Microphthalmia transcription factor regulates the expression of the novel osteoclast factor GPNMB

Bone, resorption, regulation, osteoblast

Vera M. Ripoll^{*1}, Nicholas A. Meadows^{*1}, Liza-Jane Raggatt¹, Ming K. Chang¹, Allison R. Pettit¹, Alan I. Cassady¹ and David A. Hume².

¹Institute for Molecular Biosciences, Co-operative Research Centre for Chronic Inflammatory Diseases, The University of Queensland, St. Lucia, QLD 4072, *Australia*.

²The Roslin Institute, University of Edinburgh, Roslin, Edinburgh, EH25 9PS Scotland, *United Kingdom*.

***** First co-authors.

Footnote: Vera M. Ripoll, Nicholas A. Meadows are now affiliated with Medical Research Council, Mammalian Genetics Unit, Harwell, Oxon OX11 0RD

Abbreviations: *acp5*, tartrate-resistant acid phosphatase; bp, base pairs; CAGE, cap analysis gene expression; *clcn7*, chloride channel 7; CSF-1, macrophage-colony stimulating factor 1; *csflr*, colony stimulating factor 1 receptor; *ctsk*, cathepsin K; cDNA, DNA complementary to RNA; G418, geneticin; *hprt*, hypoxanthine-guanine phosphoribosyl transferase; EMSA, electrophoretic mobility gel shift assay; IL, interleukin; MEM, Minimal Essential Medium; MITF, microphthalmia transcription factor; OCL, osteoclast; *ostm1*, osteopetrosis-associated transmembrane protein 1; NFAT, nuclear factor of activated T cells; PBS, phosphate buffered saline; RANKL, receptor activator of NF- κ B ligand; qPCR, quantitative real time polymerase chain reaction; RAW/C4, RAW264.7 cell line subclone C4;

Corresponding Author: Dr Nicholas Meadows, Mammalian Genetics Unit, Medical Research Council, Harwell, Oxfordshire OX11 0RD, England, United Kingdom

Phone +44-1235-841246

N.Meadows@har.mrc.ac.uk

ABSTRACT

Microphthalmia transcription factor (MITF) regulates bone homeostasis by inducing expression of critical genes associated with osteoclast function. *Gpnmb* is a macrophage-enriched gene that has also been shown to be expressed in osteoblasts. Here, we have shown *gpnmb* to be highly induced in maturing murine osteoclasts. Microarray expression profile analysis identified *gpnmb* as a potential target of MITF in RAW264.7 cells, subclone C4 (RAW/C4), that overexpress this transcription factor. Electrophoretic mobility shift assays identified a MITF binding site (M-box) in the *gpnmb* promoter that is conserved in different mammalian species. Anti-MITF antibody supershifted the DNA-MITF complex for the promoter site while MITF binding was abolished by mutation of this site. The *gpnmb* promoter was transactivated by co-expression of MITF in reporter gene assays while mutation of the *gpnmb* M-box prevented MITF transactivation. The induction of *gpnmb* expression during osteoclastogenesis was shown to exhibit similar kinetics to the known MITF targets, *acp5* and *clcn7*. GPNMB expressed in RAW/C4 cells exhibited distinct subcellular distribution at different stages of osteoclast differentiation. At days 5 and 7, GPNMB protein co-localized with the osteoclast/macrophage lysosomal/endocytic marker MAC-3/LAMP-2, suggesting that GPNMB resides in the endocytic pathway of mature macrophages and is possibly targeted to the plasma membrane of bone resorbing osteoclasts. The inclusion of *gpnmb* in the MITF regulon suggests a role for GPNMB in mature osteoclast function.

1. INTRODUCTION

Osteoclasts are large multinucleated cells that are derived from the haematopoietic myeloid/monocyte lineage and are specialised for bone resorption. Normal bone remodelling requires tight coupling between the activity of osteoclasts and the primary bone-synthesising cells, osteoblasts. Receptor activator of NF- κ B ligand (RANKL) and macrophage-colony stimulating factor (CSF-1) are osteoblast products that initiate osteoclastogenesis and functional maturation of bone-resorbing activity (Kodama et al. 1991, Suda et al. 1999, Yasuda et al. 1999, Boyle et al. 2003). The use of transgenic gene knock-out mice has elucidated the role of several transcription factors in regulating osteoclast differentiation and function. Null mutations in PU.1 (Tondravi et al. 1997), c-Fos (Wang et al. 1992) and NF- κ B p50/p52 (Franzoso et al. 1997, Iotsova et al. 1997, Xing et al. 2002) all result in osteopetrosis, which is defined by a failure of bone resorption. Amongst these regulators, the microphthalmia transcription factor (MITF) controls the expression of genes associated with late stage osteoclast maturation.

MITF has been shown to regulate the expression of tartrate-resistant acid phosphatase (*acp5*) (Luchin et al. 2000), cathepsin K (*ctsk*) (Motyckova et al. 2001), chloride channel 7 (*clcn7*) and osteopetrosis-associated transmembrane protein 1 (*ostm1*) (Meadows et al. 2007) in osteoclasts. All of these genes are required for osteoclast function and bone resorption. Together with the Ets transcription factor PU.1, MITF acts downstream of CSF-1 and RANKL to activate target genes. This complex is also involved with recruiting another key osteoclast transcription factor NFATc1, to maintain target gene expression in differentiated cells (Sharma et al. 2007). Similarly the zinc finger protein Eos, an Ikaros family member, forms a complex with MITF and PU.1 to repress target genes (Hu et al. 2007). MITF is a member of the basic helix-loop-helix-leucine zipper (b-HLH-ZIP) family of transcription factors and together with TFEB, TFEC and TFE3, MITF is part of the MiT sub-group of the b-HLH-ZIP family. All of the MiT factors are expressed in cells of the mononuclear phagocyte lineage with TFE3 and TFEB being widely expressed in other cell types but TFEC being restricted to myeloid cells (Rehli et al. 1999). We have employed the identification of novel genes regulated by factors like MITF to elucidate the mechanism of osteoclast function.

One gene that was identified was *gpnmb*, (also referred to as *dc.hil* and *osteoactivin*) which encodes for a transmembrane glycoprotein, first described in human melanoma cell lines (Weternan et al. 1995). Murine GPNMB was identified as a candidate adhesion molecule in myeloid dendritic cells (Shikano et al. 2001). The rat GPNMB orthologue, termed osteoactivin, has been attributed a role in osteoblast differentiation and function (Selim et al. 2003, Abdelmagid et al. 2007) while murine osteoactivin was identified in primary calvarial osteoblasts cultures (Bachner et al. 2002). Since GPNMB also shares significant sequence homology to melanosomal proteins including quail neuroretina clone 71 (QNR-71) (Turque et al. 1996), it was proposed that GPNMB may have a role in melanin biosynthesis and the development of retinal pigment epithelium and the iris (Bachner et al. 2002). Correlated with this, mutation of the *gpnmb* gene has been found to be responsible for iris pigment dispersion disorder in DBA/2J mice (Anderson et al. 2002). The pathogenesis of the pigmentary glaucoma in DBA/2J mice has also been associated to immune dysfunction and chronic inflammatory responses involving IL-18 (Mo et al. 2003, Libby et al. 2005, Zhou et al. 2005). In spite of these observations the mechanism of GPNMB function and its role in different cell types remains unclear.

We have previously shown that *gpnmb* expression is highly macrophage-enriched, elevated in inflammatory macrophages and functions as a feedback repressor of inflammation (Ripoll et al. 2007). In the current study, we have identified *gpnmb* as a target of MITF and provide evidence for a possible function in osteoclasts.

2. MATERIALS AND METHODS

2.1 General reagents. Recombinant human RANKL (Peprotech), human colony-stimulating factor-1 (a gift from Chiron Corp.). pEF6 (Invitrogen), pGL2-Basic (Promega). Mouse monoclonal anti-MITF antibody (NeoMarkers), rat anti-mouse MAC-3 monoclonal antibody (BD Pharmingen), mouse anti-V5 tag monoclonal antibody (Invitrogen). C57BL/6 mice were obtained from a specific pathogen free (SPF) colony at the Animal Breeding House (IMB, University of Queensland). Experiments were conducted in accordance with local animal ethics guidelines.

2.2 Cell lines and cell culture. RAW/C4 cells: a subclone of the RAW264.7 macrophage-like cell line (ATCC) (Cassady et al. 2003). RAW/C4 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 5% heat-inactivated fetal calf serum (FCS) (Biowhittaker). For osteoclast differentiation, 1×10^4 RAW/C4 or bone marrow cells were cultured on 0.1% gelatin pre-coated plates for 7 days in the above medium supplemented with 50 $\mu\text{g/mL}$ ascorbic acid (Sigma), 40 ng/mL RANKL and 10^4 units/mL CSF-1. Medium was changed every 2 - 3 days. Osteoclasts were observed after 5 - 7 days in culture. Primary osteoblasts were isolated from the calvarial of 2-day-old neonatal mice. Calvarial were dissected, rinsed with PBS and digested six times for 10 min at 37 °C in Hanks solution containing 0.25 U/ml collagenase D (Roche) and 2.2 U/mL dispase (Invitrogen). 6.9×10^4 cells were cultured in Minimal Essential Medium (MEM, Invitrogen) containing 10 % heat-inactivated FBS (Invitrogen). Cells were cultured at 37°C and 5% CO₂. Cells were differentiated in BGJb medium (Invitrogen) supplemented with 50 $\mu\text{g/mL}$ ascorbic acid (Sigma) and 10 β -glycerolphosphate (Sigma) from day 7. MC3T3-E1-S14 cells were maintained in MEM with 10% heat-inactivated FBS. After confluence (day 2) the cells were cultured in differentiation medium, with medium changes every 2-3 days.

2.3 Enrichment of primary osteoblasts and depletion of macrophages from calvarial preparations. Osteoblasts were purified from freshly digested primary preparations using the mouse lineage cell depletion kit supplemented with mouse CD11b microbeads (Miltenyi, Biotec.). Approximately 1×10^7 calvarial digested cells were incubated with lineage cell depletion antibody cocktail supplemented with CD11b beads. Cells were sorted using a MACS separation column where lineage positive haematopoietic cells were retained and enriched osteoblasts collected. Cells were counted and seeded for differentiation experiments.

2.4 Generation of RAW/C4 cell lines stably overexpressing GPNMB. Mouse *gpnmb* was PCR amplified from cDNA (forward primer: 5'-TCGGAGTCAGCATGGAAAGT-3'; reverse primer: 5'-GAGTGTCTTGGCTTGTCCT-3') and cloned into the pEF6/V5-His TOPO TA vector (Invitrogen). RAW/C4 cells were plated at a density of 3×10^5 cells/ml and cultured for 24 h before transfection. RAW/C4 cells (5×10^6 cells/transfection) were electroporated at 0.28 kV and 1000 μF using a Gene Pulser (BioRad). Cells were co-transfected with the selection plasmid, pNT-Neo, at a 3:1 ratio, plated into tissue culture plates and grown for 48 h before selection with G418 (Invitrogen) at 450 $\mu\text{g/mL}$. The stable transfectant cell lines, RAW/C4-*gpnmb*-pEF6 and RAW/C4-pEF6 were maintained in the presence of the G418 selective agent during routine culture.

2.5 RNA isolation and quantitative PCR. Total cellular RNA was extracted and purified to make cDNA. The PCR amplicon was quantitated using SYBR Green (Applied Biosystem) using an ABI Prism 7000 sequence detection system (Applied Biosystem). Sample amplicon levels during the linear phase of amplification were normalized against hypoxanthine

phosphoribosyl transferase (*hprt*) control PCR product. Assays were performed in triplicate and the means $\pm$ SD were determined. The specific primers used for qPCR were as follows: mouse TRAP1C (GB: NM_007388) forward primer (5'-ACCTGTGCTTCCTCCAGGAT-3'), reverse primer (5'-TCTCAGGGTGGGAGTGGG-3'); mouse Clcn7 (GB: NM_011930) forward primer (5'-GACTGGCTGTGGGAAAGGAA-3'), reverse primer (5'-TCTCGCTTGAGTGATGTTGACC-3'); mouse Gpnmb (GB: NM_053110) forward primer (5'-AGCACAACCAATTACGTGGC-3'), reverse primer (5'-CTTCCCAGGAGTCCTTCCA-3'); mouse csflr (GB: NM_007779) forward primer (5'-CCACCATCCACTTGTATGTCAAAGAT-3'), reverse primer (5'-CTCAACCACTGTCACCTCCTGT-3'); mouse Runx2 (GB: NM_009820.3) forward primer (5'-ACAAACAACCACAGAACCACAAGT-3'), reverse primer (5'-GTCTCGGTGGCTGGTAGTGA-3'). Relative expression levels were calculated using experimentally determined primer efficiency and the ΔC_T method (Pfaffl 2001).

2.6 Analysis of promoter activity. Endotoxin-free plasmid DNA was prepared for transfection into RAW/C4 cells by electroporation (280 V/1000 μ F, Gene-Pulser, Bio-Rad). 5×10^6 RAW/C4 cells were transfected with 10 μ g of reporter plasmid and 2 μ g of expression plasmid. Cellular harvest and luciferase activity assay were performed according to Meadows et al. 2007. Luciferase activity was normalised to the total protein concentration of the cell lysate to give relative light units (RLU). The standard error of the mean was calculated within, and between experiments.

2.7 Electrophoretic mobility gel shift assay (EMSA). Nuclear extracts were prepared according to the protocol described by Meadows et al. 2007. Complementary oligonucleotides used for double-stranded probe preparation were as follows with mutated residues shown in lower case: Gpnmb oligo (5'-CTGCTTAAA ACATCACATGATCTCCC-3') and Gpnmb Δ E (5'-CTGCTTAAAACATctcgagATCTCCC-3'). Oligonucleotides were 5' end-labeled with γ - 32 P-ATP and T4 polynucleotide kinase for 30 min at 37°C. Nuclear extract proteins were bound to the DNA probe in a 10 μ L reaction containing 20 mM HEPES pH 7.9, 500 nM DTT, 2 mM EDTA, 40 mM KCl, 12% glycerol, 1 μ g salmon sperm DNA, 0.04 pmol purified probe and 2 μ g nuclear extract. Reactions in which competitor probes were added included 10-, 50- or 100-fold molar excess of unlabelled competitor probe (0.4, 2 or 4 pmol, respectively). The super-shift reaction included the anti-Mitf antibody (NeoMarkers). The Tris-glycine-EDTA gel system was used for EMSA analysis.

2.8 Immunofluorescence. RAW/C4 cells overexpressing GPNMB were grown on coverslips and differentiated in the presence of RANK-L and CSF-1 for 7 days, as described above. At indicated time points, cells were washed twice with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 whereupon the cells were washed in PBS containing 0.5% bovine serum albumin (BSA) and labelled with primary anti-V5 antibody and anti-MAC-3 for 1 h. Primary antibodies were detected with secondary Alexa 488-conjugated (for V5) and Alexa 594-conjugated (for MAC-3) (Invitrogen). Cells were mounted onto glass coverslips using DAKO Cytomation fluorescent mounting media (Dako Corporation). Slides were photographed using a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

3. RESULTS

3.1 *Gpnmb* is highly enriched in both macrophage and osteoclast lineages.

In mice, *gpnmb* is highly enriched in macrophages (Ripoll et al. 2007). However, both mouse *gpnmb* and its rat homolog (osteosclerost) have been attributed a role in osteoblast differentiation and function (Safadi et al. 2001, Bachner et al. 2002, Abdelmagid et al. 2007). Microarray data that we have published as part of the Novartis symatlas project (symatlas.gnf.org) supports published evidence that *gpnmb* mRNA is detectable in primary mouse calvarial osteoblasts, and increases, as they are stimulated towards matrix calcification *in vitro*. Nevertheless, such primary cultures may be contaminated with myeloid cells, and known macrophage-specific genes (*emr1*, *csflr*) can be detected in the osteoblasts. To assess whether the osteoblasts themselves express *gpnmb*, magnetic assisted cell sorting (MACS) was used to deplete macrophages and other haematopoietic cells from the calvarial cell preparation and enrich osteoblasts. Cell populations were cultured over a standard 21-day time course, RNA was harvested and gene expression profiles were determined by quantitative real time PCR (qPCR). The expression of *gpnmb* increased during differentiation of both the unsorted and enriched osteoblast cultures but was at least 6-fold higher in the unsorted population compared to enriched osteoblasts (Fig. 1a). Similarly, the expression of the macrophage gene, *csflr* was induced during unsorted osteoblasts differentiation and expressed at minimal levels in enriched osteoblasts osteoblast cultures (Fig. 1a). *Runx2* mRNA levels increased in the enriched osteoblast cells over the time course of differentiation, confirming that the sorting process enriched for osteoblasts (Fig. 1a). These data support that the reported expression of *gpnmb* in osteoblasts is largely, if not exclusively, due to macrophage contamination of primary cultures.

The microarray data in the Novartis symatlas project also revealed that *gpnmb* mRNA is expressed in differentiated osteoclasts. Considering the lineage relationship between macrophages and osteoclasts we sought to compare the expression of *gpnmb* in osteoclasts and osteoblasts. A time course for *gpnmb* mRNA levels was performed in osteoclast and osteoblast cells using both primary cells and cell lines. Whilst *gpnmb* is up-regulated during differentiation of both cell types, maximal expression of *gpnmb* in primary osteoclasts is at least 4-fold above the maximal expression of *gpnmb* in enriched primary osteoblasts (Fig. 1b). Maximal expression of *gpnmb* in osteoclast-like cells differentiated from the RAW/C4 cell line is at least 3-fold above the maximal expression of *gpnmb* in the osteoblast cell line MC3T3 (Fig. 1c). This is the first evidence that suggests that *gpnmb* expression in osteoclasts is significantly higher than osteoblasts. In fact *gpnmb* could be described predominantly as a mature myeloid cell expressed gene.

3.2 Overexpression of MITF increases basal *gpnmb* expression and superinduces *gpnmb* during osteoclastogenesis.

We showed previously that overexpression of MITF in the precursor RAW/C4 line increases OCL number and the endogenous expression of typical osteoclast markers following treatment with sRANKL and CSF-1. Based on this approach, two novel targets of MITF (*clcn7* and *ostm1*) were identified (Meadows et al. 2007). These genes have a known function in bone resorption (Lange et al. 2006). Moreover, the co-expression of these MITF targets with the MITF-responsive genes, *acp5* and *ctsk*, suggests that the MITF regulon is associated generally with mature osteoclast function. Within the list of potential MITF transcriptional targets, a number of genes with relatively unknown roles in osteoclast biology were also identified, and by association, these genes are likely candidates to be involved with osteoclast function. One such gene was *gpnmb*, which increased in expression in RAW/C4-GFP-*mitf*/pEF6 cells compared to RAW/C4-GFP-pEF6 cells in the microarray analysis (Fig. 2). This result was validated using qPCR for *gpnmb* with RNA prepared from the same

timecourse. Given that *gpnmb* is also expressed in melanocytes (Weterman et al. 1995), which require MITF for differentiation (Steingrímsson et al. 2004), we reasoned that *gpnmb* was likely to be a direct MITF target and a contributor to osteoclast function.

3.3 The *gpnmb* proximal promoter region is conserved between mouse and human.

The transcription start site of *gpnmb* was identified from an extensive genome-wide promoter analysis using CAGE (cap analysis gene expression) data for both mouse and human (Fig. 3a) (Carninci et al. 2006). In both mouse and human there is a major peak of transcription initiation associated with a conserved CA initiator (Carninci et al. 2006). Of the 3206 tags mapped in mice, two thirds were derived from bone marrow and myeloid specific tissues (primary data can be accessed at www.macrophages.com). The promoter for *gpnmb* was annotated using a Clustal W alignment of the mouse and human conserved regions (Fig. 3b). Several features are relevant to the regulation of *gpnmb*. Unlike many myeloid-specific promoters, *gpnmb* has a single dominant start site around 30 bp downstream of a TATA-like element in both mouse and human. A conserved AP-1 (TGAGTCA) site was identified in the *gpnmb* promoter as well as a conserved core-binding site (CACCA) for the RUNX family of transcription factors, which could contribute to expression in both macrophages/osteoclasts (through AML1/runx1) and osteoblasts (through runx2). The conserved region contains a consensus M-box element (TCACATGA) for binding of MITF. This MITF binding site is aligned and conserved between at least 11 different species (Fig. 3c). The clear conservation of these elements suggests that *gpnmb* has similar regulation in all mammals.

3.4 MITF binds and transactivates the *gpnmb* promoter.

To establish whether MITF directly transcriptionally regulates *gpnmb* expression in osteoclasts, the ability of MITF to bind and transactivate the *gpnmb* promoter in RAW/C4-derived OCLs was determined. Radiolabeled oligonucleotide probes containing the *gpnmb* promoter M-box sequence were designed for EMSA, under conditions previously optimized with the *acp5* promoter M-box (Meadows et al. 2007). Endogenous MITF from nuclear extracts of RAW/C4 cells cultured with sRANKL and CSF-1 for 5 days was found to bind specifically to the *gpnmb* probe, shown by the supershift that occurs in the presence of a mouse anti-MITF antibody (Fig. 4a). Binding specificity was demonstrated using cold competition assays performed with the wild type *gpnmb* M-box and a mutated M-box oligonucleotide in which the CACATG core was mutated to CTCGAG.

Transient transfections of RAW/C4 cells with a *gpnmb* promoter (-250 to +73) luciferase reporter construct and a MITF expression plasmid were performed to assess the activity and responsiveness of the proximal *gpnmb* promoter to MITF. The *gpnmb* promoter was transactivated by the wild type *mitf* expression plasmid (Fig. 4b) and mutations of the conserved M-box not only ablated this transactivation but also reduced basal promoter activity. These data indicate that MITF regulates the *gpnmb* promoter *in vitro* via the conserved M-box. Consequently these experiments identify a specific MITF-binding site within the *gpnmb* promoter and suggest that the *gpnmb* promoter is responsive to wild type MITF promoter transactivation.

3.5 *Gpnmb* expression is induced with similar kinetics to *clcn7* and *acp5* during osteoclastogenesis.

The regulation of *gpnmb* expression during osteoclastogenesis was compared to known MITF targets, *acp5* and *clcn7* (Fig. 5c). Over a 7-day timecourse with sRANKL and CSF-1, qPCR showed that *gpnmb* was up-regulated with similar kinetics to *clcn7* and *acp5* which suggests a common pattern of regulation.

3.6 RANKL induces a change in GPNMB localization during osteoclastogenesis.

Although the expression and regulation of *gpnmb* in osteoclasts has been demonstrated, nothing is known about its function in osteoclast biology. To characterize the role of GPNMB in osteoclasts, its subcellular localization in different stages of osteoclast differentiation was examined by immunofluorescence using confocal microscopy. RAW/C4-*gpnmb*-pEF6 cells overexpressing GPNMB, were treated with RANKL over 7 days, and were stained with anti-V5 antibody to detect V5-tagged GPNMB. As we previously described in macrophages (Ripoll et al. 2007), at day 0 and day 3 osteoclasts, GPNMB was confined to the membrane compartments around the nuclei typical of the Golgi network. GPNMB staining in both undifferentiated and day 3 did not coincide with that of the endosomal marker, MAC-3/LAMP-2 as shown in Figure 6. This indicates that GPNMB is not present in lysosomes or late endosomal compartments at these time points. After further stimulation with RANKL, GPNMB cellular distribution dramatically changed from a single perinuclear Golgi compartment to dot-like organelles throughout the cytoplasm in multinuclear osteoclast-like cells. At 5 and 7 days after stimulation, MAC-3/LAMP-2 overlapped with GPNMB, which indicated it was localized to late endosomes and lysosomes (Figure 6). Intracellular membrane trafficking and endocytic pathways are essential for osteoclast function and are regulated by factors such as RANKL (Sakai et al. 2001). Many other proteins that are enriched in osteoclasts such as TRAP, CTSK and CLCN7 are closely associated with these pathways and some of them are known to be transported from late endosomes/lysosomes or recycling compartments to the cell periphery, extracellular space and plasma membrane (ruffled border) (Sahara 2001, Hollberg et al. 2002, Lange et al. 2006). Our findings suggests that GPNMB resides in the endocytic pathway of mature osteoclast-like cells and is possibly targeted to the plasma membrane or extracellular space upon osteoclast terminal differentiation.

4. DISCUSSION

Genes regulated by MTF have a strong association with osteoclast function. As late markers of osteoclast differentiation, the direct MTF targets TRAP, Cathepsin K, CLCN7, OSTM1, E-cadherin and OSCAR have all been implicated with either osteoclast resorption or late stages of osteoclast maturation (Luchin et al. 2000, Motyckova et al. 2001, Mansky et al. 2002, So et al. 2003, Meadows et al. 2007). In light of this, the identification of novel targets of master regulators of osteoclast function like MTF and NFATc1 may offer further insights into the mechanism of osteoclast function. This study has identified GPNMB as a novel candidate for osteoclast function by direct determination of MTF regulation of *gpnmb* transcription.

In a previous study, we showed that *gpnmb* expression is highly macrophage-enriched and further elevated in inflammatory macrophages (Ripoll et al. 2007). In bone, *gpnmb* has been observed to be inducibly expressed specifically in primary osteoblast differentiation cultures (Safadi et al. 2001, Bachner et al. 2002, Abdelmagid et al. 2007). Our cell-specific analyses reveal that *gpnmb* is highly expressed in both macrophages and osteoclasts. We were alerted to this pattern of expression through detection of inducible expression of many macrophage specific genes within heterogeneous populations of primary osteoblast cultures. The macrophage marker gene, *csf1r*, is one gene we have observed to have disproportionately high expression in RNA harvested from primary osteoblast populations. Similarly, we find that at least some of the *gpnmb* expression in primary osteoblast cultures can be attributed to macrophage contamination, and that macrophages and osteoclasts express the mRNA at much higher levels. This data is in agreement with an early report from Nomiya et al. (Nomiya et al. 2005) which also identified *gpnmb* as a gene differentially expressed in

osteoclast-like cells. This finding is not surprising as macrophages and osteoclasts share the same lineage and a requirement for CSF-1.

Gpnmb has been identified in low-metastatic melanoma cell lines and its expression pattern is reminiscent of migrating neural cell populations identified with *c-kit* and *trp-2* markers. MITF has been shown to regulate the expression of both *c-kit* and *trp-2* (Tsujimura et al. 1996, Ferguson and Kidson 1997) in melanocytes. MITF is a master regulator of melanocyte gene expression and its dysregulation has been linked to skin cancer melanomas (Garraway et al. 2005). Furthermore, in a homology search using the protein-protein BLAST database, the amino acid sequence of GPNMB shares identity to the melanoma markers QNR-71 (quail) SILV (mouse) and PMEL17 (human homologue of SILV). QNR-71, SILV and PMEL17 have all been shown to be regulated by MITF (Turque et al. 1996, Du et al. 2003).

Microarray and qPCR data from RAW/C4 cells that constitutively overexpress MITF revealed that *gpnmb* is up-regulated both before and during osteoclast differentiation (Meadows et al. 2007). Examination of the *gpnmb* promoter showed a strong conservation in the proximal region amongst at least 11 different mammalian species. This suggests that a common regulator of *gpnmb* is necessary for its expression in humans as in other mammals. The presence of a conserved consensus MITF binding site (M-box) within this promoter region proposes that regulation by MITF plays a critical role in directing *gpnmb* expression in osteoclasts. We have shown a clear association of MITF with the *gpnmb* promoter. Furthermore, the conserved *gpnmb* promoter region was activated by co-transfection of MITF.

GPNMB localisation in mature osteoclasts differs from its distribution in macrophages, suggesting a cell type specific function. We showed that during the differentiation of multinuclear osteoclast-like cells, GPNMB translocates from a perinuclear location to lysosomes/late endosomes with MAC-3/LAMP-2. The localisation of GPNMB to these compartments is consistent with the presence of a predicted endosomal/lysosomal-sorting signal located immediately after the transmembrane domain on the C terminal region of GPNMB (Anderson et al. 2002), (smart.embl-heidelberg.de). Intracellular membrane trafficking and endocytic pathways are regulated by the critical osteoclastogenic factor RANKL and are crucial for osteoclast function (Sakai et al. 2001). An important number of osteoclast molecules such as CTSK, CLCN7 and v-ATPase are transported from late endosomes/lysosomes and recycling compartments to the extracellular space and ruffled border to carry out their function (Sahara 2001, Toyomura et al. 2003, Lange et al. 2006). There is an exciting possibility that like these molecules, GPNMB is also transported to the vicinity of the cell surface to undertake its role. It is possible that as has been shown for GTP-binding RAB proteins, the localisation of some proteins to the ruffled border can only be observed in resorbing osteoclasts cultured on bone slices and not in non-resorbing osteoclast cultured on glass coverslips (Zhao et al. 2002). In addition to the structural and regulatory relationship between GPNMB and PMEL17, GPNMB subcellular location in osteoclasts is also reminiscent of PMEL17. Just as GPNMB moves from a perinuclear location in immature osteoclast-like cells to endosome-related structures, PMEL17 is a major component of melanosomes in melanocytes, and traffics from a perinuclear location to the intraluminal vesicles of multivesicular endosomes in differentiating melanocytes. A mutation in PMEL17 (silver) disrupts melanosome formation (Theos et al. 2006). By extension, we predict that GPNMB will have a function in formation of a population of endosome-like structure involved in bone resorption and is probably also involved in melanosome formation in melanocytes. Further functional studies are required to understand GPNMB intracellular sorting.

The mutation of the *gpnmb* gene has been found to be responsible for an iris pigment dispersion disorder in DBA/2J mice (Anderson et al. 2002). Because of the melanocyte involvement in iris pigment development and the critical role of MITF in melanocyte biology, this phenotype supports our observation of MITF regulation of *gpnmb* expression. There is no evident bone phenotype in DBA/2J mice. This does not necessarily imply that there is no function in osteoclasts; the TRAP and cathepsin K knockouts have relatively mild phenotypes, partly due to the presence of compensatory phosphatases and protease activities from other loci, and partly because bone turnover is balanced by competing actions of osteoblasts and osteoclasts. It will clearly be of interest to examine OCL resorptive function in the mutant mice.

5. REFERENCES

- Abdelmagid, S.M., et al., 2007. Osteoactivin acts as downstream mediator of BMP-2 effects on osteoblast function. *J Cell Physiol.* 210(1), 26-37.
- Anderson, M.G., et al., 2002. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. *Nat Genet.* 30(1), 81-5.
- Bachner, D., Schroder, D.Gross, G., 2002. mRNA expression of the murine glycoprotein (transmembrane) *nmb* (*Gpnmb*) gene is linked to the developing retinal pigment epithelium and iris. *Brain Res Gene Expr Patterns.* 1(3-4), 159-65.
- Boyle, W.J., Simonet, W.S.Lacey, D.L., 2003. Osteoclast differentiation and activation. *Nature.* 423(6937), 337-42.
- Carninci, P., et al., 2006. Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet.* 38(6), 626-35.
- Cassady, A.I., Luchin, A., Ostrowski, M.C.Hume, D.A., 2003. Regulation of the murine TRACP gene promoter. *J Bone Miner Res.* 18(10), 1901-4.
- Du, J., et al., 2003. MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am J Pathol.* 163(1), 333-43.
- Ferguson, C.A.Kidson, S.H., 1997. The regulation of tyrosinase gene transcription. *Pigment Cell Res.* 10(3), 127-38.
- Franzoso, G., et al., 1997. Requirement for NF- κ B in osteoclast and B-cell development. *Genes Dev.* 11(3482-3496).
- Garraway, L.A., et al., 2005. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature.* 436(7047), 117-22.
- Hollberg, K., et al., 2002. Osteoclasts from mice deficient in tartrate-resistant acid phosphatase have altered ruffled borders and disturbed intracellular vesicular transport. *Exp Cell Res.* 279(2), 227-38.
- Hu, R., et al., 2007. Eos, MITF, and PU.1 recruit corepressors to osteoclast-specific genes in committed myeloid progenitors. *Mol Cell Biol.* 27(11), 4018-27.
- Iotsova, V., et al., 1997. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med.* 3(11), 1285-9.
- Kodama, H., Nose, M., Shumpei, N.Yamasaki, A., 1991. Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. *J. Exp. Med.* 173(1291-1294).
- Lange, P.F., Wartosch, L., Jentsch, T.J.Fuhrmann, J.C., 2006. *Clc-7* requires *Ostm1* as a beta-subunit to support bone resorption and lysosomal function. *Nature.* 440(7081), 220-3.
- Libby, R.T., Gould, D.B., Anderson, M.G.John, S.W., 2005. Complex genetics of glaucoma susceptibility. *Annu Rev Genomics Hum Genet.* 6(15-44).

- Luchin, A., et al., 2000. The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. *J Bone Miner Res.* 15(3), 451-60.
- Mansky, K.C., et al., 2002. The microphthalmia transcription factor (MITF) contains two N-terminal domains required for transactivation of osteoclast target promoters and rescue of mi mutant osteoclasts. *J Leukoc Biol.* 71(2), 295-303.
- Meadows, N.A., et al., 2007. The expression of *clcn7* and *ostm1* in osteoclasts is coregulated by microphthalmia transcription factor. *J Biol Chem.* 282(3), 1891-904.
- Mo, J.S., et al., 2003. By altering ocular immune privilege, bone marrow-derived cells pathogenically contribute to DBA/2J pigmentary glaucoma. *J Exp Med.* 197(10), 1335-44.
- Motyckova, G., et al., 2001. Linking osteopetrosis and pycnodysostosis: regulation of cathepsin K expression by the microphthalmia transcription factor family. *Proc Natl Acad Sci U S A.* 98(10), 5798-803.
- Nomiyama, H., et al., 2005. Identification of genes differentially expressed in osteoclast-like cells. *J Interferon Cytokine Res.* 25(4), 227-31.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29(9), e45.
- Rehli, M., et al., 1999. TFEC is a macrophage-restricted member of the microphthalmia-TFE subfamily of basic helix-loop-helix leucine zipper transcription factors. *J. Immunol.* 162(3), 1559-1565.
- Ripoll, V.M., et al., 2007. Gpnmb is induced in macrophages by IFN-gamma and lipopolysaccharide and acts as a feedback regulator of proinflammatory responses. *J Immunol.* 178(10), 6557-66.
- Safadi, F.F., et al., 2001. Cloning and characterization of osteoactivin, a novel cDNA expressed in osteoblasts. *J Cell Biochem.* 84(1), 12-26.
- Sahara, N., 2001. Cellular events at the onset of physiological root resorption in rabbit deciduous teeth. *Anat Rec.* 264(4), 387-96.
- Sakai, E., et al., 2001. Characterization of phagosomal subpopulations along endocytic routes in osteoclasts and macrophages. *J Biochem (Tokyo).* 130(6), 823-31.
- Selim, A.A., et al., 2003. Anti-osteoactivin antibody inhibits osteoblast differentiation and function in vitro. *Crit Rev Eukaryot Gene Expr.* 13(2-4), 265-75.
- Sharma, S.M., et al., 2007. MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation. *J Biol Chem.* 282(21), 15921-9.
- Shikano, S., Bonkobara, M., Zukas, P.K., Ariizumi, K., 2001. Molecular cloning of a dendritic cell-associated transmembrane protein, DC-HIL, that promotes RGD-dependent adhesion of endothelial cells through recognition of heparan sulfate proteoglycans. *J Biol Chem.* 276(11), 8125-34.
- So, H., et al., 2003. Microphthalmia transcription factor and PU.1 synergistically induce the leukocyte receptor OSCAR gene expression. *J Biol Chem.*
- Steingrímsson, E., Copeland, N.G., Jenkins, N.A., 2004. Melanocytes and the microphthalmia transcription factor network. *Annu Rev Genet.* 38(365-411).
- Suda, T., et al., 1999. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev.* 20(3), 345-57.
- Theos, A.C., et al., 2006. A lumenal domain-dependent pathway for sorting to intraluminal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev Cell.* 10(3), 343-54.
- Tondravi, M.M., et al., 1997. Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature.* 386(81-84).

- Toyomura, T., et al., 2003. From lysosomes to the plasma membrane: localization of vacuolar-type H⁺ -ATPase with the a3 isoform during osteoclast differentiation. *J Biol Chem.* 278(24), 22023-30.
- Tsujimura, T., et al., 1996. Involvement of transcription factor encoded by the mi locus in the expression of c-kit receptor tyrosine kinase in cultured mast cells of mice. *Blood.* 88(4), 1225-33.
- Turque, N., et al., 1996. Characterization of a new melanocyte-specific gene (QNR-71) expressed in v-myc-transformed quail neuroretina. *Embo J.* 15(13), 3338-50.
- Wang, Z.Q., et al., 1992. Bone and haematopoietic defects in mice lacking c-fos. *Nature.* 360(741-745).
- Weternan, M.A., et al., 1995. nmb, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. *Int J Cancer.* 60(1), 73-81.
- Xing, L., et al., 2002. NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J Bone Miner Res.* 17(7), 1200-10.
- Yasuda, H., et al., 1999. A novel molecular mechanism modulating osteoclast differentiation and function. *Bone.* 25(1), 109-13.
- Zhao, H., Ettala, O.Vaananen, H.K., 2002. Intracellular membrane trafficking pathways in bone-resorbing osteoclasts revealed by cloning and subcellular localization studies of small GTP-binding rab proteins. *Biochem Biophys Res Commun.* 293(3), 1060-5.
- Zhou, X., et al., 2005. Involvement of inflammation, degradation, and apoptosis in a mouse model of glaucoma. *J Biol Chem.* 280(35), 31240-8.

FIGURE LEGENDS

Figure 1. *Gpnmb* expression in macrophages and osteoclasts is dominant over *gpnmb* expression in osteoblasts. qPCR for *gpnmb*, *csf1r* and *runx2* in primary osteoblasts before and after cellular sorting to remove contaminating macrophages (A). Whilst *gpnmb* is up-regulated over the timecourse in both populations, both *csf1r* and *gpnmb* expression is markedly lower in the sorted osteoblast population. *Runx2* expression is increased in the sorted population. qPCR for *gpnmb* in primary osteoclasts and enriched osteoblasts (B), and in osteoclast and osteoblast cell lines over a time course of differentiation (C). *Gpnmb* expression in osteoclasts is at least 3-fold above the expression detected in osteoblasts in both primary and cell line derived cells. Experiments were performed in triplicate and the bars represent the standard deviation for one experiment. Statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analysed by paired t-test. Statistical significance across a timecourse was performed using one way ANOVA (# $p < 0.05$).

Figure 2. Microarray and qPCR data for *gpnmb* expression in cells overexpressing MITF. Expression of *gpnmb* mRNA is induced by sRANKL. Treatment of RAW/C4 cells for 5 days and superinduced by MITF overexpression. The qPCR was consistent with the microarray data. Vector refers to RAW/C4 cells stably transfected with the empty pEF6 control. Statistically significant difference (** $p < 0.01$, *** $p < 0.001$) was analysed by paired t-test.

Figure 3. Promoter annotation and Clustal W alignment of the *gpnmb* promoter with other mammalian species. CAGE analysis has been used to identify the transcription start sites of *gpnmb* in both mouse (red bars) and human (blue bars) (A). There is a clear dominant CA initiator site (3206 tags) for mouse that is relatively conserved with the human *gpnmb* transcription start site. The conserved *gpnmb* promoter region for mouse and human has been aligned (B). Aligned and conserved consensus binding sites for MITF, RUNX and AP-1 have been boxed. Conserved TATA-like elements proximal to the transcription start sites have also been represented. Transcription start sites have been marked by an arrow and the ATG start codon is marked by *. The M-box in the *gpnmb* promoter is conserved between at least 11 different mammalian species (C).

Figure 4. EMSA and luciferase transfection assays confirm that MITF binds and transactivates the *gpnmb* promoter. (A) Nuclear extracts from RAW/C4 cells treated with sRANKL and CSF-1 (5 days) incubated with probes containing the M-box from the *gpnmb* promoter region. MITF binding specificity was demonstrated with cold competition with both WT and M-box mutation oligonucleotides (arrow). Incubation with an anti-MITF antibody produces a supershifted band (*). Selective loss of the MITF complex by wild type, and not mutant, competitors suggest that MITF has specificity for the M-box probe. (B) Co-transfections with *mitf*/pEF6 and a *gpnmb* promoter-reporter construct produce at least a 6-fold induction in relative luciferase activity compared to basal levels and transfections with *mi*/pEF6. There was no transactivation of the reporter construct in which the M-box site was mutated. Bars represent means $\pm$ standard errors of the mean ($n = 9$).

Figure 5. *Cln7*, *acp5* and *gpnmb* are up-regulated with similar kinetics during osteoclastogenesis. qPCR for *clcn7*, *acp5* and *gpnmb* in RAW/C4 cells cultured with sRANKL and CSF-1 over a timecourse of 7 days. Expression of *clcn7*, *acp5* and *gpnmb* was induced after 3 days reaching maximal expression between 5 – 7 days. All three genes display similar kinetics of expression during the timecourse. Experiments were performed in triplicate and the bars represent the standard deviation for one experiment.

Figure 6. GPNMB localisation in RAW/C4 cells treated with RANKL over 7 days. GPNMB/V5 (green) localisation shifts from the Golgi apparatus to a diffuse vesicular pattern following culture with RANKL over a timecourse of 7 days. MAC-3 (red) has been included as a lysosomal and late endosome marker. The 2 proteins occupy different cellular compartments at day 0 and 3. By day 5 and 7 the 2 proteins co-localize to similar cellular compartments (yellow).

Figure 1.

Figure 2.

Figure 3.

A

B

C

Figure 4.

Figure 5.

Figure 6.