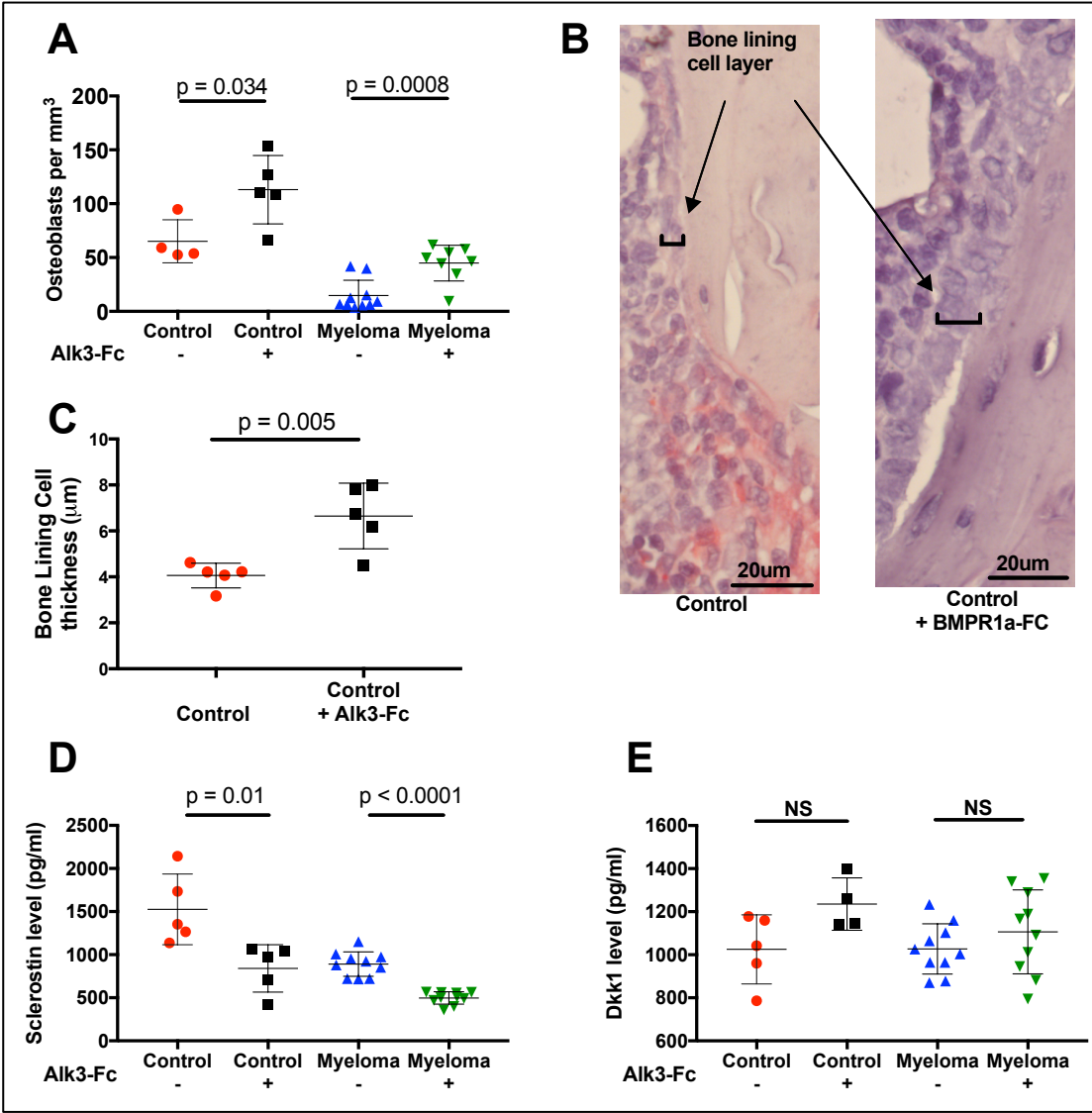


Figure 5.13: Alk3-Fc use increases bone lining cell number and width, and causes a significant fall in bone marrow plasma sclerostin.

A: Osteoblasts per mm³ (Bone lining cell density, N.Ob/T.Ar) in mice treated with vehicle vs Alk3-Fc
 B: Images of bone lining cell layer (haematoxylin and TRAP stained femur sections) in healthy mice treated with vehicle or Alk3-Fc
 C: Bone lining cell layer thickness (µm) in healthy mice treated with vehicle vs Alk3-Fc
 D: Bone marrow plasma sclerostin level (pg/ml)
 E: Bone marrow plasma Dkk1 level (pg/ml)
 p values generated using unpaired T tests.

Blinded measurement of the width of the bone lining cell layer demonstrated increased bone lining cell thickness with Alk3-Fc treatment. Although the effect was also present with LDN193189 treatment, the difference was again greater with Alk3-Fc (Fig 5.13 B-C, compare to Fig. 4.11 B-C).

Also of note on observing the histological appearance of marrow space in Alk3-Fc-treated mice, compared to the expected loss of trabecular thickness with myeloma infiltration, were large areas of thick trabeculae, in which osteocytes were easily visible (Fig. 5.11 A).

Unfortunately in this experiment mice developed neurological symptoms and had to be culled before they had received two calcein injections to enable bone formation rate calculation, therefore this analysis could not be performed.

As with LDN193189, further investigations were performed to elucidate mechanisms by which Type 1 BMP inhibition achieved increased bone mass. In contrast to the findings with LDN193189, bone marrow plasma sclerostin level decreased significantly with Alk3-Fc use in both healthy and myeloma-bearing animals (Fig. 5.13 D). However as with LDN193189 use, bone marrow plasma Dkk1 levels were not significantly different between Alk3-Fc treated and untreated animals (Fig. 5.13 E).

In chapter 4, LDN193189 was shown to cause a drop in *Dkk1* but not *Sost* gene expression by the rat osteosarcoma UMR106.01 cell line, and a drop in *Dkk1* expression in myeloma patient-derived stromal cells. On repeat of these experiments using murine and human Alk3-Fc, again marginal reduction in *Dkk1* expression was seen in myeloma patient-derived stromal cells ($p = 0.036$), and also in UMR106.01 cells (Fig. 5.14 A, B i.), where LDN193189 was again included for comparison. However this time, the UMR106.01 cell cultures were all treated with additional recombinant BMP4 to see if an explicit BMP-induced gene expression was reversed. Unexpectedly, with BMP4 added to cultures, LDN193189 did cause a significant reduction in *Sost*, but Alk3 ligand traps did not (Fig. 5.14 B ii.).

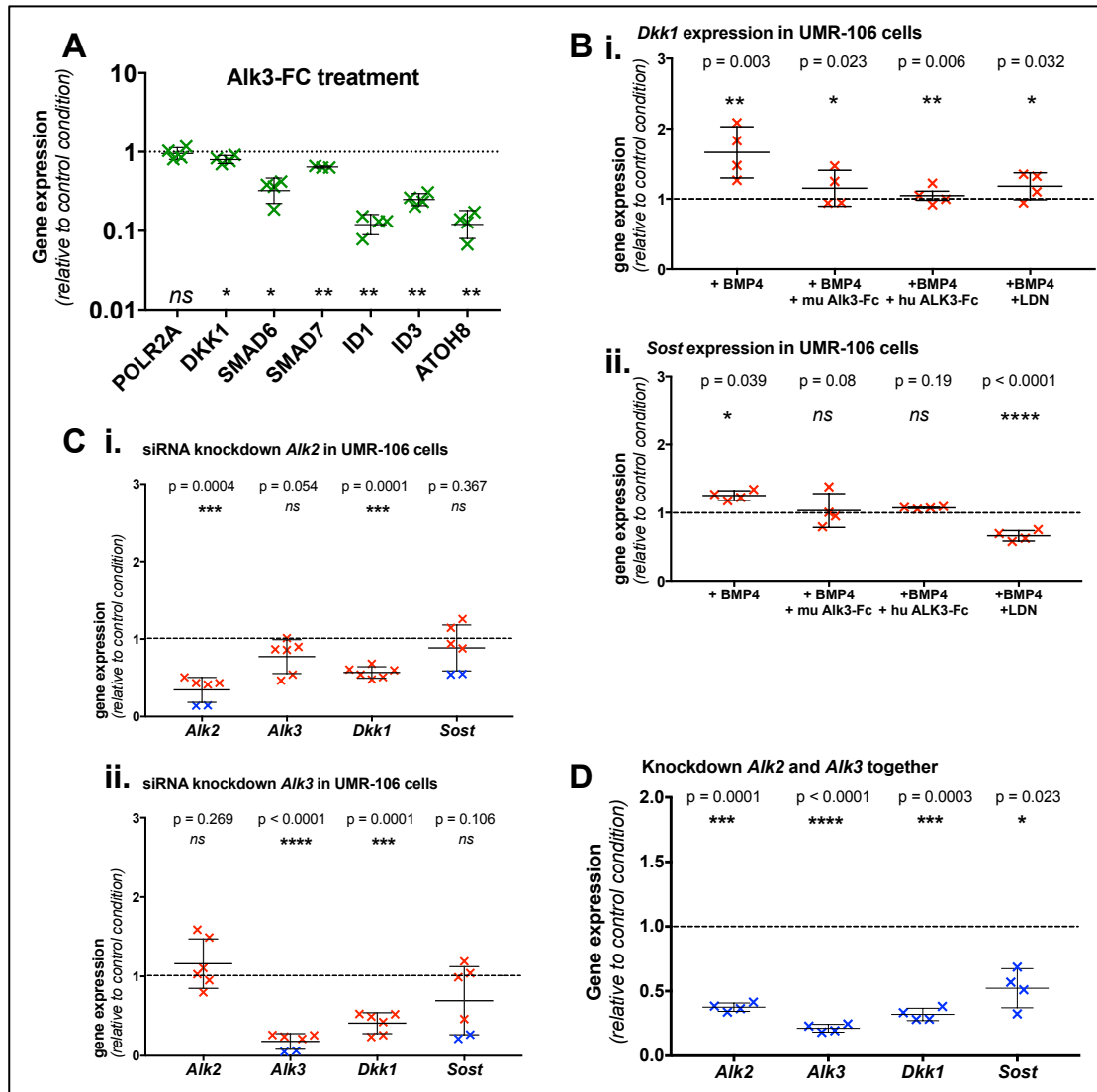


Figure 5.14: Alk3-Fc treatment marginally decreases *Dkk1* gene expression in human stromal and UMR106.01 cells; knockdown of *Alk2* and *Alk3* reduces *Dkk1* and *Sclerostin* expression in UMR106.01 cells.

A: Expression of *POLR2A* (control) and BMP-response genes *SMAD7*, *SMAD6*, *ID1*, *ID3*, *ATOH8*, and *DKK1* by patients' bone marrow stromal cells after 24h incubation with Alk3-Fc (10 μ g/ml). $2^{-\Delta CT}$ method using *GAPDH* as endogenous control ($n = 4$ patient samples). P values generated with paired ratio t-tests as each sample was individually normalised to control due to baseline variation between patient samples, with correction for false discovery rate by Benjamini & Hochberg method: *POLR2A* $p = 0.670$; *DKK1* $p = 0.036$; *SMAD6* $p = 0.014$; *SMAD7* $p = 0.002$; *ID1* $p = 0.002$; *ID3* $p = 0.002$, *ATOH8* $p = 0.003$.

B: Effect of 24h incubation with Alk3-Fc (10 μ g/ml) or LDN193189 (100nM) on expression of i. *Dkk1* and ii. *Sost* genes in rat osteosarcoma line UMR-106.01, with addition of BMP4 50ng/ml/well. $2^{-\Delta CT}$ method using *Hprt* as endogenous control, p values generated using one-way ANOVA using Sidak's multiple comparisons test: BMP4 alone condition compared to that of untreated control (indicated by dotted line), BMP4 plus additional compounds compared to BMP4 treatment alone ($n = 4$ replicates).

C: Effect of siRNA knockdown of i. *Alk2* and ii. *Alk3* on *Alk2*, *Alk3*, *Dkk1* and *Sost* gene expression in rat osteosarcoma line UMR-106.01 ($n = 6$ replicates). Blue points indicate where most complete knockdown correlate to lowest *Sost* expression.

D: Effect of siRNA double knockdown of *Alk2* and *Alk3* (rat osteosarcoma line UMR-106.01) on *Alk2*, *Alk3*, *Dkk1* and *Sost* gene expression ($n = 4$ replicates).

C-D: $2^{-\Delta CT}$ method using *Hprt* as endogenous control. P values generated using one-way ANOVA with multiple comparisons to control condition (scramble siRNA) using Dunnett's multiple comparisons test.

Whether Alk3 behaves in a physiological manner in the malignant UMR106 cell line is not known. All compounds achieved a significant reduction in *Dkk1* expression, but this could be Alk2-mediated (Kamiya *et al*, 2011). BMPs have been shown to promote *Sost* expression in the UMR106.01 cell line (Sedaghati *et al*, 2016), but there are no reports of inhibitors achieving the reverse. *Sost* expression in particular is reported to be regulated by Alk3 (Bmpr1a)-mediated BMP2/4 signalling in osteocytes (Delgado-Calle *et al*, 2013; Kamiya *et al*, 2016). To address this lack of clarity, further investigation was performed using Alk2 and Alk3 siRNA mediated knockdown in the UMR106.01 cell line (Fig. 5.14 C-D). This showed significantly reduced *Dkk1* expression with knockdown of either *Alk2*, or *Alk3*, or both. *Sost* suppression on single receptor knockdown was more variable, and not significant overall (possibly related to KD efficiency, as shown by best receptor knockdowns, highlighted blue, correlating with lowest *Sost* expression), but was significantly reduced on double knockdown.

5.3.5 ALK3-FC reduces hepcidin transcription *in vitro*, but has no effect *in vivo*

As with LDN-193189 and Anti-BMP6, the effect of Alk3-Fc on hepcidin expression and anaemia was assessed. Although *in vitro* Alk3-Fc (human and mouse) significantly suppressed *HAMP* (hepcidin) gene expression in HuH7 human hepatoma cells (Fig. 5.15 A), *in vivo* there was no significant difference between treated and untreated groups, either in *Hamp1* gene expression, serum hepcidin or haemoglobin (Fig. 5.15 B-D).

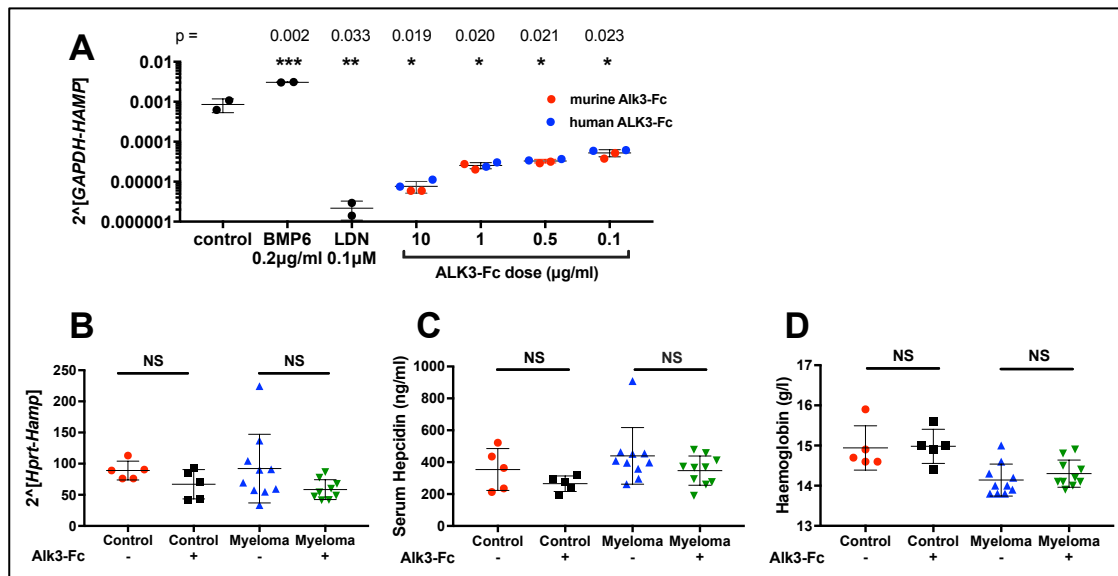


Figure 5.15: Alk3-Fc suppresses hepcidin (*HAMP*) gene expression *in vitro*, but *in vivo* in myeloma-bearing and control mice, has no effect on hepcidin or haemoglobin.

A: *In vitro* suppression of *HAMP* (hepcidin) gene expression in HuH7 human hepatoma cells by human and murine Alk3-Fc (dose response curves comparing potency to LDN-193189).

B-D: Indices of anaemia and hepcidin in healthy and myeloma-bearing mice, treated with Alk3-Fc or vehicle.

B: *Hamp1* gene expression in liver tissue samples. $2^{-\Delta CT}$ method using *Hprt* as endogenous control.

C: Serum hepcidin level in ng/ml

D: Plasma haemoglobin at cull (g/dL)

5.4 Discussion

The experiments detailed in this chapter were performed with the aim of examining the effect of Type 1 BMP receptor inhibitors designed for use in humans on the KaLwRij 5TGM1 murine model of myeloma. First, I tested a fully humanised neutralising anti-BMP6 antibody, because this BMP reported to be the only one secreted by myeloma cells (Grcevic *et al*, 2010; Seckinger *et al*, 2009). In this experiment, anti-BMP6 significantly reduced hepcidin expression and protein level, and significantly increased haemoglobin.

As BMP6 is the main iron-sensing signalling mediator of hepcidin expression (Andriopoulos Jr *et al*, 2009; Meynard *et al*, 2009), the hepcidin fall observed on BMP6 removal by anti-BMP6 is not surprising. This experiment has not shown that either BMP6 or hepcidin were abnormally elevated in myeloma-bearing mice; whatever the starting point, suppressing the hepcidin level prevents ferroportin degradation, so enabling iron absorption to increase and iron release from cellular sequestration to occur. Serum iron therefore rises and is available for use, enabling erythropoiesis to increase and haemoglobin to rise. Serum transferrin, to which iron must be bound to be absorbed by cells, rises in response to unmet erythropoietic iron requirements; when the iron supply is adequate, it falls. In the myeloma-bearing mice, serum iron rose and transferrin fell in response to anti-BMP6 treatment. This same iron and transferrin response is seen clinically when an iron-deficient patient receives iron; in these mice, the drop in hepcidin increasing iron availability had the same effect. It is pertinent to consider to what degree the moderately low haemoglobin in myeloma-bearing mice is caused by loss of erythropoietic reserve and to what lack of iron availability? And if lack of iron availability, is this due to increased competition for iron from the growing tumour, or to inflammatory cytokine-mediated hepcidin elevation? Data available from this experiment do not answer all these questions but some observations are possible. If the haemoglobin drop induced by myeloma were purely secondary to loss of erythropoietic reserve, anti-BMP6 would not have ameliorated it. However, although anti-BMP6 improved the haemoglobin from a mean of 13.5 g/dL to mean 14.2g/dL, it did not quite return to our observed normal range of 14.5-16 g/dL; therefore a contributing element of limiting erythropoietic reserve is also likely. Inflammatory cytokines or acute phase proteins were not measured here to assess the degree of systemic inflammation, but transferrin was above, not below the normal range seen in age and sex-matched mice (see section 5.3.1), as would be expected in inflammation. The abnormal hepcidin elevation reported in human myeloma has been

attributed to BMPs 2 and 4 (Maes *et al*, 2010); however had they been the cause of the haemoglobin drop in this model, anti-BMP6 would not have corrected it.

Therefore these results suggest, but do not conclusively show, that the myeloma-bearing mice in this model are subject to a multifactorial anaemia of iron sequestration or lack, in addition to a degree of erythropoietic reserve compromise. Anaemia in another murine model of myeloma has been described; the Vk*MyC model was shown to have mild progressive anaemia which accompanied indolent disease development. It was shown that increased iron uptake, both by the malignant plasma cells and by macrophages in tumour-bearing mice, left limiting levels of iron for erythropoiesis. Increased death of erythroblasts was also noted, but no rise in hepcidin expression (Bordini *et al*, 2015). Our results are compatible with a similar pathophysiology in the KaLwRij 5TGM1 model, where anti-BMP6 has corrected a functional iron deficiency to contribute to an improved haemoglobin, as indicated by the transferrin drop observed, an indicator of adequate correction. To test this hypothesis, a comparator group of myeloma-bearing mice could be treated with iron supplementation to compare the relative effects on haemoglobin correction of iron and anti-BMP6.

Anti-BMP6 had no effect on tumour burden or bone disease in myeloma-bearing mice. As stated in the hypothesis at the start of this chapter, there had been a possibility it could have caused increased tumour burden, possibly by improving iron availability to the tumour for growth, and because BMP6 expression is associated with a better prognosis in myeloma (Seckinger *et al*, 2009). However there is no evidence to support these suggestions. The finding that it did not improve bone disease implies that removing tumour-derived BMP6 does not mitigate either upregulated BMP pathway activity observed in niche

cells or myeloma-induced sclerostin or Dkk1 expression, and therefore other mechanisms of BMP pathway dysregulation are implicated.

Alk3-Fc had very different effects to anti-BMP6 in myeloma-bearing mice. Its use led to a dramatic effect of increasing bone volume, increasing osteoblasts and reducing osteoclasts, and a significant fall in bone marrow plasma sclerostin. No mouse that received it exhibited signs of neurological compromise due to myeloma bone disease, although 5 out of 10 of the non-treated myeloma-bearing mice did so on the day of cull. It was unfortunate that dynamic bone formation could not be assessed due to cull requirement before double calcein injection had occurred, but in all microCT and histomorphometric parameters except for cortical lesion number, the bone mass increase observed was greater than that seen with LDN193189. It is not clear therefore why LDN193189 should cause a significant fall in the number of cortical lesions, but not Alk3-Fc. However, reports of differential effects on cortical and trabecular bone of *Bmpr1a* (Alk3) knockout mice may be relevant here. When the increased bone mass phenotype in adult mice with conditionally-deleted *Bmpr1a* in osteoblasts was first described, it was noted that the effect on trabecular and cortical bone was different (Kamiya, N. *et al*, 2008), with increased density occurring in the trabecular compartment, but increased porosity and decreased density in cortical bone. The same group later investigated these observations further, finding that the osteoblast-specific *Bmpr1a* knockout mice had increased collagen cross-linking in the trabecular compartment, but not the cortical. Collagen cross-linking promoted mineralisation, meaning that the mineral-to-matrix ratio was higher in trabeculae. The result of this was increased trabecular hardness and elastic modulus (elasticity). The femoral cortex however had a 9% lower cortical area and 7% lower thickness. In the femur, the cortical thickness is key to withstanding lateral force; on 4-point bending tests at the mid-diaphysis of femoral cortical compartments, osteoblast *Bmpr1a* knockout femorae had a lower ability to withstand

force (Zhang *et al*, 2016). Of note, vertebral bone strength and load resistance was not tested; in contrast to the femur, vertebral resistance to collapse relies more on trabecular strength than cortical (McDonnell *et al*, 2007). In myeloma-bearing Alk3-Fc-treated mice, there was a lack of neurological compromise suggestive of vertebral compression fracture, but no reduction in tibial cortical lesions; these findings may be compatible with the differential effect of Bmpr1a inhibition in the cortical and trabecular compartments. Higher trabecular collagen cross-linking has also been noted to occur with alfacalcidol (vitamin D₃ prodrug) dosing in osteopenic glucocorticoid-treated rats, so reversing the osteopenic phenotype (Saito *et al*, 2011). As discussed in chapter 4, the transcriptome of MSCs from LDN193189-treated showed significant enrichment of the nuclear receptors gene set, most strongly the vitamin D receptor (*Vdr*). Whether the mechanism by which type 1 BMP signalling modulates collagen cross-linking is dependent upon vitamin D-controlled transcriptional changes has not been investigated. Further work should include strength and elasticity test comparisons of femora and vertebrae from myeloma-bearing mice treated with Alk3-Fc or control, as well as investigation of the relationship between vitamin D signalling, BMP signalling, collagen cross-linking and sclerostin. As discussed in chapter 4, these four features have already been linked in the transition of osteoblast to osteocyte (St. John *et al*, 2014; Sutherland *et al*, 2004), but have not been considered as a mechanism of dysregulation in cancer-induced bone disease.

An important point to note when comparing findings in mice treated with Alk3-Fc to findings reported in conditional Bmpr1a knockouts in adult mice is that these genetic deletions occur in one cellular compartment at a time, either osteoblasts, osteocytes or osteoclasts. In each of these cell types, Bmpr1a deletion was shown to have a bone-mass enhancing effect mediated by different mechanisms; Rankl and Dkk1 suppression in osteoblasts (Kamiya *et al*, 2010; Kamiya, N. *et al*, 2008), sclerostin and Rankl in osteocytes (Kamiya *et al*, 2016), and as

yet unidentified signalling in already-mature osteoclasts (Okamoto *et al*, 2011). *In vivo*, Alk3-Fc use affects all cells concurrently, meaning the phenotype should not be expected to be identical to cell-specific gene deletions. Unlike osteoblast-specific *Bmpr1a* deletion, Alk3-Fc-treated myeloma-bearing mice did have increased cortical bone mass, even though lesions were not reduced. Increased cortical bone mass with Alk3-Fc was also described in an osteoporosis model, and importantly, femoral diaphysis bone strength was tested in that model, and was significantly increased above baseline (Baud'huin *et al*, 2012).

Why are there differences in findings between LDN193189 and Alk3-Fc? The magnitude of the bone mass improvement was greater with Alk3-Fc, which could be explained by better pharmacokinetics (section 5.1), but LDN193189 was also found to better reduce cortical lesions (Fig. 4.7 F), whereas Alk3-Fc did not (Fig. 5.9 F). Alk3-Fc but not LDN193189 significantly reduced sclerostin levels in the bone marrow plasma of myeloma-bearing mice; this requires acting on BMP ligands binding matrix-embedded osteocytes, which LDN193189 may not have sufficient half-life, or high enough Alk3 affinity, to achieve. LDN193189 however suppressed its expression *in vitro* in UMR cells, whereas Alk3-Fc did not (Fig. 5.14 B). Due to this lack of clarity, knockdown of *Alk2* and *Alk3* was performed in the UMR106.01 cell line, in mimicry of the inhibitory effects of these drugs. Again, knockdown of either receptor alone suppressed *Dkk1* expression, but not *Sost*; however, double knockdown markedly suppressed the production of both (Fig. 5.14 C-D). Various explanations may be proposed for all these *in vivo* and *in vitro* discrepancies. In the (not necessarily physiologically representative) UMR cell line, redundancy in contribution to signalling initiation by each receptor may be greater for control of sclerostin expression than *Dkk1*. The different mechanisms of the two drugs should also be remembered: Alk3-Fc has been shown to most efficiently eliminate ligands BMP2 and BMP4; the next most efficiently bound BMPs, 6 and 7, had a 50-fold lower affinity (Baud'huin *et al*, 2012). LDN193189

inhibits phosphorylation of Smad1 by the intracellular kinase domain of type 1 BMP receptors (IC_{50} 5nM for Alk2, 30nM for Alk3) (Yu *et al*, 2008), therefore is more or just as likely to inhibit the effects of BMPs 6 and 7, which preferentially bind Alk2. However it can therefore only inhibit canonical Smad1/5/8 signalling, and BMPs also have non-canonical, Smad-independent activities (Cook, 2014), which Alk3-Fc would inhibit. It is also possible, certainly *in vivo*, that alterations in experimental conditions or technique may explain differences, as LDN193189 and Alk3-Fc experiments were not performed concurrently, and tumour burden, which cannot be controlled in this model, was moderately different in each experiment. Repeat of the experiment with concurrent treatment groups for each drug should be performed to address this, and close comparisons of bone strength and microstructure could be made. Comparison of transcriptome and proteome from MSCs, osteoblasts and osteocytes from mice treated with each drug in such an experiment would demonstrate in which cell type, and to what extent, their effects are different. A further experimental group with the two treatments being used together would demonstrate if there are additive effects by inhibiting both mechanisms concurrently; however these experiments are not straightforward to perform with large groups of mice, particularly niche sorting.

The difference between the effect of Alk3-Fc and ActRIIa-Fc (sotatercept) is important to consider, as ActRIIa-Fc blockade has been shown to improve myeloma bone disease and anaemia (Abdulkadyrov *et al*, 2014; Chantry *et al*, 2010). Although these two receptors commonly heterodimerise to allow signalling *in vivo*, as ligand traps their affinities are very different, with Alk3-Fc removing primarily BMP2 and BMP4, and ActR11a-Fc mainly binding activin A and activin B with high affinity, but also GDF-8, GDF-11, BMP6 and BMP10 (Aykul & Martinez-Hackert, 2016). Therefore even though there are phenotypic similarities in their effects in myeloma, the mechanism is not the same. ActR11a-Fc was found not to inhibit

osteoclastogenesis *in vitro* (Chantry *et al*, 2010) or reduce bone resorption in patients (Abdulkadyrov *et al*, 2014). In our direct osteoclastogenesis assays, ActR11a-Fc promoted osteoclastogenesis. A possible explanation for this is the observation that activins compete for type 1 BMP receptor binding but cannot activate signalling, therefore acting as BMP signalling inhibitors. ActR11a-Fc has been shown to overcome this antagonism by removing the competing activins, so enhancing BMP signalling (Aykul & Martinez-Hackert, 2016).

With Alk3-Fc use in this model, hepcidin was not suppressed and haemoglobin unchanged. The reason for this is not clear, as it suppressed hepcidin expression *in vitro*, its use in an inflammatory anaemia model has demonstrated this effect *in vivo* (Steinbicker *et al*, 2011b), and knockout studies have shown it to be an important receptor of BMP6 in the liver (Steinbicker *et al*, 2011a). As there is no evidence that myeloma-bearing mice in this model have an elevated hepcidin, nor are they significantly anaemic, it is not the best model in which to assess the potential of Alk3-Fc in this regard; as it has been shown to work in inflammatory models it may well also work in a myeloma model with a strong inflammatory anaemia component and this effect cannot be ruled out on the limited data from this experiment. However, no murine myeloma model has yet been shown to have an inflammation-dependent anaemia, despite hepcidin elevation occurring in myeloma patients (Sharma *et al*, 2008).

Both Alk3-Fc and anti-BMP6 were well tolerated and did not affect disease burden or have obvious unexpected off-target or side effects. The widespread physiological roles of BMPs in tissue maintenance make this consideration vital. An exception to this claim is the significantly higher weight gain seen in Alk3-Fc-treated myeloma-bearing mice compared to untreated myeloma-bearing mice. In the context of treating a disease known for presenting

with weight loss, this is unlikely to be a negative effect, but the cause remains unknown. The reduced skeletal compromise with Alk3-Fc treatment, with potential accompanying pain reduction, may have enabled better access to food. Another possible explanation is that the increased bone volume in these mice, dramatic as it is, could be responsible. Whether a drug this potent could lead to bone volume being increased above a desirable level, if used over a prolonged period of many years, is worth asking, even if not likely to be relevant to the myeloma population – after all, the autosomal recessive disease sclerosteosis, caused by complete loss of sclerostin expression, leads to many unpleasant symptoms associated with bone overgrowth (Balemans *et al*, 2001). Muscle mass has been shown to be under BMP signalling control, with transfection of constitutively active Alk3 to muscle causing massive hypertrophy, and LDN193189 causing muscle wasting (Sartori *et al*, 2013). It does not seem likely therefore that the weight gain should be due to increased muscle bulk. A further possibility is dysregulation of adipogenesis; BMP4 increases adipocyte recruitment and BMP inhibition is associated with reduced adipocyte recruitment but hypertrophy of existing adipocytes, which has a role in obesity (Gustafson *et al*, 2015). Further work on the body composition in Alk3-Fc-treated mice is needed to understand why their weight gain is greater than control mice, and whether this would pose any potential risks in humans.

While there is not in this data the evidence of a ‘wonder-drug’ that will eradicate anaemia and bone disease in myeloma in one go, there are findings that appear to be, none the less, novel and not without clinical potential. Inhibiting the BMP pathway has the potential for clinical utility in inflammatory anaemia (Drakesmith & Prentice, 2012), but an effect of anti-BMP6 has not previously been published *in vivo*. There are many other diseases as well as cancers where a clear potential for such a compound exists, for example autoimmune diseases or renal failure. A drug such as anti-BMP6 has the potential to improve an anaemia caused by either inflammation-mediated hepcidin elevation or iron-restricted

erythropoiesis, or, as so often occurs in clinical practice, a combination of both together. Turning attention to Alk3-Fc, although osteoporosis remains the most obvious target of a drug such as this, it could offer potential in cancer-induced bone disease if it does indeed have both the catabolic and anabolic effects that this work suggests. The novel potential of BMP inhibition in this regard will be discussed in more detail in chapter 6.

Chapter 6 Summary and Future Perspectives

6.1 Summary of findings

Myeloma cells cause bone destruction in order to enhance tumour proliferation. The relationship between bone homeostasis and tumour survival is increasingly recognised to be important, maybe even vital to the problem of incurability. Thus, not only is bone damage and pain the symptom of myeloma reducing quality of life most profoundly, it may also be inextricably linked to why this disease is still usually fatal. By improving the currently inadequate treatments for bone disease, we may therefore not only improve quality of life, but also potentially alter myeloma prognosis.

This project sought to find new targets for disrupting the relationship between myeloma and its bone lining niche, with the aim of identifying novel means of altering outcomes in bone damage and disease behaviour. Although tumour-induced permanent alterations in the behaviour of stromal, osteoblast and endothelial cells from the bone marrow niche of myeloma patients have been shown (Arnulf *et al*, 2007; Corre *et al*, 2007; Dotterweich, J. *et al*, 2016; Garcia-Gomez *et al*, 2014; Ria *et al*, 2009; Todoerti *et al*, 2010), these investigations have only been performed in cells cultured *in vitro* over significant time periods, not directly from the *in vivo* niche, where contacts with the great variety of cellular and matrix components influence the nature of the dysregulation that occurs. Therefore such experiments cannot be relied upon to capture the range of complexity that produces the end result of bone destruction. The traditional method of retrieving stromal cells, namely to expand by culture cells that adhere to a plastic cell culture dish, allows for survival and overgrowth of a minority rather than representation of the whole, so heterogeneity is lost. For this reason I performed transcriptomic profiling of *in vivo* FACS-sorted components of the bone lining niche using a murine myeloma model. Markers of bone marrow niche cell types have been extensively validated in the mouse in the field of haematopoietic stem cell

research (Nakamura *et al*, 2010; Yu & Scadden, 2016), but not applied to investigations of the same niche in myeloma. Such isolation techniques or validation of cell-specific markers of components of the human bone marrow niche has not been performed, hence the need to use a mouse model.

Chapter 3 reports results of the transcriptomic profiling of bone-lining niche cells, where significantly different gene expression between cellular components of the bone lining niche of myeloma-bearing and healthy mice was assessed. Due to the experimental aims expressed above, I wished to focus on transcriptomic changes associated with specific association to disruption of bone homeostasis, so designed a priori gene sets to assess bone formation, bone loss and bone remodelling, focusing on mesenchymal stromal progenitor cells (MSCs) (CD45-CD31-Sca1+ALCAM-) rather than osteoblasts (CD45-CD31-Sca1-ALCAM+) because they had the greater number of differentially expressed genes. Although the 'bone remodelling' set (FDR 0.066) did not reach significance, the 'bone loss' and 'bone formation' gene sets were significantly enriched in MSCs from myeloma-bearing mice. This confirmed my hypothesis that transcriptomes in these cells would show changes due to myeloma-induced bone pathology, so I looked for evidence of dysregulated bone homeostasis pathways not previously described in myeloma bone disease pathogenesis.

Bmpr1b (*Alk6*) was one of 18 significantly differentially expressed genes identified by overlap between two different methods of assessing differential gene expression, and of these 18 the one with strongest evidence of a role in bone homeostasis. I therefore examined evidence of BMP pathway dysregulation in MSCs. A BMP pathway gene set was significantly enriched in MSCs from myeloma-bearing mice (FDR = 0.006). To assess if this enrichment was equivalent in magnitude to that of our bone homeostasis sets, the bone remodelling gene set was expanded to include the BMP pathway gene set members. In this

expanded gene set, now significantly enriched (FDR = 0.007), 13/41 of the leading edge genes driving enrichment were upregulated BMP gene set members, supporting a role for the BMP pathway in the disordered bone homeostasis of myeloma bone disease.

Chapter 4 presented work based on the hypothesis that, in view of the finding of BMP pathway enrichment in bone-lining MSCs in myeloma-bearing mice, inhibition of BMP signalling would improve myeloma bone disease.

Previously published findings relating to BMP signalling in myeloma are limited to observations that BMPs are toxic to myeloma cells in vitro (Hjertner *et al*, 2001; Holien *et al*, 2012; Kawamura *et al*, 2000), that BMPs may increase hepcidin levels and contribute to anaemia (Maes *et al*, 2010) and certain ligands and receptors may be increased in myeloma marrow samples, including BMP6 (Grcevic *et al*, 2010; Seckinger *et al*, 2009). It has been proposed that BMP ligands could have a therapeutic role in myeloma due to their myeloma-toxic and pro-osteogenic effects (Holien *et al*, 2012; Seher *et al*, 2017). In view of the above reports about roles of BMP signalling, I also hypothesized that BMP inhibition would improve anaemia, if these mice indeed had anaemia, by reducing hepcidin, but could increase tumour burden. However I found BMP toxicity to myeloma cells to be abrogated by stromal contact, and *in vivo* no difference in overall tumour burden with BMP inhibition by any of the three molecules tested.

BMP pathway inhibition using LDN193189 (LDN, an inhibitor of all BMP type 1 receptors, but with highest affinity for Alk2 and Alk3) (Cuny *et al*, 2008; Mohedas *et al*, 2013) increased trabecular and cortical bone volume, reduced cortical lesions, decreased osteoclastogenesis and increased osteoblast differentiation and bone formation in myeloma-bearing mice. *In vitro*, osteoclastogenesis from purified CD14+ PBMC populations was directly inhibited by

LDN. Aberrant osteoclastic activity in myeloma bone disease is associated with an increase in the RANKL:OPG ratio; we found this was not corrected in osteoblasts following LDN treatment *in vivo*. The direct inhibition of osteoclastogenesis *in vitro* by LDN suggested the effect is microenvironment-independent, consistent with a previous description of direct osteoclastogenic activity of BMPs (Jensen *et al*, 2010).

To better understand how LDN promoted new bone formation, repeat RNASeq experiments were performed to find pathways upregulated in MSCs of the bone lining layer on LDN treatment. BMPR1a (Alk3) inhibition has been previously noted to improve collagen structure and strength (Zhang *et al*, 2016), and in line with this, our RNASeq data showed upregulation of collagen genes in MSCs from LDN-treated mice. The additionally enriched 'Nuclear Receptor Transcription' gene set represents a group of steroid hormone receptors and their associated nuclear binding apparatus, including the vitamin D receptor and retinoid X receptors. The *Vdr* gene was highly enriched in MSCs from LDN-treated myeloma-bearing mice. *Vdr* is a marker of developing osteoblasts (Wang, Yongji *et al*, 2014), reported to have a role in ensuring osteogenic rather than adipogenic differentiation (Cianferotti & Demay, 2007), and osteoblast to osteocyte transition (St. John *et al*, 2014). Our GSEA data supports the hypothesis that BMP inhibition alters the transcriptome of MSCs in favour of osteoblastic differentiation and bone matrix formation.

Interestingly, on FACS sorting for RNASeq, we detected an increase in the small proportion of myeloma cells adherent to the BLC layer on treatment with LDN193189. Since a proposed purpose of bone lining niche disruption by myeloma cells is deregulation of the dormant tumour fraction to favour proliferation (Lawson *et al*, 2015a), it is intriguing to speculate that the increase in BLCs in response to BMP blockade may facilitate an increase in the dormant myeloma population, potentially desirable during MGUS or remission states.

Chapter 5 presented the use of two further BMP inhibitors better suited to use in humans with different mechanisms of action to LDN193189 in the myeloma mouse model. Firstly, a blocking antibody to BMP6 did not have anabolic effects on bone homeostasis, but did improve haemoglobin by the inhibition of hepcidin expression and increase in serum iron. There was no evidence that these mice had an elevated hepcidin associated with their mild anaemia, but the improvement of the anaemia with increased iron availability suggested that it was at least in part caused by competition for available iron. Serum transferrin appeared to be raised in these mice, and dropped upon anti-BMP6 treatment, again suggesting that iron supply and demand had been mismatched, but anti-BMP6 restored the balance.

Alk3-Fc (a ligand trap with the Fc domain of IgG1 attached to Alk3, leading to ligand removal primarily of BMP2 and BMP4) was the final BMP inhibitor tested. It caused a marked increase in trabecular and cortical bone mass in healthy and myeloma-bearing mice, demonstrating, as with LDN193189, reduced osteoclast numbers in vivo and increased bone lining cells and bone lining cell thickness, although dynamic bone formation could not be assessed. Direct inhibitory effects on osteoclastogenesis in vitro were also demonstrated, again as with LDN193189. In marked contrast, in the same experimental conditions, inhibition of the related activin pathway using ligand trap ActRIIa-Fc increased osteoclast number. This highlights that the mechanism of bone mass increase seen on BMP blockade is not biologically interchangeable with that seen on activin blockade.

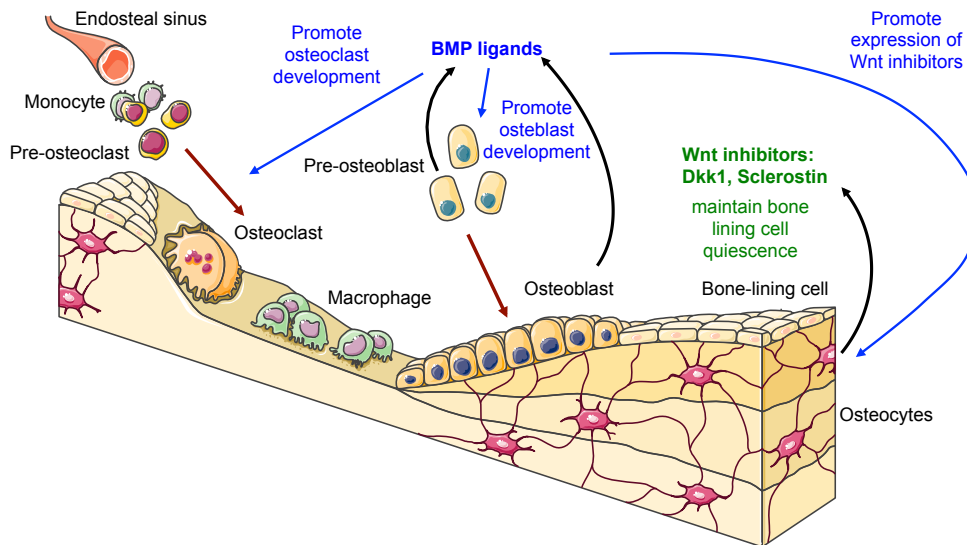
In vitro, BMP inhibition does not directly stimulate osteoblast precursors to promote bone matrix formation (Supplementary Figure 9), suggesting the observed bone anabolic effect is environmentally mediated. I therefore finally hypothesized that BMP inhibition might recapitulate the anabolic effects of anti-sclerostin antibodies in myeloma bone

disease (McDonald *et al*, 2017) since: a) osteocyte-secreted sclerostin and Dkk1 are major mediator of osteogenesis block in myeloma (Delgado-Calle *et al*, 2016a; Tian *et al*, 2003); b) anti-sclerostin antibodies ‘activate’ quiescent bone lining cells to become thicker, active and produce bone matrix *in vivo* (Kim *et al*, 2017) and c) both *Acvr1* and *Bmpr1a* knockout *in vivo* decreased *Sost* and *Dkk1* transcription (Kamiya *et al*, 2011; Kamiya, N. *et al*, 2008). The receptor deletion studies indicate that loss of type 1 BMP signalling reduces expression of these Wnt inhibitors; probably most pertinently *in vivo* not by osteoblasts, but by the far more numerous osteocytes (Delgado-Calle *et al*, 2016a). My *in vivo* and *in vitro* results pertaining to this hypothesis were mixed, as has been extensively discussed in chapter 5. However Alk3-Fc treatment resulted in a clear fall in bone marrow plasma sclerostin *in vivo*, and the ‘activated’, thicker appearance of the bone lining cells in the same Alk3-Fc treated mice was highly reminiscent of that described by Kim and colleagues as an effect of anti-sclerostin antibodies, as above.

In conclusion, the data presented here supports the hypothesis that BMP signalling is upstream of Wnt inhibitor signalling in mature bone cells, and therefore inhibiting BMP signalling reverses the osteogenic block that prevents bone formation in myeloma bone disease (Fig. 6.1).

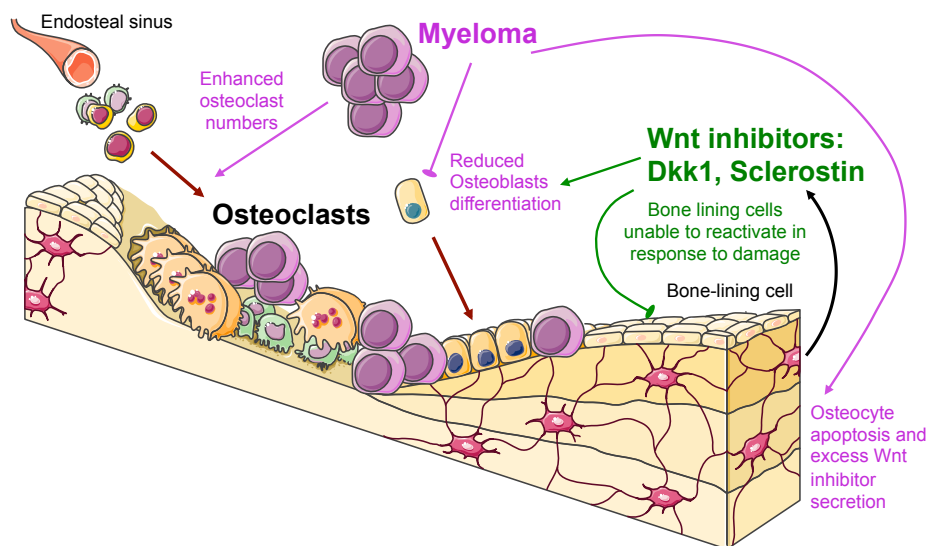
A. Healthy adult bone homeostasis:

Key role for BMPs in coupling osteoblast development, osteoclast recruitment and maintenance of Wnt inhibitor levels



B. Myeloma disrupts bone homeostasis:

Balance uncoupled: high osteoclast recruitment, high Wnt inhibitors and blocked osteoblast development



C. BMP inhibition in myeloma restores bone homeostasis:

Reduced osteoclast recruitment, reduced Wnt inhibitors and bone lining cell conversion to active osteoblasts.

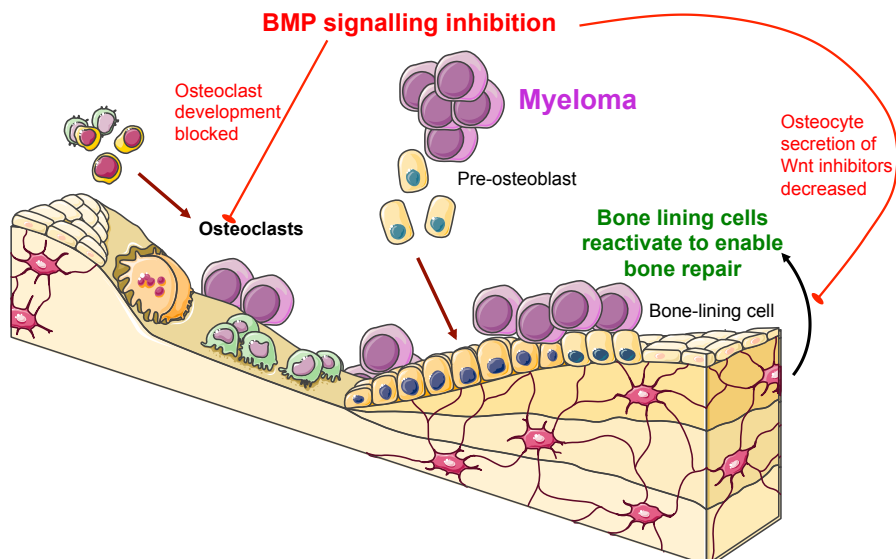


Figure 6.1: Mechanistic model of the involvement of BMP signalling inhibition in the restoration of bone mass in multiple myeloma

A: BMP signalling in adult bone homeostasis couples osteoblast and osteoclast development. By controlling expression of Wnt inhibitors, BMP signalling also prevents excess matrix deposition. Wnt inhibitors are negative regulators of osteoblast development and bone lining cell reactivation.

B: Myeloma overrides the balance between osteoclast and osteoblast leading to excess bone resorption without repair. High Wnt inhibitor levels, induced by myeloma-induced osteocyte toxicity and apoptosis, are critical to this uncoupling by preventing osteoblast development and bone lining cell activation.

C: Inhibition of BMP signalling reverses the excess Wnt inhibitor levels and osteoclast recruitment, thereby allowing bone lining cells to reactivate to matrix-secreting osteoblasts to restore damage.

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6.2 Future Perspectives

Increased bone mass has been previously reported in inducible Alk3-knockout mice, and with type 1 BMP receptor blockade using Alk3-Fc in an osteoporosis model (Baud'huin *et al*, 2012; Kamiya *et al*, 2011; Kamiya, N. *et al*, 2008). Our findings expand these observations to the severe and complex bone disease found in myeloma. Here, I will firstly discuss further work that would take these findings forward and address further hypotheses generated; secondly I will discuss considerations regarding the potential use of these compounds clinically.

6.2.1 Further work

How could findings of BMP inhibition suppressing Wnt inhibitors be integrated with transcriptome analyses results indicating enhanced differentiation of LDN193189-treated bone lining MSCs *in vivo*? These MSCs, at a stage of pre- or early commitment to osteoblastic lineage, are not the cells that express the physiologically important Wnt inhibitors – neither *Dkk1* nor *Sost* transcripts were identified in these cells on our RNASeq

investigations. This leads to an assumption, which requires testing, that BMP inhibitors are not affecting BMP signalling in the MSCs directly, but mediating their differentiation by promoting shifts in the microenvironmental balance of Wnt ligands and inhibitors (Baron & Kneissel, 2013). Entire microenvironments are not easy to recreate *in vitro*, particularly matrix-embedded osteocytes. An experiment addressing this would be to FACS sort (using markers as described here) into culture medium, MSCs from three concurrent experimental groups: healthy mice, myeloma-bearing mice, and myeloma-bearing Alk3-Fc treated mice. After initial proliferation, these cells would be allowed to undergo osteogenic differentiation in osteogenic media (described in chapter 2), after which mineralization would be quantified. Myeloma-exposed MSCs may or may not differentiate less well than controls (reports differ in this regard, myeloma cells could be added to the culture to enhance effect (Arnulf *et al*, 2007; Corre *et al*, 2007), but *in vivo* Alk3-Fc treatment during the myeloma exposure would promote differentiation. To show that this is *in vivo* environment-mediated rather than direct, negative control wells would have Alk3-Fc added directly to the osteogenic media, which prevents differentiation (Supplementary Figure 9). Positive control wells would contain BMP2, which promotes differentiation. A final condition would be the addition of sclerostin to MSCs from the Alk3-Fc-treated mice, to demonstrate reversal of the Alk3-Fc 'rescue' of differentiation block. A simpler version of this experiment might be the treatment of differentiating MSCs with bone marrow plasma from myeloma-bearing mice with and without Alk3-Fc treatment. However the presence of the drug in the plasma may well inhibit the differentiation more strongly than the drop in sclerostin would enhance it, so I would hypothesize that this technique would not work.

An analysis of the *in vivo* transcriptional changes that occur in adult murine osteoblast precursors on reduction of either Dkk1 or sclerostin levels has not to my knowledge been performed. Following on from the experiment described above, comparison of the results of

MSC transcriptomic analysis on Alk3-Fc and anti-sclerostin treatment in this *in vivo* model would highlight whether osteoblast differentiation occurs with common or different transcriptome perturbations with these two treatments. Due to technological advances that have occurred since these experiments were devised, a better way to do this experiment would be high throughput single cell transcriptomics, for example using a dropseq platform (Prakadan *et al*, 2017), which would enable the great heterogeneity of “MSCs” to be examined, and whether a particular subset, of differentiation stage, were responding to treatment with these drugs. This would not just answer questions about mechanisms of these drugs, but have greater use in the field of myeloma bone disease, as the precise stage at which differentiation block occurs is not defined, and such an experiment would be expected to shed light on this.

We were also interested in the overlap of effect of BMP pathway inhibition on bone disease and anaemia: firstly, both are important complications in patients with myeloma; and secondly other ligand traps of the TGF β superfamily reverse anaemia (Platzbecker *et al*, 2017), or in the case of ActRIIa-FC, improve anaemia and myeloma bone disease (Abdulkadyrov *et al*, 2014). However, the haemoglobin-increasing mechanisms of these drugs are not reported to be hepcidin-mediated, whereas antagonism of Type 1 BMP receptor signalling reduces hepcidin (Steinbicker *et al*, 2011b). Anaemia in the 5TGM1 model of myeloma is variable and unpredictable, likely reflecting the speed of disease development. Although LDN and Alk3-Fc reduced hepcidin expression *in vitro*, and have reduced hepcidin in inflammatory anaemia models (Steinbicker *et al*, 2011b), neither approach increased haemoglobin in myeloma-bearing mice. In contrast, anti-BMP6, which is highly effective at suppressing hepcidin, increased iron availability and improved haemoglobin in myeloma-bearing mice. Further work to clarify whether iron availability limits erythropoiesis in this model, and whether iron replacement would enhance tumour

growth, would be to have concurrent experimental groups treated with anti-BMP6, isotype control and iron dextran, to see if iron supplementation had the same effect as anti-BMP6. Iron storage, erythropoietic response (CD71+ erythroblast quantification) and a better assessment of inflammatory status (acute phase proteins CRP, IL6) should also be performed. This work would address an area that is poorly explored, namely, is treatment of anaemia in the cancer setting with iron beneficial, or potentially dangerous due to favouring tumour proliferation.

Finally, further work on the effect of tumour niche dynamics beyond osteoblast development is indicated by preliminary data in these experiments. We found that LDN193189 increased the proportion of tumour cells adherent to the bone lining. It has previously been shown in this same myeloma model that dormant tumour cells, which segregate to the bone lining, have a transcriptional signature consistent with dormancy (Lawson *et al*, 2015a) when compared to bulk tumour. Although I wished to compare bone-adherent myeloma cell transcriptomes with those of the bulk myeloma, the difference in preparation techniques (collagenase digestion vs. flushing) confounded this data. Preparing both fractions with collagenase digestion should be performed. In addition, experiments testing duration of premalignant MGUS phases of disease (probably using a model with a more indolent disease progression), or relapse from a state of minimal residual disease post chemotherapy in the presence of BMP inhibition should be performed, to examine whether a more intact and active bone lining layer will prolong the remission or MGUS state. The 'reverse' experiment to this, investigating a less desirable outcome, would be to test the hypothesis that a more robust bone lining would increase the drug resistant fraction (Chen *et al*, 2014; Croucher *et al*, 2016). By comparing the effect on disease progression dynamics of drugs that 'enhance' the bone lining (Alk3-Fc, anti-sclerostin) with drugs that promote its degradation (RANKL, BMP2 or 4, sclerostin) at three different stages:

pre-malignant MGUS, during active treatment and post treatment remission – a picture could be formed of the role of bone lining integrity in the control and treatment in myeloma.

6.2.2 Clinical perspectives

Successful myeloma bone disease therapeutics may achieve higher bone mass either by reducing bone resorption, akin to current clinical therapies that target osteoclasts alone (bisphosphonates and denosumab), or by increasing bone anabolism through modification of myeloma-induced osteoblast differentiation block. Although there is hope that anti-sclerostin antibodies may offer this type of bone repair potential (McDonald *et al*, 2017; Saag *et al*), as they increase bone formation and also indirectly inhibit osteoclasts via downregulation of RANKL secretion, there is no such therapy currently available for clinical use, nor is it clear that such a mechanism would have a prolonged duration.

The ideal drug for cancer induced bone disease would have long-lasting anti-resorptive and anabolic effects that increased bone strength, repaired lytic areas and prevented fractures, without leading to excess or abnormal bone formation, or unexpectedly weakening bones as has been suggested with long term bisphosphonate use (Ma *et al*, 2017). It would also have to either impede, or at least not promote, cancer growth in the bone marrow microenvironment, and not induce drug resistance. We are still a considerable distance from achieving such a 'wonder-drug', if indeed achieving such a balance is possible. It is interesting to note that combinations of anti-resorptive and anabolic agents have been successfully trialled in osteoporosis (Tsai *et al*), although teriparatide, the PTH analogue licensed as a bone anabolic agent and used in such trials, is contraindicated in cancer-associated bone disease due to a potential pro-tumourigenic and pro-osteoclastic effect.

This has never been demonstrated in myeloma, which does not express the PTH receptor, but nonetheless it has not been trialled (Silbermann & Roodman, 2013).

Could BMP antagonism represent progress towards better myeloma bone disease therapy?

Two important questions remain unanswered here; firstly, can a sustained effect be achieved with a single pathway inhibiting mechanism? Over time, homeostatic mechanisms can overcome the effects of blocking a single pathway, and the trial of Alk3-Fc in an osteoporosis model appeared to suggest that the anabolic and anti-resorptive effects were not concurrent (Baud'huin *et al*, 2012), but an initial increase in osteoblasts was followed later by a decrease in osteoclasts. Secondly, and more importantly, what is the potential for unintended effects of inhibiting BMP signalling that may be less desirable? As one of the key morphogenetic pathways governing embryo patterning and cellular differentiation, BMP signalling has fundamental roles in many tissues (Wang, Richard N. *et al*, 2014).

Of concern regarding the use of type 1 BMP receptor inhibitors in clinical practice is the role of Alk3 / Bmpr1a signalling in the development of colon cancer, with mutations in this pathway linked to the development of juvenile polyposis (Howe *et al*, 2001) and BMP mutations present in the majority of sporadic colon cancer cases (Kodach *et al*). Ectopic expression of BMP antagonist gremlin has been shown to initiate colon cancer development (Davis *et al*, 2015), and the transgenic expression of Bmpr1a-Fc in mice to lead to the development of stomach adenomas (Yamawaki *et al*, 2016). However the story is not entirely clear cut; whereas orally administered LDN193189 has been shown to increase *de novo* intestinal adenoma formation in conditional *Apc* knockout mice (Whissell *et al*, 2014), in another model the *in vivo* growth of implanted colon cancer cells reduced in size with LDN193189 treatment (Yokoyama *et al*, 2017). As with bone development, the

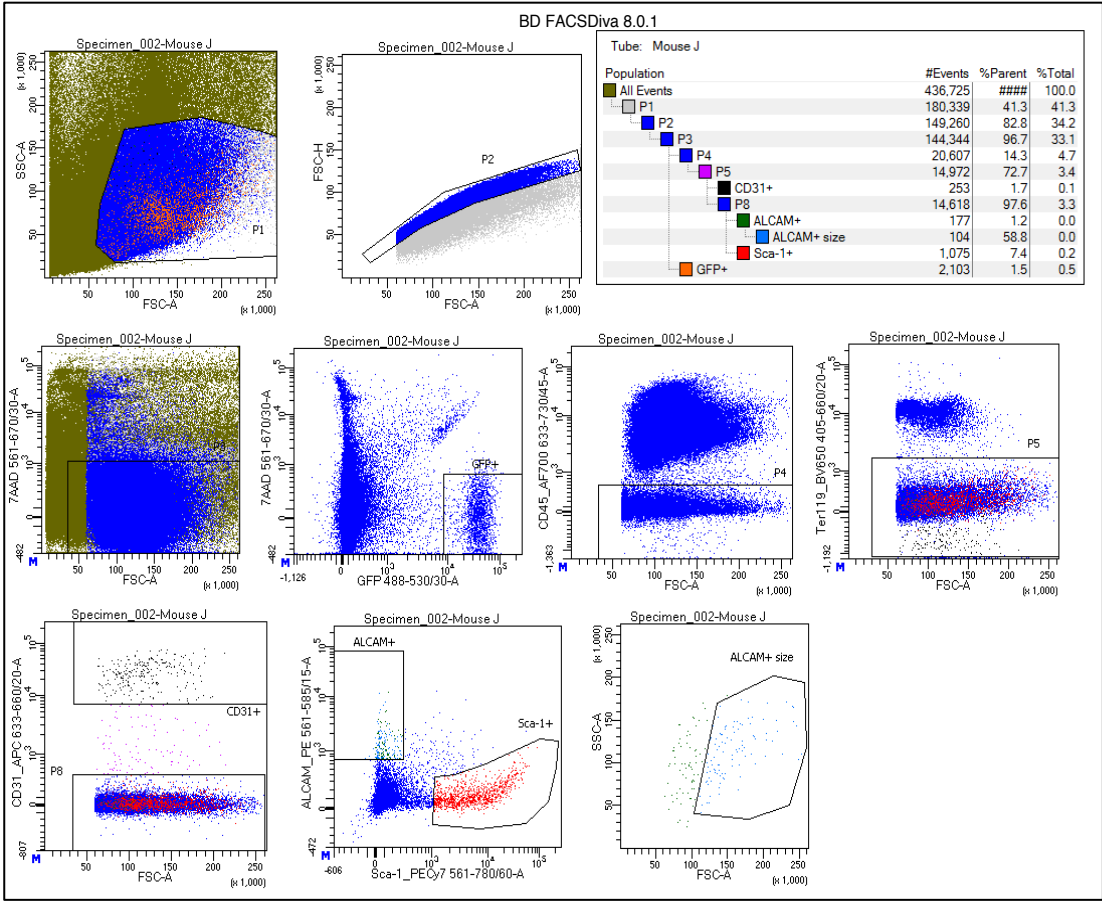
effects of BMP signalling inhibition cannot be assumed to be consistent across the different stages and compartments of colonic tumours.

This body of evidence places a potential burden of caution on using inhibitors of BMP signalling for prolonged periods in humans, although prolonged treatment would not necessarily be required in the treatment of cancer-induced bone disease. If inhibiting the BMP pathway can be used to demonstrate in model systems the benefits in cancer-induced bone disease of direct combined osteoclast inhibition and bone anabolism, or as a tool to investigate the effect on tumour behaviour of maintaining the bone lining layer intact, then further more targeted therapies achieving the same purpose can be developed.

6.3 Summary statement

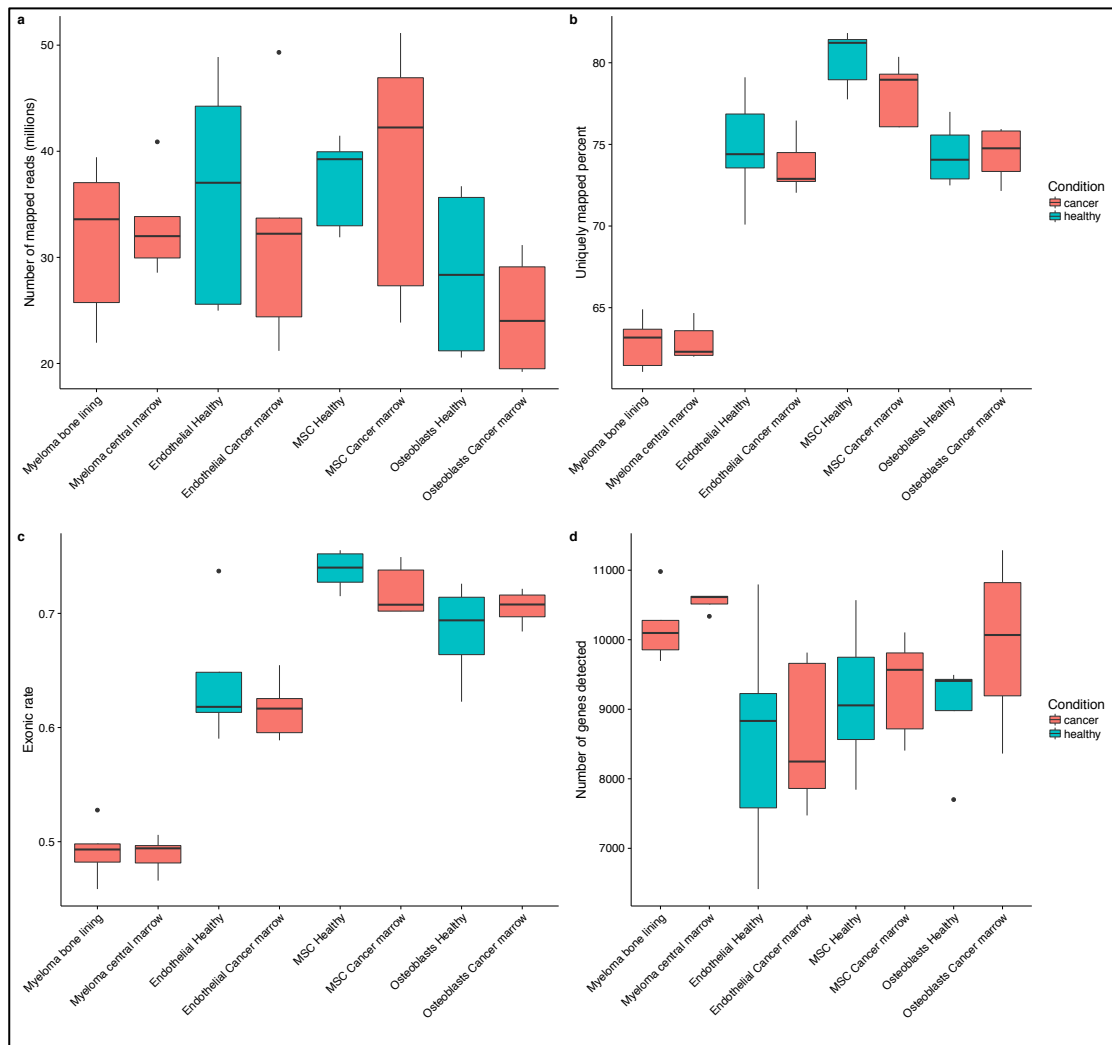
In summary, in bone lining MSCs, we found BMP pathway members, particularly *Bmpr1b*, to be enriched in myeloma-bearing mice. BMP inhibition led to increased bone mass, through a direct anti-osteoclast effect and a reduction in Wnt inhibitor production, particularly sclerostin by osteocytes, rather than through a direct effect on MSCs or pre-osteoblasts. The bone mass-enhancing effect of the BMP blockade described here occurs in control and diseased mice, so is a generic effect on both diseased and healthy bones. However, by causing a reduction in pathologically high sclerostin levels and so promoting osteoblast differentiation, whilst also inhibiting osteoclast development, pharmacological BMP inhibition has the potential to overcome the uncoupling of bone homeostasis that drives myeloma bone disease, and also to improve disease-related anaemia.

Supplementary Data



Supplementary Figure 1 A: Real-time FACS gating strategy (BD FACSDiva V 8.0.1 Application) used to sort cell populations from bone-lining niche of healthy and 5TGM1-GFP myeloma-bearing KaLwRij mice.

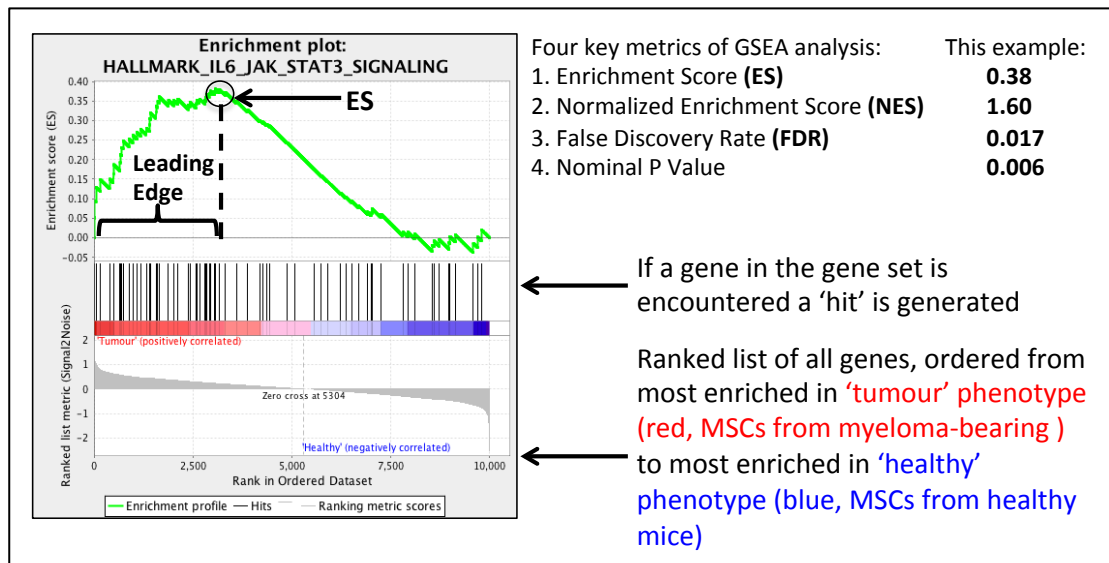
[Referenced from section 3.2.1 Fig. 3.1].



Supplementary Figure 2: Quality assessment of RNASeq mapping using RNA-SeQC (DeLuca *et al*, 2012) in healthy vs. myeloma-bearing mice.

a) Number and b) percentage of reads mapping uniquely to the genome, c) proportion of reads that map to annotated exonic regions and d) number of genes that are expressed in each group of samples.

[Referenced from section 3.2.3].



Supplementary Figure 3: Guide to interpreting GSEA results, using MSCs from 5TGM1-GFP myeloma bearing and healthy KaLwRij mice, and using the MSigDB Hallmark IL6-JAK-STAT3 gene set as an example. After

http://software.broadinstitute.org/gsea/doc/GSEAUUserGuideFrame.html?_Interpreting_GSEA_Results

The **Enrichment Score (ES)** is generated as the GSEA software 'walks' down the list of pre-ranked genes, adding to the score of the gene set every time a member of that set is encountered, and subtracting from it when non-set members are encountered. Therefore the more gene 'hits' closer to the top of the ranked list, the higher the ES, visualised as the maximum deviation from zero.

The **Normalised Enrichment Score (NES)** is more robust: by normalising the ES for gene set size, the scores can be compared between different gene sets.

The **False Discovery Rate (FDR)** is the estimated probability that the NES of a gene set is false. It takes the actual ES and compares it to the ES of all gene sets, against all permutations of the dataset. For a small dataset such as this one with 5 samples in each phenotype, the NES is judged to be significant if the FDR is < 5% / 0.05.

The **Nominal P Value** estimates the significance of the enrichment score for a single gene set, i.e. no correction for multiple hypothesis testing. It may be relevant for a gene set specifically designed to test a single hypothesis.

The **Leading Edge subset** is the gene set members contributing most to the ES: i.e. the members of the set that appear prior to the peak score, and have the highest fold change difference between the 2 phenotypes.

[Referenced from section 3.2.4].

Bone Formation	Bone Loss	Bone Remodelling	BMP signalling pathway	Bone Remodelling BMP supplemented
Abl1	2410016006Rik	Ache	Acvr1	Ache
Actn3	Ache	Acp5	Acvr1b	Acp5
Acvr1	Acp5	Acvr1	Acvr2a	Acvr1
Acvr2a	Acvr1	Adam8	Acvr2b	Acvr1b
Acvr2b	Adam8	Adrb2	Acvr1	Acvr2a
Adrb2	Adrb2	Ahsg	Bmp1	Acvr2b
Alpl	Ager	Ankh	Bmp15	Acvr1
Ano6	Ahsg	Atf4	Bmp2	Adam8
Asxl2	Ankh	Atp6ap1	Bmp2k	Adrb2
Atf4	Areg	Atp6v0a4	Bmp4	Ahsg
Atp6ap1	Asxl2	Atp6v1b1	Bmp5	Ankh
Atraid	Atp6ap1	Bmp4	Bmp6	Atf4
Bmp2	Atraid	Calca	Bmp7	Atp6ap1
Bmp4	Axin2	Calcr	Bmp8a	Atp6v0a4
Bmp6	Bcl2	Car2	Bmper	Atp6v1b1
Bmp7	Bcor	Cartpt	Bmpr1a	Bmp1
Bmpr1a	Calca	Casr	Bmpr1b	bmp10
Bmpr1b	Calcr	Cd38	Bmpr2	Bmp15
Bmpr2	Car2	Cdk6	Cer1	Bmp2
Calca	Ccl3	Ceacam1	Cerl	Bmp2k
Cartpt	Ccl5	Cldn18	Cerr1	Bmp3
Casr	Ccr1	Col13a1	Chrdl1	Bmp4
Ccl3	Cdk6	Csf1r	Chrdl2	Bmp5
Cd276	Chrd	Csk	Dand5	Bmp6
Cd38	Cited1	Cthrc1	Eng	Bmp7
Ceacam1	Creb1	Cthrc1	Grem1	Bmp8a
Cebpa	Csf1	Ctnnb1	Id1	Bmper
Cebpb	Csf1r	Ctsk	Id2	Bmpr1a
Cebpd	Ctsk	Ctss	Id3	Bmpr1b
Cldn18	Ctss	Cyp19a1	Nog	Bmpr2
Clec2d	Dcstamp	Dcstamp	Runx2	Calca
Clec2g	Dkk1	Dkk1	Smad1	Calcr
Clec2i	Dkk2	Dkk2	Smad2	Car2
Clec5a	Ecm1	Dlx3	Smad3	Cartpt
Clic1	Efna2	Dlx5	Smad4	Casr
Col10a1	Egfr	Dmp1	Smad5	Cd38
Col13a1	Eif2ak2	Dmp3	Smad6	Cdk6
Col1a1	Enpp1	DsppE	Smad7	Ceacam1
Col2a1	Epha2	Efna2	Smad9	Cer1
Csgalnact1	Fam101b	Egfr	Smurf1	Cerl
Csk	Fgf23	Eif2ak2	Smurf2	Cerr1
Cthrc1	Fgfr1	Enpp1	Tnfrsf11b	Chrdl1
Ctnnb1	Flt3l	Epha2	Tnfsf11	Chrdl2
Ctnnbip1	Flt3lg	Fgfr3	Twsg1	Cldn18
Cyp19a1	Fndc3b	Fos		Col13a1
Cyr61	Fos	Fosl1		Csf1r
Ddr2	Fshb	Fosl2		Csk
Dlx3	Gata1	Fshb		Cthrc1
Dlx5	Gdf10	Fshr		Ctnnb1
Dmp1	Gnas	Gja1		Ctsk
Dspp	Gpr68	Gli1		Ctss
Fam20c	Grem1	Gli2		Cyp19a1
Fbn1	Gsk3b	Gpr55		Dand5
Fbn2	Hand2	Grem1		Dcstamp
Fgf18	Hdac4	Gsk3b		Dkk1
Fgf2	Hdac5	Hamp		Dkk2
Fgfr3	Hdac7	Hivep3		Dlx3
Fosl1	Hif1a	Hnf1a		Dlx5
Fosl2	Hivep3	Htr1b		Dmp1
Fshr	Hnf1a	Iapp		Dmp3
Fstl3	Hoxa2	Ifnar1		DsppE
Fzd9	Id1	Ifnar2		Efna2
Gata1	Id2	Ifnb1		Egfr
Gdf10	Id3	Ihh		Eif2ak2
Gdf2	Ifng	Il20ra		Eng
Gdpd2	Igfbp5	Il7		Enpp1
Gja1	Il12b	Inpp4b		Epha2
Gli1	Il17a	Inpp5d		Fgfr3
Gli2	Il20	Irf9		Fos
Gli3	Il20ra	Itgav		Fosl1
Gnas	Il23a	Itgb3		Fosl2
Gpm6b	Itgb3	Jun		Fshb
Gpr55	Klf10	Jund		Fshr
Grem1	Limd1	Kl		Gja1
Hamp	Lrp5	Lef1		Gli1
Hdac4	Lrp6	Lep		Gli2
Hdac5	Lrrk1	Lepr		Gpr55
Hey1	Ltbp3	Lgr4		Grem1
Hoxa2	Mc4r	Lrp5		Gsk3b
Hpse	Men1	Lrp6		Hamp
Iapp	Mepe	Lrrk1		Hivep3
Id4	Mgp	Ltbp3		Hnf1a
Ifi204	Mir133a-1	Mapk8		Htr1b
Ifnar1	Mir133a-2	Mc4r		Iapp
Ifnar2	Mir135a-1	Mgp		Id1
Ifnb1	Mir137	Mitf		Id2
Igf1	Mir204	Msx1		Id3
Igf1r	Mir205	Msx2		Ifnar1
Ihh	Mir214	Nbl1		Ifnar2
Il4	Mir217	Ncdn		Ifnb1
Il6	Mir23a	Nf1		Ihh
Il6st	Mir30c-1	Nfatc1		Il20ra
Ilk	Mir30c-2	Nfkb1		Il7
Impad1	Mir338	Notch2		Inpp4b
Inpp4b	Mitf	Nox4		Inpp5d
Inpp5d	Nbl1	Ostf1		Irf9
Inpp1	Nbr1	P2rx7		Itgav
Irf9	Ncdn	Pdk4		Itgb3
Isg15	Nell1	Ppargc1b		Jun
Itgav	Nfatc1	Prg		Jund
Itgb3	Nfe2	Prg1		Kl
Jag1	Nfkb1	Ptger4		Lef1

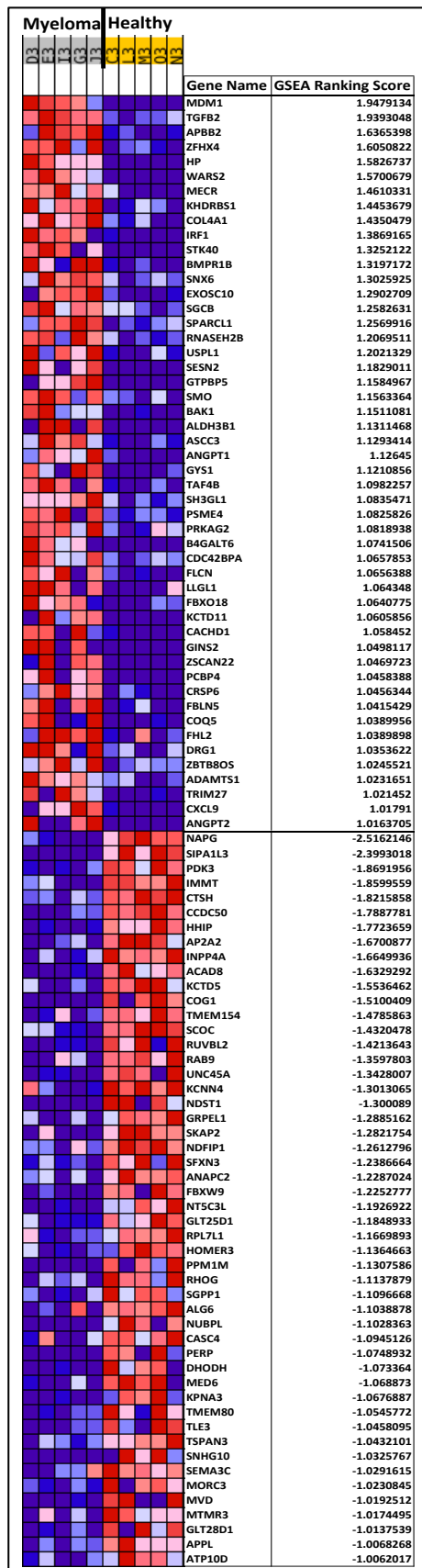
Bone Formation	Bone Loss	Bone Remodelling	BMP signalling pathway	Bone Remodelling BMP supplemented
Jun	Noct	Pth		Lep
Jund	Nog	Pth1r		Lepr
Kl	Notch1	Ptk2b		Lgr4
Lef1	Notch2	Rab3d		Lrp5
Lgr4	Nox4	Rab7		Lrp6
Lilrb3	Ocstamp	Rac1		Lrrk1
Lilrb4	Ostf1	Rac2		Ltbp3
Limd1	Ostn	Rassf2		Mapk8
Lmna	Pdk4	Rela		Mc4r
LOC100909750	Plxnb1	Rps6ka3		Mgp
Lrp5	Ppargc1b	Runx2		Mitf
Lrp6	Ptch1	Runx3		Msx1
Lrrc17	Ptger4	S1pr1		Msx2
Ltf	Pth	Sfrp1		Nbl1
Mafb	Ptk2b	Siglec15		Ncdn
Mapk8	Rela	Slc34a1		Nf1
Mef2c	Rflna	Snai2		Nfatc1
Mef2d	Rflnb	Sost		Nfkb1
Men1	Rorb	Sp7		Notch2
Mia3	Sema4d	Sparc		Nox4
Mir210	Sfrp1	Spp1		Ostf1
Mir3960	Ski	Spp2		P2rx7
Mmp13	Slc9b2	Src		Pdk4
Mmp14	Smad3	Srgn		Ppargc1b
Mmp16	Sost	Stath		Prg
Msx1	Sox2	Suco		Prg1
Msx2	Sox9	Syk		Ptger4
Nab1	Spp1	Syt7		Pth
Nab2	Src	Tcf7l2		Pth1r
Nell1	Srgn	Tfrc		Ptk2b
Nf1	Syk	Tgfb1		Rab3d
Notch1	Tf	Tgfb3		Rab7
Npnt	Tfrc	Tmem64		Rac1
Nppc	Tgfb1	Tnfrsf11a		Rac2
Osr1	Tgfb3	Tnfrsf11b		Rassf2
Osr2	Tmem64	Tnfsf11		Rela
P2rx7	Tnf	Tph1		Rps6ka3
Pdlim7	Tnfsf11	Tpp1		Runx2
Pex7	Tob1	Traf6		Runx3
Phospho1	Tph1	Trf		S1pr1
Pik3r1	Tpp1	Twist1		Sfrp1
Pilrb1	Traf6	Twist2		Siglec15
Pkccc	Trf	Txlng		Slc34a1
Pla2g4a	Trpm4	Ubash3b		Smad1
Prkd1	Twist1	Vegfa		Smad2
Ptger4	Twist2	Wif1		Smad3
Pth	Twsg1	Wnt16		Smad4
Pth1r	Txlng	Wwp1		Smad5
Pthlh	Ucma	Xdh		Smad6
Rab3d	Wif1			Smad7
Rac1	Wwp1			Smurf1
Rac2	Zfp932			Smurf2
Rassf2				Snai2
Runx2				Sost
Runx3				Sp7
S1pr1				Sparc
S1pr2				Spp1
Satb2				Spp2
Scube2				Src
Sxcb				Srgn
Sfrp1				Stath
Sfrp2				Suco
Shh				Syk
Slc34a1				Syt7
Slc8a1				Tcf7l2
Smad1				Tfrc
Smad3				Tgfb1
Smad5				Tgfb3
Snai1				Tmem64
Snai2				Tnfrsf11a
Sox11				Tnfrsf11b
Sox8				Tnfsf11
Sp7				Tph1
Sparc				Tpp1
Suco				Traf6
Syt7				Trf
Tcf7l2				Twist1
Tek				Twist2
Tfap2a				Twsg1
Tgfb1				Txlng
Tgfb3				Ubash3b
Tjp2				Vegfa
Tmem119				Wif1
Tmem178				Wnt16
Tmem178a				Wwp1
Tnfaip3				Xdh
Tnfrsf11b				
Tob2				
Trp63				
Ubash3b				
Vegfa				
Wnt10b				
Wnt16				
Wnt4				
Wnt7b				
Zhx3				

Supplementary Table 1: Bone homeostasis gene sets. [Referenced from section 3.2.5].

	Geneid	logFC	AveExpr	adj.P.Val
1	Mecr	9.30969581	2.71232738	0.00290236
2	Plac8	9.24031048	0.17831415	0.00637087
3	Mdm1	9.215764	-0.8125408	0.0019761
4	Ms4a4d	8.80526068	-0.7362837	0.03161473
5	Wars2	8.65561329	-0.8810462	2.37E-06
6	Aldh3b1	8.28974357	0.1722427	0.01869896
7	Hp	8.26796796	1.66845504	0.05184994
8	Angpt1	8.16676239	-1.6060239	0.00270691
9	Trappc11	7.82196345	2.25948609	0.02339064
10	Abcc9	7.74122748	2.26513757	0.05509534
11	Rbm15b	7.64401303	-0.6564667	0.02339064
12	Ligl1	7.61388083	2.29178103	0.08942851
13	Kctd11	7.50108494	-1.5039675	0.06724004
14	Slc25a27	7.50050066	-2.6617028	0.06823136
15	Ap5m1	7.45462903	0.39984701	0.02707995
16	Cacna1c	7.02875846	-3.1758729	0.06695199
17	Ttc13	6.85425612	2.94126974	0.02587257
18	2310047M10	6.79207632	-0.220057	0.08707915
19	Foxred1	6.71989971	1.64015168	0.09451089
20	D1Ert622e	6.64748623	2.27781752	0.0959781
21	Stk40	6.64744013	0.55870571	0.02339064
22	Zfp235	6.61833944	-1.54768	0.06548293
23	S1pr3	6.58687319	-0.4657326	0.08883865
24	Per1	6.46739498	2.24952425	0.06724004
25	Cdk16	6.45932551	3.19438701	0.00847268
26	Get4	6.44667292	1.80220299	0.0433465
27	Smardc1	6.43465746	3.73579022	0.08538388
28	Ppl	6.35406191	-2.6568429	0.06559785
29	Pcbp4	6.34336545	-1.3646383	0.02339064
30	Tlcl1	6.33803506	1.74281627	0.08883865
31	Cdk12	6.21218379	2.1788233	0.06522205
32	Exosc10	6.2116958	2.66128905	0.0695972
33	Cyp4f16	6.19719095	2.53757148	0.06941292
34	Cd320	6.19567268	2.0953002	0.09692822
35	Larp6	6.18526551	-1.7836013	0.02613167
36	Car15	6.0535738	-1.8178548	0.02480463
37	5930412G12	5.95929847	-4.0230937	0.0153444
38	Ccl9	5.90730291	-4.1941488	0.03891035
39	Bmpr1b	5.90716772	-2.7104428	0.05548694
40	Helz2	5.82219533	2.10226587	0.07959748
41	Mfsd9	5.79318272	-3.825041	0.05548694
42	Ptpn13	5.68390962	-1.8689648	0.06724004
43	Ccl19	5.68215779	-3.8483977	0.05323557
44	Tnfrsf11	5.62814344	-2.8261096	0.09451089
45	Shkbp1	5.47014314	-1.0839812	0.06067361
46	Cpne1	4.83585792	5.05867132	0.03239344
47	Igf2bp3	4.82275281	-0.0465079	0.02339064
48	Dnajc1	4.13572098	4.58189267	0.08883865
49	1500015O10	1.95405933	7.82495221	2.37E-06
50	Mgp	-1.3308348	10.6829471	0.00637087
51	Cilp	-1.3981095	7.37613455	1.18E-10
52	Hhip	-3.2521038	0.53817063	0.03161473
53	Ttll3	-5.1294305	-2.3198454	0.09692822
54	Gm14322	-5.359896	-1.977972	0.08682905
55	Epb4.1l4b	-5.3766731	0.32580825	0.08883865
56	Zc3h15	-5.3926657	5.27837267	0.03345424
57	Git1	-5.4270876	3.78549764	0.05669379
58	Tcf7	-5.7678884	-1.8958261	0.06522205
59	Pla2g5	-5.8730088	0.51129302	0.02569499
60	Agbl2	-6.0777104	-2.7582184	0.06559785
61	Agxt2l2	-6.0955682	0.71589745	0.08278638
62	Cel	-6.1748763	-3.9335822	0.05548694
63	Gareml	-6.1871899	-3.1872654	0.06941292
64	Slc30a1	-6.4250395	3.14262792	0.06479343
65	Ajap1	-6.5827028	-3.6718417	0.01302406
66	Sipa1l3	-6.6344324	-0.8200233	0.02339064
67	Ctif	-6.7271788	-2.6235405	0.06824647
68	Gstt2	-6.7995007	2.62212779	0.06724004
69	Snhg10	-6.8400579	-1.3108079	0.00637087
70	Zfp385c	-6.8681126	-3.3688283	0.01869896
71	Serpinb7	-6.8916049	-2.1746786	0.06238048
72	Rcan3	-6.908746	1.40406719	0.09451089
73	Acad8	-6.9426726	2.03753149	0.05155652
74	Zc3hav1l	-6.9546932	0.59541435	0.03891035
75	Cog1	-7.0909688	-0.6112152	0.07639145
76	Dapp1	-7.125938	1.25131151	0.08874741
77	Nelfcd	-7.2752166	1.22927833	0.03161473
78	Tmem246	-7.644228	-2.2529665	0.06559785
79	4632415L05f	-7.8076006	-0.2487441	0.00637087
80	C2cd3	-7.8744143	0.95609202	0.03022146
81	Mib1	-8.1189622	0.62801329	0.02339064
82	Perp	-8.1902587	-3.3452068	0.00016002

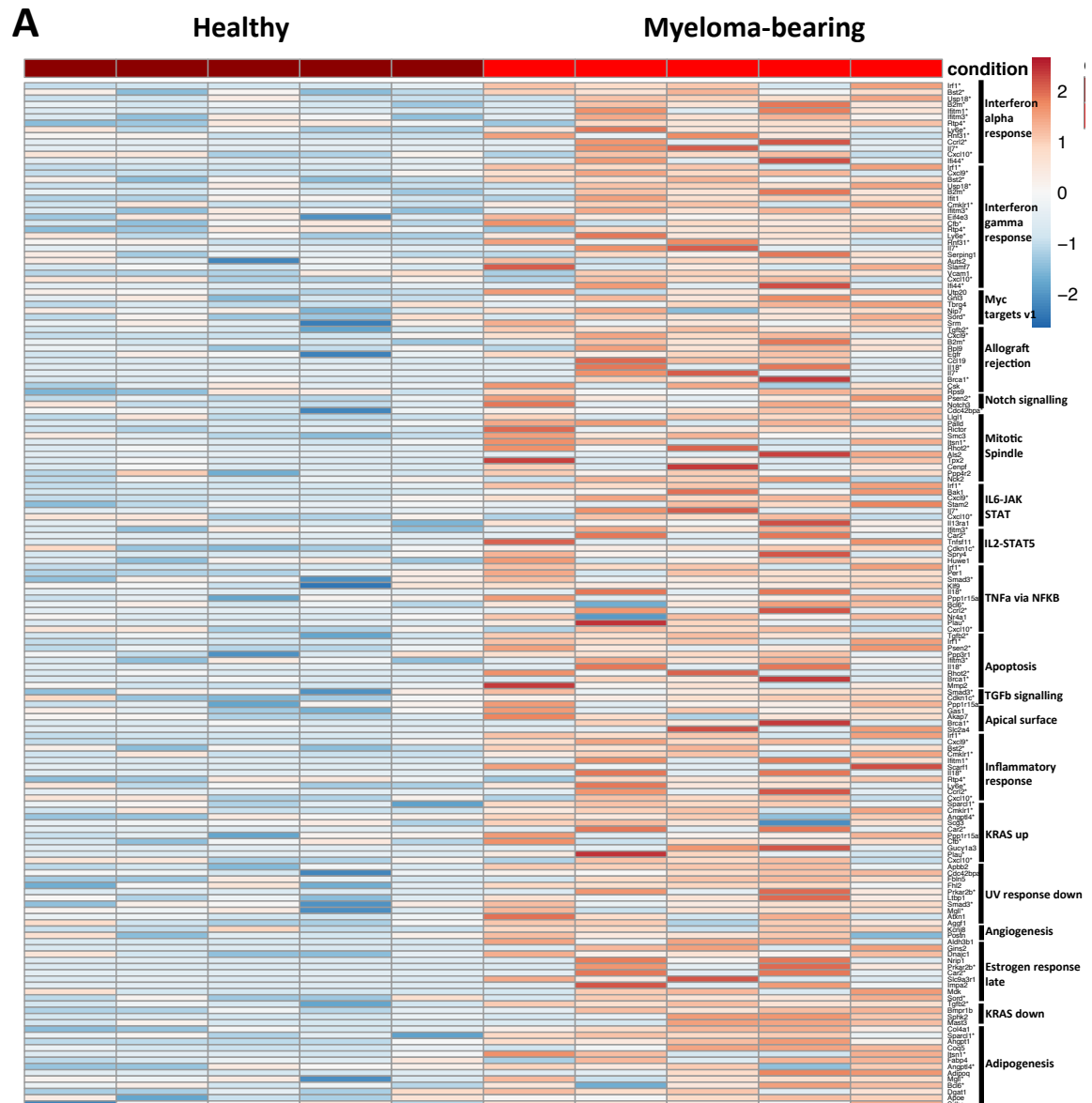
Supplementary Table 2: Significantly differentially expressed genes in bone-lining MSCs

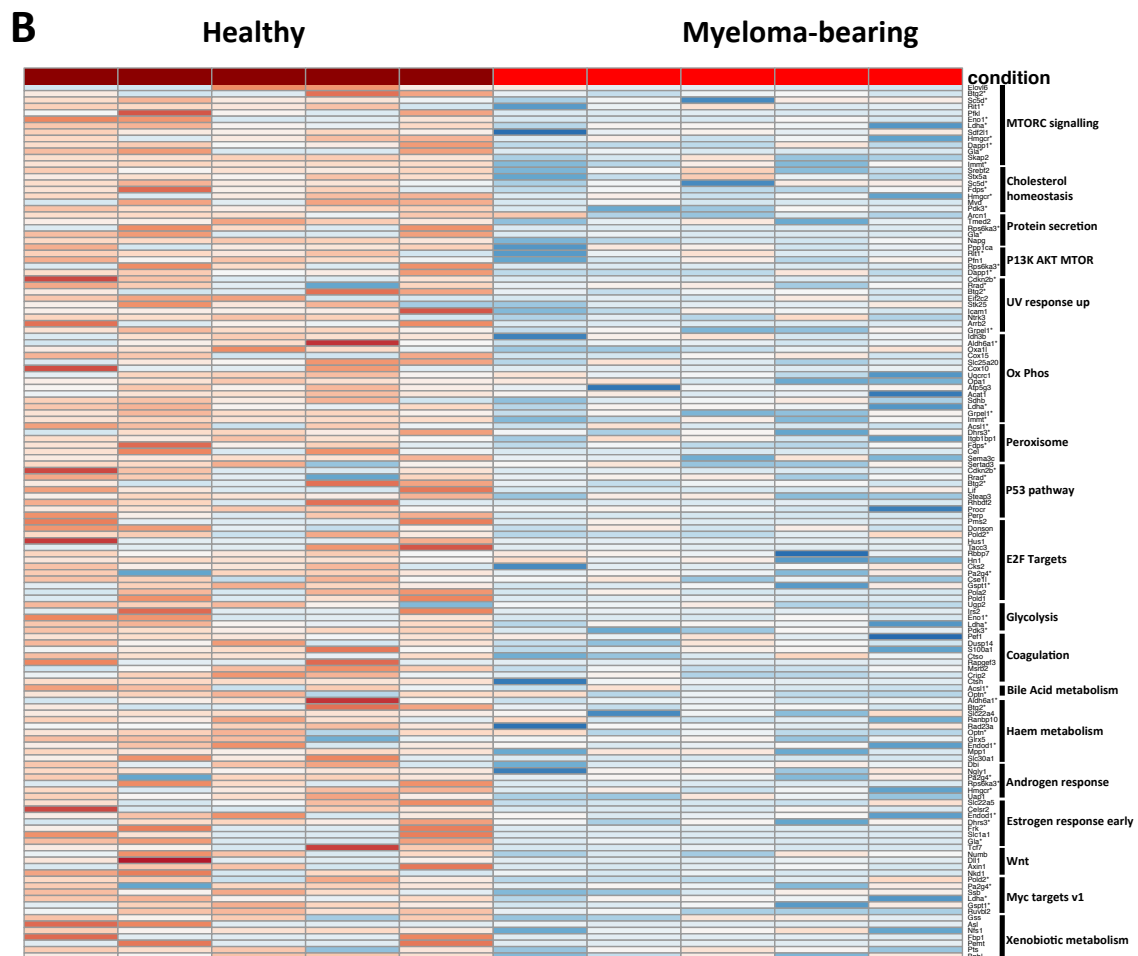
Adjusted p-value (FDR)<0.1 and abs(logFC)>1, using voom-limma method. N = 82 genes. AveExpr = normalised expression. [Referenced from section 3.2.5].



Supplementary Figure 4: GSEA software-generated heatmap.

Differential gene expression in MSCs ranked by GSEA software using RPKM values according to the 'signal2noise' algorithm (100 top ranked genes). [Referenced from section 3.2.5].





Supplementary Figure 5: Cluster Heatmap of all genes in the leading edges of enriched MSigBD Hallmark gene sets, that also fell in the top 1000 differentially regulated genes in the transcriptome of MSCs from myeloma-bearing mice (red top bar) vs healthy mice (maroon top bar).

Each column represents data from a single 100-cell MSC sample from one mouse. Each row represents relative expression of the gene as labelled. Genes are clustered by Hallmark gene set, as labelled by adjacent bars. If a gene is a member of more than one of these gene sets, it is repeated. LogCPM values, internally scaled per gene (centralised by subtracting the mean and dividing with the standard deviation) to highlight the relative differences between the two phenotypes were used to create this heatmap. The red/blue scale represents these scores.

A: Genes upregulated in MSCs from myeloma-bearing mice

B: Genes upregulated in MSCs from healthy mice

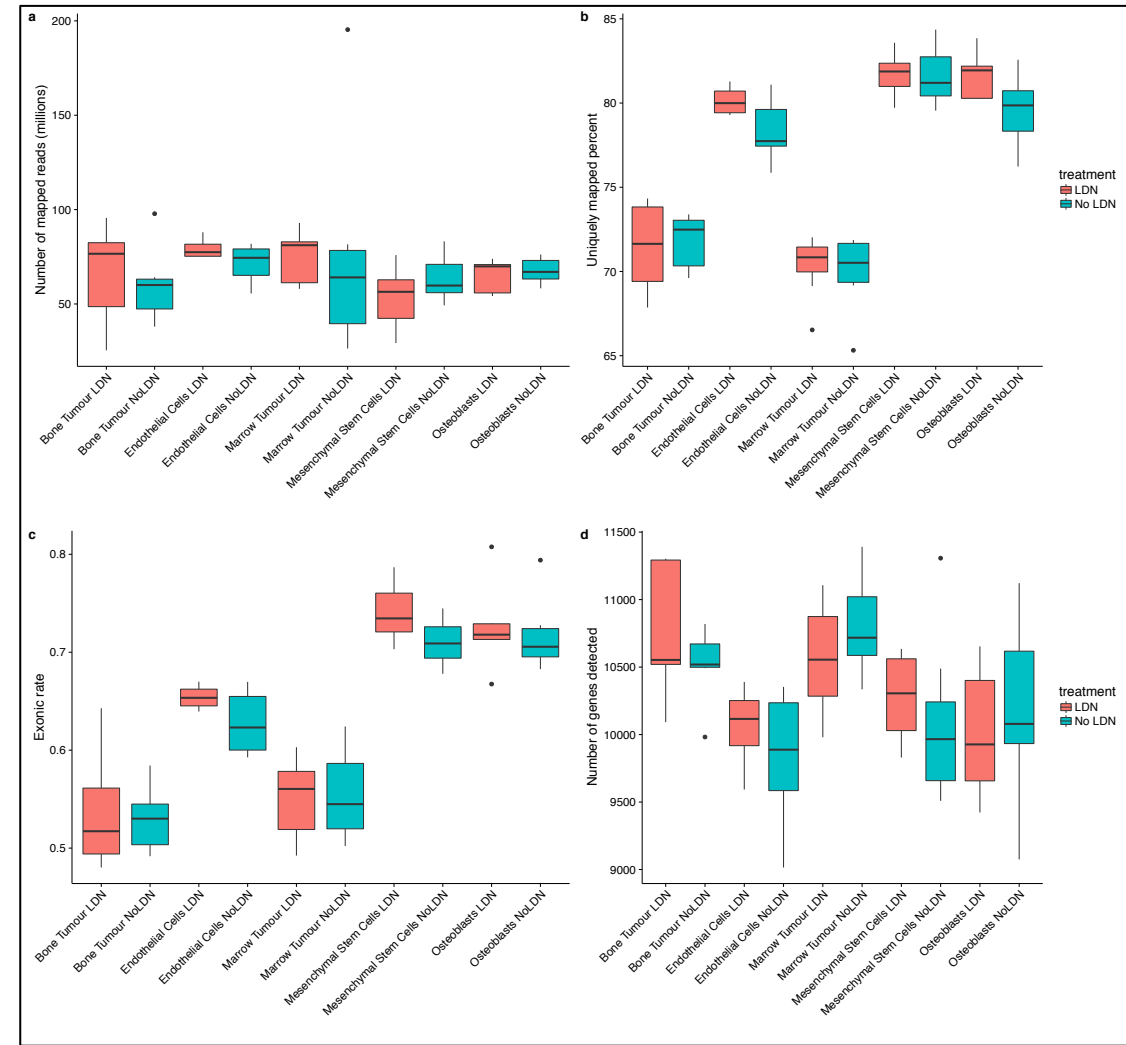
[Referenced from section 3.2.5].

Patient sex, age	Clinical diagnosis	Previous treatments	Aspirate sample Plasma cell %
m, 74	Myeloma post induction	VCD induction	2
m, 68	New myeloma without bone lesions	Nil	10
m, 65	Myeloma, post high dose melphalan autograft	Induction + ASCT	2
m, 70	Myeloma extensively pretreated	RCD, 2x ASCT, R maintenance	10
m, 77	New myeloma without bone lesions	Nil	15
m, 75	New myeloma with bone lesions	Nil	20
m, 69	MGUS	Nil	10

Supplementary Table 3: Patient characteristics associated with clinical samples used to generate BMSC cultures.

Diagnosis and plasma cell percentage of patient samples used to generate bone marrow stromal cell cultures used to measure gene expression changes in response to LDN (n=7) or BMPR1a treatment (n=4, same patients). [Referenced from section 2.2.7, Figs. 4.5 E, 5.14 A].

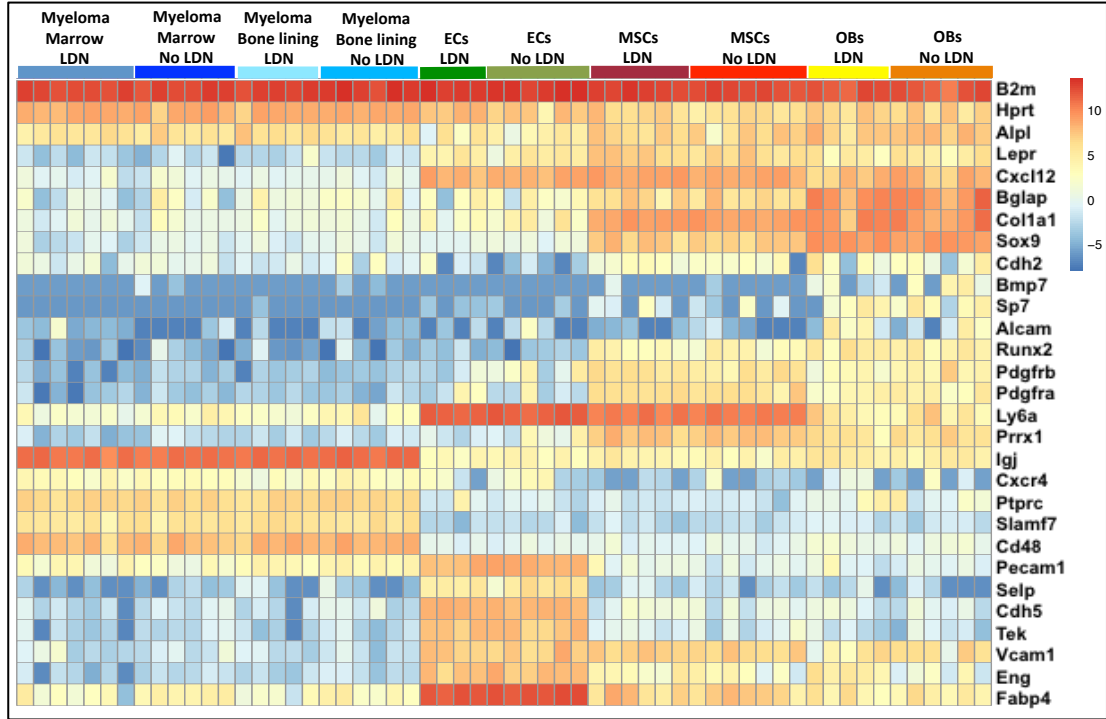
VCD = Bortezomib, Cyclophosphamide and Dexamethasone treatment; ASCT = autologous stem cell transplant; RCD = Lenalidomide, Cyclophosphamide and Dexamethasone treatment; R = Lenalidomide monotherapy.



Supplementary Figure 6: Quality assessment of RNASeq mapping using RNA-SeQC (DeLuca *et al*, 2012) in control (NotLDN) vs. LDN193189-treated (LDN) myeloma-bearing mice.

A) Number and b) percentage of reads mapping uniquely to the genome, c) proportion of reads that map to annotated exonic regions and d) number of genes that are expressed in each group of samples.

[Referenced from section 4.2.3].



Supplementary Figure 7: Heatmap demonstration of the relative expression of selected genes that identify the phenotype of sorted cell populations in LDN193189 ('LDN') vs. control- treated ('NoLDN') myeloma-bearing mice.

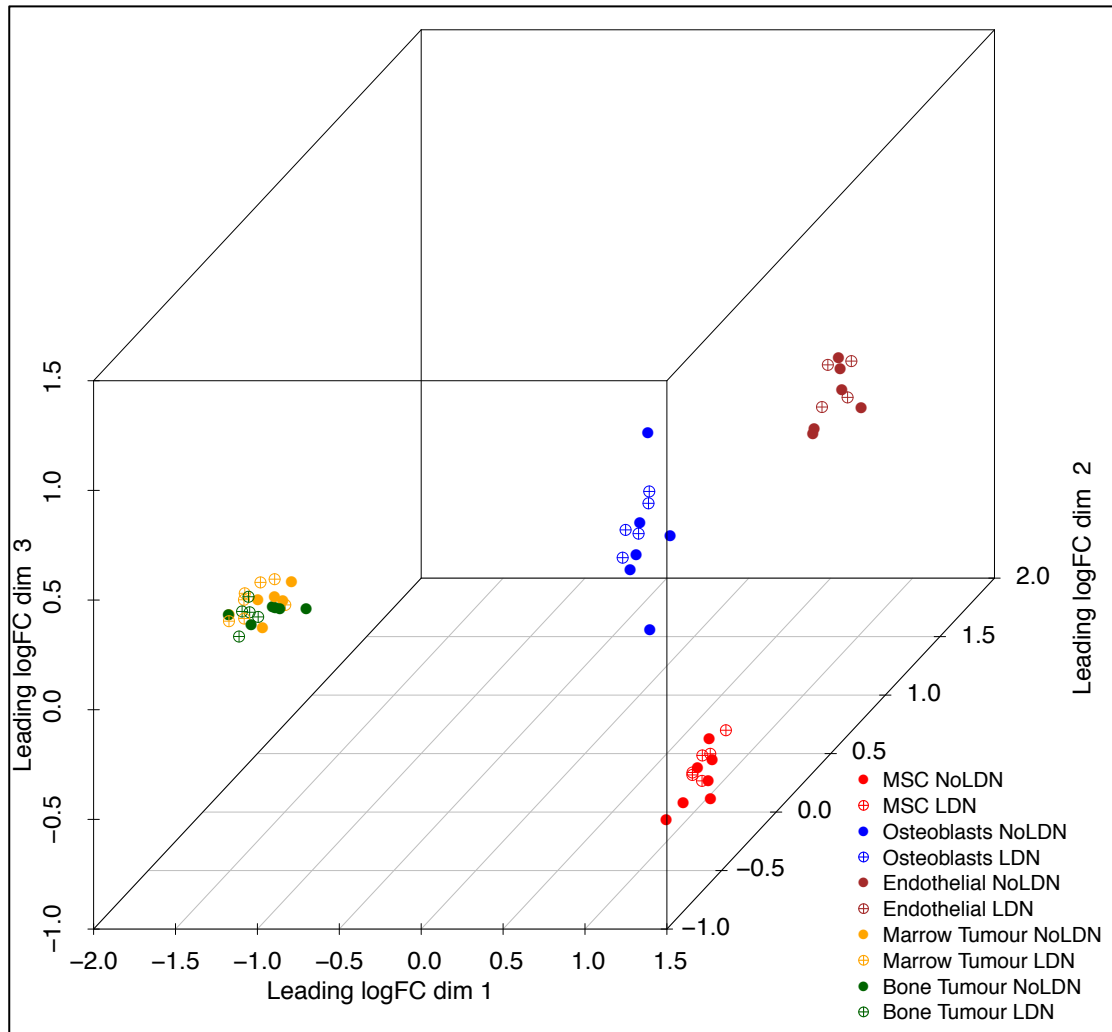
Each column represents a 100-cell sorted sample from an individual mouse, the top colour block legend providing reference of cell type.

Each row represents a particular gene, the colour of each block representing the expression of that gene (logRPKM values) in that cell population, as per reference scale strip.

B2m and *hprt*: selected endogenous control genes.

ECs = endothelial cells; MSCs = mesenchymal stem / progenitor cells; OBs = osteoblasts.

[Referenced from section 4.2.3].



Supplementary Figure 8: RNASeq-generated gene expression data visualisation by MDS plot, to demonstrate unsupervised clustering of bone-lining cell types in LDN193189 ('LDN') vs. control-treated ('NoLDN') myeloma-bearing mice.

Distances correspond to leading log-fold-changes between each pair of RNA samples, where the leading log-fold-change is the average (root-mean-square) of the largest absolute log-fold- changes between each pair of samples.

[Referenced from section 4.2.3].

Geneid	logFC	logCPM	PValue	FDR	Notes of interest re function where known
Rn45s	0.64008891	15.4633472	8.12E-11	1.06E-06	ribosomal
Alad	4.43477126	7.00030492	1.30E-09	8.47E-06	metal ion response
Mcm3	4.50616308	6.19250521	2.62E-07	0.00113747	DNA rep / cell cycle
Zfp952	8.2936565	4.25076682	3.81E-07	0.00124316	?? Transcriptional regulator
Mcoln2	-5.9238312	5.29280361	6.52E-07	0.00169947	?? membrane ion channel
Gins2	7.82956521	4.86198077	8.04E-07	0.00170216	DNA rep / cell cycle
Mbnl3	6.13350186	5.61371282	9.14E-07	0.00170216	metal (zinc) binding; RNA processing
Entpd1	3.89636118	7.81629891	1.35E-06	0.00195107	ATP binding Gprotien coupled
Incenp	5.9927463	4.49384119	1.20E-06	0.00195107	mitotic regulator, cell cycle
Blm	5.74056544	4.82041588	1.90E-06	0.00205956	cell cycle neg regulator
C920009B18Rik	-6.332019	4.79463615	1.65E-06	0.00205956	
Qtrt1	5.79619547	4.65158906	1.89E-06	0.00205956	tRNA modification (queuosine)
2500004C02Rik	7.72599613	3.35566836	2.50E-06	0.00250558	
Retsat	4.64729231	5.52107221	3.95E-06	0.00324266	retinol metabolism
Slc25a29	9.20449218	2.28411564	3.53E-06	0.00324266	AA membrane transporter
Xpr1	-3.1927856	6.57995463	3.98E-06	0.00324266	phosphate homeostasis
2810442N19Rik	-8.889673	0.83596862	5.44E-06	0.00417249	
Ralgps2	5.59020986	4.4282506	6.19E-06	0.00448746	?? Cytoskeleton organisation
Vdr	8.1206932	3.09518525	7.80E-06	0.00535463	Vit D receptor doi: 10.1016/j.jsbmb.2015.08.009.
Cenpl	6.46482101	5.01281608	1.01E-05	0.00651037	centromere component
Tram2	6.04518405	3.94104982	1.05E-05	0.00651037	Ca for collagen synthesis
1110008L16Rik	-5.1212877	3.80433139	1.25E-05	0.00742249	
Card6	-7.6395591	4.35844652	1.60E-05	0.00905273	apoptosis regulator
Col9a1	3.23217165	8.32615215	2.04E-05	0.00960703	metal ion binding, collagen
D2Wsu81e	5.83072153	4.26285902	1.98E-05	0.00960703	cell cycle
Fgf13	7.96104279	2.46999303	1.93E-05	0.00960703	microtubule / cell migration
Gale	7.12223187	4.28037274	2.06E-05	0.00960703	galactose metabolism
Lym7	7.52168445	3.50379863	2.02E-05	0.00960703	mitochondrial
Uhrf1	5.55959705	4.17932315	2.47E-05	0.01108941	ubiquitin ligase
Lyz2	5.8087039	6.80916165	2.68E-05	0.01166491	Lysozyme (antibact)
Itga3	6.19597691	4.16375629	3.62E-05	0.01521091	Integrin alpha 3
2610305D13Rik	-5.2043652	6.14863557	4.23E-05	0.01597277	
Chl1	7.66971073	3.91698567	4.26E-05	0.01597277	cell adhesion / migration
Nelfe	4.12767546	5.51067255	4.23E-05	0.01597277	Negatively regulates transcription (ERK)
Zfand1	6.97651705	4.51493848	4.29E-05	0.01597277	
Lrg1	4.16648217	6.41847663	4.69E-05	0.016987	TGFb receptor binding
Thnsl1	-7.1176584	3.84600566	4.84E-05	0.01704867	?? Pyridoxal phosphate binding
Nabp1	5.7712401	5.54010214	5.04E-05	0.01730147	cell cycle checkpoint / dna repair
Rinl	7.13941281	3.7364995	5.26E-05	0.01758135	
BC030867	6.29569315	2.60294198	6.07E-05	0.01975738	
Knstrn	-6.1608485	4.65774497	6.21E-05	0.01975738	
Lrrc8b	-9.8233231	2.330541	7.18E-05	0.0222993	maintaining osmotic potential
Tnfrsf18	5.05448118	5.0105932	8.09E-05	0.02454542	inflammatory response
Pat1	-4.4396476	5.09603095	8.47E-05	0.02511003	mRNA processing
Cyp4b1	5.93288585	4.92992868	8.67E-05	0.02512979	
Gnb1l	-8.1044441	3.52335853	9.17E-05	0.0254491	
Gucy1a2	7.20809656	3.76603605	9.13E-05	0.0254491	
Neil1	-8.1638302	3.15068576	0.00010727	0.02914488	DNA repair
Copg2	-8.5738116	3.41717134	0.00011187	0.02977237	protein transport
Nrp	4.9588694	2.61288213	0.00012264	0.03135955	VEGF binding
Rars	-3.0370126	6.99753325	0.00012052	0.03135955	tRNA activity
Mcm9	-6.8016355	3.74416409	0.00012654	0.03157105	DNA damage response
Ttn	5.29136664	4.30478895	0.00012831	0.03157105	
Khk	5.96379227	3.85938475	0.00014522	0.03209924	glycolysis
Mgme1	5.24501218	3.92966716	0.00014331	0.03209924	mitochondrial DNA repair
Prkrip1	5.79339572	3.66640692	0.00013314	0.03209924	neg reg cyclin-dep kinases
Shq1	7.27494325	3.52061516	0.00014392	0.03209924	pos regulator apoptosis
Zfp120	-5.1745208	4.82744107	0.00013788	0.03209924	transcriptional regulator
Zfp455	10.0559373	3.09180969	0.00013937	0.03209924	
2610020H08Rik	6.13437339	3.4416289	0.00015436	0.03324147	
Spp12b	5.76624556	3.06323591	0.00015549	0.03324147	
Tmem106c	-4.7972287	4.25100323	0.00016152	0.03397314	
Zfp668	-8.3531918	2.22044162	0.00017065	0.0353241	
Atad5	-5.251425	4.65089697	0.00018488	0.03657445	
Kcnb1	-5.7734926	3.94505607	0.00018302	0.03657445	
Klf16	4.85882295	2.37133714	0.00018746	0.03657445	
Tubgcp6	-6.5044913	3.29725778	0.00018791	0.03657445	
AI467606	-5.2516606	3.66375458	0.00019425	0.03711332	
Nsun6	6.90033427	3.46856607	0.00019637	0.03711332	
Nat9	4.7748232	4.41756718	0.00020498	0.03818816	
Skida1	-8.4637168	1.75537983	0.00021391	0.03891374	
Tsga14	5.45176734	3.57114812	0.00021702	0.03891374	
Wdr12	3.40611137	6.26877982	0.00021783	0.03891374	
Pde2a	4.01287732	6.22908853	0.00022474	0.039077	
Phyhd1	-7.7603801	2.82115385	0.0002234	0.039077	
2310042D19Rik	7.30988333	2.33072752	0.00022869	0.03924155	
Cenpk	4.73364345	4.87788458	0.00023195	0.03928453	
Inadl	7.57983913	2.90496139	0.00023786	0.03976818	
Pim2	5.13647538	4.80861671	0.00026333	0.04346938	
Rhbdf2	-5.1337884	3.58504105	0.00026678	0.04348871	
Cd177	5.82845998	2.73090839	0.00027555	0.0442602	
Lin54	-5.0416767	5.0932226	0.0002802	0.0442602	
Raver2	8.54326068	2.08445774	0.0002817	0.0442602	
Gcat	3.87630018	5.19407212	0.00029827	0.04630695	
Pmvk	4.53573775	5.50136243	0.0003089	0.0473918	
Bysl	5.1196813	4.84559111	0.00032969	0.04999469	

Supplementary Table 4: Significantly differentially expressed genes in bone-lining MSCs from myeloma-bearing mice treated with LDN193189 vs vehicle.

Adjusted p-value (FDR)<0.05. N = 86 genes.

Genes reported or likely to be associated with bone matrix formation are highlighted yellow. Green highlight denotes possible cell cycle regulation involvement (inferred from literature only).

[Referenced from section 4.3.7].

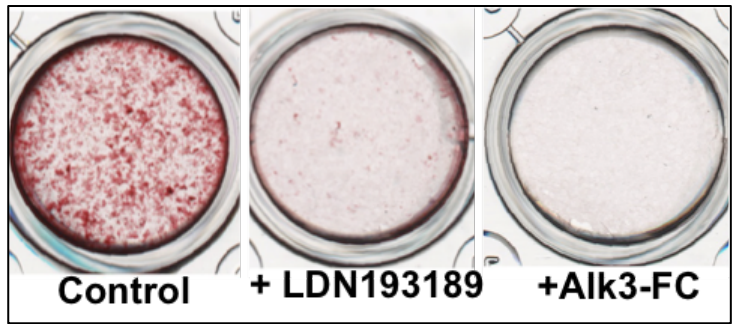
Gene sets enriched in LDN-treated Sca1+/ALCAM- BLCs vs LDN-untreated Sca1+/ALCAM- BLCs				
Enriched genes: red Non-enriched: black	Nuclear Receptor transcription pathway R-HSA-383280	Extracellular Matrix Organisation R-HSA-1474244	Collagen Formation R-HSA-1474290	NCAM1 Interactions R-HSA-419037
Original gene set size	49	87	58	39
After restricting to dataset	29	63	50	27
Genes expressed in dataset in order of enrichment score	VDR ESRRA NR6A1 PPARD NRBF2 NR2F1 NRBP1 NR1H2 RARA RXRB PPARG NR3C2 RXRA NR3C1 RORC NR1D2 THRB ESR1 NR4A2 NR1H3 NR0B2 NR4A1 NR2F6 AR NR1D1 RARG RORA THRA NR4A3	COL4A5 COL2A1 COL9A1 GLT25D1 MMP14 COL4A2 BMP1 COL9A2 COL22A1 COL5A1 COL6A3 COL9A3 ADAMTS14 COL27A1 P4HB P4HB PLOD3 COL6A1 TIMP2 COL11A2 COL5A3 COL6A2 COL4A1 SERPINH1 PCOLCE PLOD1 COL12A1 COL25A1 MMP2 COL3A1 MMP13 COL16A1 MMP8 MMP9 COL7A1 COL1A1 PPIB MMP15 LEPREL2 COL8A2 COL1A2 COL8A1 COL14A1 LEPREL1 COL15A1 COL10A1 ADAMTS3 COL11A1 PCOLCE2 COL24A1 CRTAP COL4A3 PLOD2 COL13A1 COL4A4 ADAMTS2 GLT25D2 COL5A2	COL4A5 COL2A1 COL9A1 GLT25D1 COL4A2 BMP1 COL9A2 COL22A1 COL5A1 COL6A3 COL9A3 ADAMTS14 COL27A1 P4HB PLOD3 COL6A1 COL11A2 COL5A3 COL6A2 COL4A1 SERPINH1 PCOLCE PLOD1 COL12A1 COL25A1 COL3A1 COL16A1 COL7A1 COL1A1 PPIB LEPREL2 COL8A2 COL1A2 COL8A1 COL14A1 LEPREL1 COL15A1 COL10A1 ADAMTS3 COL11A1 PCOLCE2 COL24A1 CRTAP COL4A3 PLOD2 COL13A1 COL4A4 ADAMTS2 GLT25D2 COL5A2	COL4A5 COL2A1 COL9A1 COL4A2 COL9A2 ST8SIA4 COL5A1 COL6A3 COL9A3 CACNB3 COL6A1 COL6A2 COL4A1 NCAM1 COL3A1 COL1A1 GFRA1 COL1A2 CACNA1G PRNP ST8SIA2 NRTN COL4A3 COL4A4 GFRA2 CACNB4 COL5A2

Supplementary Table 5: Significantly enriched REACTOME gene sets.

Lists of genes included in REACTOME gene sets that were significantly enriched in MSCs from LDN-treated myeloma-bearing mice (met enrichment criteria of FDR < 0.25 and n > 25):

- (A) Nuclear Receptor Transcription pathway
- (B) Extracellular Matrix Formation
- (C) Collagen Formation
- (D) NCAM1 Interactions

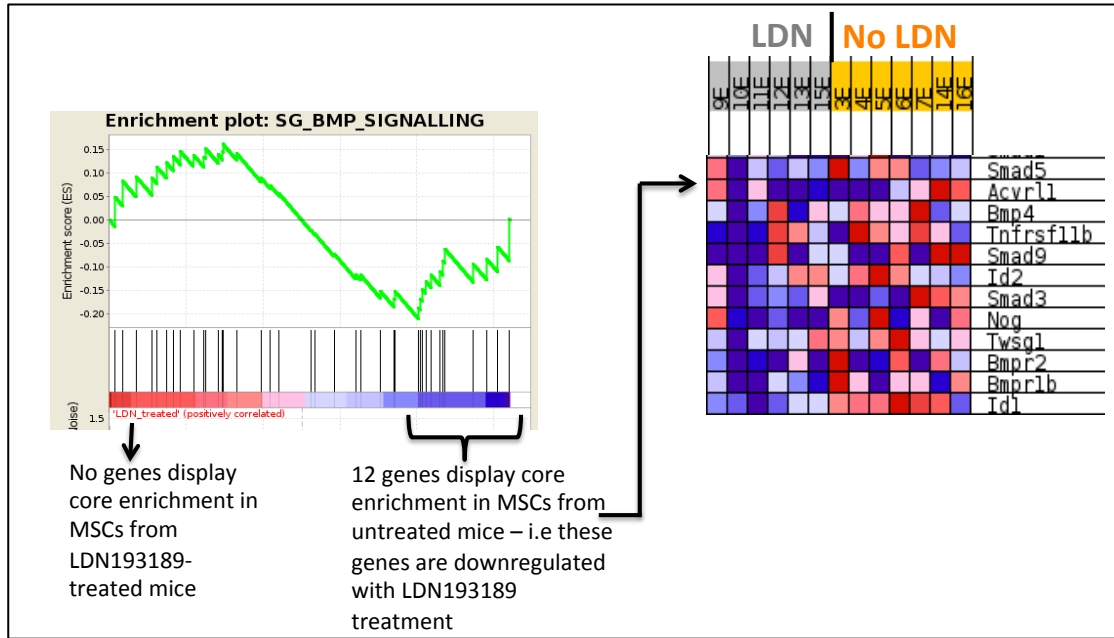
[Referenced from section 4.3.7].



Supplementary Figure 9: Mineralization of murine pre-osteoblast cell line 2T3 in presence of LDN193189 or Alk3-FC

Preliminary experiment without quantification demonstrated that either LDN193189 or Alk3-Fc added to osteogenic media completely prevented mineralisation of 2T3 pre-osteoblast cell line. Calcified matrix stained red with Alizarin Red stain.

[Referenced from chapter 6].



Supplementary Figure 10: Pharmacological effect of LDN193189 demonstrated by downregulation of known BMP target genes in MSCs from LDN193189-treated mice on RNASeq.

Using GSEA of the BMP pathway geneset described in chapter 3, known BMP target genes *Id1*, *Id2* and *Tnfrsf11b* are shown to be downregulated in MSCs from LDN193189-treated mice, as are 2 BMP inhibitors *Nog* and *Twsg1*.

Note *Bmpr1b*, upregulated in MSCs from myeloma-bearing mice vs healthy (chapter 3), is then downregulated in MSCs from LDN193189-treated myeloma-bearing mice.

[Referenced from section 4.3.7].

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