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Genetic regulatory mechanisms in human osteoclasts suggest a role for the STMP1 and DCSTAMP genes in Paget’s disease of bone

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Paget’s disease of bone (PDB) is characterised by focal abnormalities of bone remodelling, with increased osteoclastic resorption the primary feature of the disease. Genetic factors have been shown to play an important role in PDB, and genome-wide association studies (GWAS) have identified 7 genetic loci as associated with PDB at the genome-wide level. Expression quantitative trait locus (eQTL) studies using cell types that are directly relevant to the disease of interest are increasingly being used to identify putative effector genes for GWAS loci. We have recently constructed a unique osteoclast-specific eQTL resource using cells differentiated in vitro from 158 subjects for study of the genetics of bone disease. Considering the major role osteoclasts have in PDB, we used this resource to investigate potential genetic regulatory effects for the 7 PDB genome-wide significant loci on genes located within 500 kb of each locus. After correction for multiple testing, we observed statistically significant associations for rs4294134 with expression of the gene STMP1, and rs2458413 with expression of the genes DPYS and DCSTAMP. The eQTL associations observed for rs4294134 with STMP1, and rs2458413 with DCSTAMP were further supported by eQTL data from other tissue types. The product of the STMP1 gene has not been extensively studied, however the DCSTAMP gene has an established role in osteoclast differentiation and the associations seen between rs2458413 and PDB are likely mediated through regulatory effects on this gene. This study highlights the value of eQTL data in determining which genes are relevant to GWAS loci.

Paget’s disease of bone (PDB), first described by Sir James Paget in 1876, is characterised by focal abnormalities of bone remodelling. It is the most common metabolic bone disease after osteoporosis, affecting around 2% of European individuals aged 55 years and over, although both the incidence and severity of newly diagnosed cases is falling2,3. The prevalence of PDB increases with age and is slightly more common in men than in women. The abnormal bone remodelling seen in PDB disrupts normal bone architecture and structure, leading to deformity, bone pain, secondary arthritis and increased fracture risk4. The primary feature of PDB at the cellular level is increased osteoclastic bone resorption, followed by increased and disorganised bone formation that is thought to be secondary to the increased osteoclast activity. The osteoclasts in affected bone are larger in size, have more nuclei and display characteristic intra-nuclear inclusion bodies.

Genetic factors have been shown to play an important role in the pathogenesis of PDB and are generally considered to be the primary risk-factor for developing the disease, with relatives of an affected individual having around 7-fold greater risk of developing PDB than the general population5. The genetic architecture of the disease is complex, with contributions from rare mutations in the SQSTM1 gene as well as common variants with small effects located in other genes. Mutations in SQSTM1 have been found to cause autosomal dominant PDB with high but incomplete penetrance6–8, and may account for up to 40% of familial PDB cases and 10% of apparently
sporadic PDB cases. The common non-
SQSTM1 PDB risk-variants are thought to contribute to the disease in an additive manner and interact with SQSTM1 mutations to influence disease severity.

Association of PDB GWAS variants with gene expression. We analysed the 7 variants identified as significantly associated with PDB at the genome-wide level by Albagha et al., rs4294134 (7q33), rs2458413 (8q22.3), rs1561570 (10p13), rs10498635 (14q32.12), rs5742915 (15q24.1) and rs3018362 (18q21.33), for association with local gene expression (+/−500 kb) in our osteoclast eQTL dataset. All 7 variants were found to have a MAF ≥5% in the eQTL dataset, with a total of 56 expressed gene transcripts identified within the combined analysis windows (mean 8 per variant). After correction for multiple testing within each locus, we observed statistically significant associations for rs4294134 with expression of the gene STMP1 (Table 1 and Fig. 1A), and rs2458413 with expression of the genes DPYS and DCSTAMP (Table 1 and Fig. 1B,C). For rs4294134, the PDB risk-increasing G allele was associated with increased expression of STMP1 (Table 1). For rs2458413, the PDB risk-increasing T allele was associated with reduced expression of DPYS and increased expression of DCSTAMP (Table 1).

Top eQTL variants for the STMP1, DPYS and DCSTAMP genes. We next examined all variants located within +/−500 kb of the STMP1, DPYS and DCSTAMP transcription start sites (TSS) to identify the top cis-eQTL variant for each gene. For STMP1, the PDB GWAS variant rs4294134 was found to demonstrate the strongest association with expression of the gene (Fig. 1A). For the DPYS gene, rs12674899 was identified as the top eQTL variant from the region, with the more common G allele associated with increased expression of the gene (P = 7.67 × 10−6, Fig. 1B). This variant was found to exhibit a low level of linkage disequilibrium (LD) with the PDB GWAS variant rs2458413 (r2 = 0.13), suggesting that the two may represent independent eQTL association signals. For the DCSTAMP gene, rs2458418 was identified as the maximally associated eQTL variant in the region, with the more common C allele associated with increased expression of the gene (P = 5.36 × 10−11, Fig. 1C). This variant was found to exhibit a moderate degree of LD with the PDB GWAS variant rs2458413 (r2 = 0.43), suggesting that the two may be part of the same eQTL association signal.

| Variant Location | EA | OA | EAF | PDB OR (95% CI) | Gene | Expression* | Distance to TSS | P | Beta* |
|------------------|----|----|-----|----------------|------|-------------|----------------|---|-------|
| rs4294134 chr7:135608380 G A 0.82 1.45 (1.29–1.63) STMP1 10.44 ± 1.71 −54,116 3.11E−08 0.739 |
| rs2458413 chr8:104347204 T C 0.56 1.40 (1.29–1.51) DPYS 0.21 ± 0.16 −119,850 2.27E−04 −0.396 |

Table 1. PDB GWAS variants demonstrating significant eQTL associations in the osteoclast-like cells. EA: effect allele; OA: other allele; EAF: effect allele frequency (derived from osteoclast eQTL cohort); PDB: Paget's disease of bone; OR: odds ratio; CI: confidence interval; TSS: transcription start site; variant locations obtained from dbSNP build 150 (GRCh38/hg38); PDB odds ratios obtained from Albagha et al. The eQTL associations are statistically significant using a multiple testing corrected FDR of 5% (analysis corrected for the covariates RNA-Seq batch, patient age and 10 principal components). *Expression levels are stated as mean reads per kilobase million (RPKM) ± standard deviation. **Normalised effect size on gene expression for the effect allele.
Figure 1. Regional association plots generated using the eQTL association results for (A) STMP1, (B) DPYS and (C) DCSTAMP from the osteoclast-specific dataset. Genetic variants within 400 kb of rs4294134 or rs2458413 are depicted (x axis) along with their eQTL $P$ value ($-\log_{10}$). For DPYS and DCSTAMP, the maximally associated eQTL variant from the region is also indicated (rs12674899 and rs2458418, respectively). Variants are colour coded according to their LD ($r^2$) with PDB GWAS loci rs4294134 or rs2458413 (1000GP Nov 2014 EUR population). The red arrows indicate significant associations between rs4294134 or rs2458413 and expression of nearby genes, with the allelic effects on normalised gene expression presented in the box-and-whisker plots. The recombination rate (blue line) and position of genes, their exons and direction of transcription is also indicated.
Replication in other cell/tissue types. The 2 PDB GWAS/eQTL variants identified in this study, rs4294134 and rs2458413, were queried using the publicly available GTEx Portal for evidence of eQTL effects in other tissues. These variants were checked against pre-calculated eQTLs from the GTEx Portal, which were identified for tissues with ≥70 samples using a q-value threshold of 0.05 corrected for multiple-testing. Supporting evidence for regulatory effects was found for rs4294134 with the gene STMP1 in 38 tissue types (P = 4.5 × 10^-6 - 6.6 × 10^-4) and rs2458413 with DCSTAMP in lung tissue (P = 1.1 × 10^-10). For all of these significant associations, the allelic effect seen for rs4294134 and rs2458413 on gene expression was consistent with that observed in this study of osteoclasts.

The top eQTL variants identified for the DPYS and DCSTAMP genes in this study, rs12674899 and rs2458418, were also queried using the GTEx Portal for evidence of replication. No significant associations were seen between the variant rs12674899 and expression of DPYS in any of the GTEx tissues. However, the variant rs2458418 was found to be significantly associated with expression of the DCSTAMP gene in 5 tissue types (P = 2.0 × 10^-5 - 6.8 × 10^-4), and demonstrated the second strongest association of all variants in the region behind rs2514662, with which it is in strong LD (r^2_ = 0.97). For each of these tissues, the more common C allele for rs2458418 was associated with higher DCSTAMP expression, consistent with the allelic effect observed in this study.

Bioinformatics analysis. Analysis of rs4294134 using HaploReg v4.1 revealed that it is in strong LD (r^2_ > 0.8) with 10 other variants in the region. Of these, the variant rs4676063 is a strong candidate for having a regulatory role as it lies within a variety of predicted regulatory elements, including promoter histone marks (8 tissues), enhancer histone marks (15 tissues), DNase hypersensitivity sites (10 tissues) and 7 regulatory motifs. The variant rs3110823 also presents strongly from a regulatory perspective, being located in promoter histone marks (2 tissues), enhancer histone marks (13 tissues), DNase hypersensitivity sites (9 tissues), 3 regulatory motifs and a GERP (Genomic Evolutionary Rate Profiling) and SiPhy conserved region.

Analysis of rs2458413 suggested that it is in strong LD (r^2_ > 0.8) with 6 other variants, of which rs2669448 may have a potential regulatory role. The variant is located within several predicted regulatory elements, including a promoter histone mark (1 tissue), enhancer histone marks (10 tissues), DNase hypersensitivity sites (10 tissues), and is predicted to alter 2 regulatory motifs.

Discussion
We have used our unique osteoclast-specific eQTL dataset to identify a potential regulatory role for the PDB GWAS variant rs4294134 on expression of the gene STMP1 (short transmembrane mitochondrial protein 1). This finding was strongly supported by publicly available eQTL data in multiple tissue types from the GTEx Portal. The G allele at rs4294134, which is associated with an increased risk of PDB, was found to be associated with increased expression of STMP1 in our osteoclast eQTL dataset, consistent with the allelic effect observed in the GTEx tissues. The fact that rs4294134 demonstrated the strongest association with expression of STMP1 of all tested variants in our dataset situated within 500 kb of the gene TSS strongly suggests that the association between this variant and PDB is mediated through regulatory effects on STMP1. The product of the STMP1 gene is a short trans-membrane protein that may have a role as a mitochondrial respiratory complex subunit. This gene has not been extensively studied and has no obvious role in bone biology, although knockdown of the STMP1 gene in zebrafish resulted in a series of mild morphological defects, including abnormal shape of the head and jaw.

Albagha et al. initially suggested that the association between rs4294134 and PDB may be mediated through the gene NUP205, which encodes one of the main subunits of the nuclear pore complex involved in the regulation of transport between the cytoplasm and nucleus. However, that was based primarily on the location of rs4294134 within NUP205 and not on functional evidence, with the authors acknowledging that no genes in the region were known to affect bone metabolism. Our data showed no evidence of a role for rs4294134 in regulating expression of NUP205 in this cell type.

We have identified an eQTL association between the PDB GWAS variant rs2458413 and expression of the gene DPYS in this study. A potentially independent eQTL association signal relevant to the DPYS gene and led by rs12674899 was also identified in the region. The DPYS gene encodes the enzyme dihydropyrimidinase, which has a role in pyrimidine metabolism. Mutations in the DPYS gene have been implicated in dihydropyrimidinase deficiency (OMIM #222748), a very rare autosomal recessive condition characterised by accumulation of dihydroacil and dihydrothymine in the urine, blood and cerebrospinal fluid. Clinical features of this condition are highly variable and primarily include gastrointestinal abnormalities, hypotonia, seizures and neurological abnormalities. The product of the DPYS gene has no obvious role in bone biology and it should be noted that this gene was expressed at a relatively low level in the osteoclast-like cells, suggesting that the eQTL association between rs2458413 and expression of DPYS may be more relevant in other cell types and disorders unrelated to PDB.

The PDB GWAS variant rs2458413 was also identified as significantly associated with expression of the DCSTAMP (dendrocyte expressed seven transmembrane protein) gene in this study. The PDB risk-increasing T allele at rs2458413 was found to be associated with increased expression of DCSTAMP, consistent with the allelic effect observed for this gene in lung tissue from the GTEx Portal. The product of the DCSTAMP gene is a multi-pass transmembrane protein that is considered to be a master regulator of osteoclastogenesis, as it is essential for the cell fusion that occurs between osteoclast precursors. Kukita et al. demonstrated that expression of the murine Destamp gene is upregulated in mouse osteoclast precursors when cultured in the presence of the osteoclastogenic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL). They also found that knockdown of Destamp inhibited the formation of multinucleated mouse osteoclast-like cells, while over-expression enhanced osteoclastogenesis in the presence of RANKL. Yagi et al. subsequently demonstrated that Destamp-knockout mice display an osteopetrotic phenotype due to an absence of functional multinucleated osteoclasts. In humans, osteoclast-like cells derived from PDB patients that are carriers for a rare non-syndromic
coding variant in the DCSTAMP gene (rs62620995) present with increased nuclei number compared to cells
derived from healthy controls. These cells also demonstrated increased expression of the DCSTAMP gene
compared to those obtained from PDB patients that do not carry the variant. In the study by Albabha et al.,
DCSTAMP was identified as the likely effector gene from the PDB genome-wide significant locus at 8q22.3. Our
data supports this by suggesting that the PDB GWAS variant rs2458413 is exerting an effect on PDB susceptibility
though regulatory effects on the DCSTAMP gene. Considering the established role of DCSTAMP in osteocla-
togenesis, it seems likely that increased expression of DCSTAMP leads to enhanced osteoclast fusion, thereby
contributing to the focal lesions of PDB.

There are some potential limitations to this study. One of these is that the osteoclast-like cells used in these
experiments were cultured and differentiated in vitro, and therefore potentially exhibit somewhat different gene
expression profiles to osteoclasts in vivo which are subject to the local bone microenvironment. Other potential
limitations include the difficulties associated with using cells derived from the circulatory system to study a
disease like PDB with a focal nature, as well as the fact that the eQTL effects identified in this study could be more
relevant to females due to the absence of males in the study cohort. It should also be noted that the research
materials in this study were not focused on individuals with PDB. However, considering the complex nature of the
genetics of PDB and the fact that these PDB GWAS variants have a common MAF in the general population, we
consider that our findings are relevant and representative of true contributing factors to both osteoclast biology
in general and also to this disease. In the case of DCSTAMP, it is possible that increased expression of this gene
has minimal effects in the general population, but in individuals at increased risk of PDB due to environmental
or other genetic factors the increased expression facilitates enhanced fusion of osteoclast precursors and subse-
quently formation of the abnormally large, overactive osteoclasts that are characteristic of the disease.

In conclusion, we have identified regulatory effects for the PDB GWAS variants rs4294134 (STMP1) and
rs2458413 (DTPYS and DCSTAMP) in human osteoclast-like cells. Our data do not support a role for rs4294134
in the regulation of NUP205 in this cell type, as has been proposed previously. The eQTL associations observed
for rs2494134 with STMP1, and rs2458413 with DCSTAMP were supported by publicly available eQTL data from
other tissue types. The STMP1 gene has not been extensively studied to date, however the DCSTAMP gene has
an established role in osteoclast differentiation and the associations seen between rs2458413 and PDB are likely
mediated through regulatory effects on this gene.

Methods

Subject recruitment. Recruitment of participants and laboratory procedures has been previously
described. In brief, 158 female participants aged 30–70 were recruited for the study. These individuals had been
referred for dual-energy X-ray absorptiometry (DXA) BMD scanning (Hologic, Bedford, MA, USA) at the Bone
Density Unit at Sir Charles Gairdner Hospital in Western Australia. Only females were recruited for the study to
control for gender-specific differences in osteoclast gene expression. Patients completed a medical history ques-
tionnaire, with a number of exclusion criteria applied including the presence of medical conditions or use of med-
ications likely the influence the ability of their cells to differentiate into osteoclasts or resorb bone. To minimise
potential issues with population stratification, only subjects with self-reported European ancestry were included
in the study. A blood sample was obtained from each patient, which consisted of two 6 ml lithium heparin and
4 ml EDTA blood tubes obtained from each patient were used for isolation of peripheral blood mononuclear cells (PBMCs)
by density gradient centrifugation as described previously using well-established protocols in our laboratory.
Triplicate cultures were grown for each sample in 24-well cell culture plates, with each well seeded with 1.5 × 10^6
cells suspended in complete α-MEM (minimum essential medium) supplemented with 25 ng/ml macrophage
colony stimulating factor (M-CSF). After 2 days, the culture medium was replaced with complete α-MEM sup-
plemented with 25 ng/ml M-CSF and 100 ng/ml RANKL. The cells were grown in this medium formulation for
another 12 days, during which time they underwent osteoclastogenesis. We have previously demonstrated that
osteoclast-like cells cultured using this protocol are capable of resorbing bone in an in vitro pit assay. Staining of
the differentiated cells for tartrate resistant acid phosphatase (TRAP) was performed as an additional character-
isation of the osteoclast phenotype.

Nucleic acid extraction. The QIAamp DNA Blood Mini Kit (QIAGEN) was used to extract genomic DNA
from 200 μl of each EDTA blood sample. Each set of triplicate osteoclast-like cell cultures was harvested at day 14
of culture and combined into a single aliquot before genomic DNA and RNA were extracted using the AllPrep
DNA/RNA Mini Kit (QIAGEN). An on-column DNAase digestion step was performed for the RNA fraction of
each sample to prevent DNA contamination of the RNA samples. Each RNA sample was subjected to quality
assessment using the Agilent 2100 Bioanalyzer, with all samples generating RNA integrity numbers (RINs) ≥9.7.

Genotyping and imputation. Each genomic DNA sample extracted from EDTA blood was subjected to
genotyping using the Illumina Infinium OmniExpress-24 BeadChip array. Pre-imputation QC criteria were
applied to the genotype data, including removal of individuals and variants with a call rate <90%, removal of var-
iants that were monomorphic, unmapped, had a MAF <5% or Hardy-Weinberg equilibrium P < 5 × 10^{-8}. After
these QC criteria had been applied, 572,898 variants remained in the genotype dataset. The Sanger Imputation
Service was then used to perform genotype imputation using the Haplotype Reference Consortium (HRC) release
1.1 reference panel. Post-imputation QC included removal of variants with an IMPUTE2 info score <0.4.
Relatedness testing and principal components analysis. Principal components analysis and identity-by-descent (relatedness) testing were performed on the genotype data using Plink v1.92. We generated 10 principal components for the study cohort, which were used to check for outliers and were included as covariates in the eQTL analysis to correct for any population stratification that may be present.

Gene expression analysis. Gene expression was quantitated in the RNA samples extracted from the osteoclast-like cells using 50 bp single-end RNA-Seq as described previously. Truncated mean of M-values (TMM) normalisation and correction of the data for total read count by conversion to counts per million (CPM) was performed using the edgeR package. This software was also used to calculate reads per kilobase million (RPKM) values to allow comparison of expression levels between different genes and with other tissues.

eQTL analysis. The TMM normalised CPM values were used for the eQTL analysis, which was performed using the FastQTL software. This software efficiently implements linear regression to identify associations between genotypes and gene expression phenotypes. A hypothesis-driven association analysis was performed for the quantile-normalised gene expression levels (generated using the rtrtransform function of the GenABEL package) with imputed genotype probabilities for the 7 PDB GWAS variants. The covariates RNA-Seq batch, patient age and 10 principal components were included in the eQTL analysis. All expressed genes with a TSS located within 500 kb of a PDB GWAS variant were included in the analysis (cis-eQTLs). Each locus was corrected for multiple testing using the Benjamini-Yekutieli procedure with a false discovery rate (FDR) of 5%.

Bioinformatics analysis. HaploReg v4.1 and RegulomeDB were used to perform bioinformatics analysis of individual eQTL-variants to identify other variants in strong LD and to characterise potential regulatory effects. Analysis of LD between eQTL variants within the same locus was performed using LDlink (1000G Phase 3 EUR population).

Data Availability The osteoclast eQTL data described in this study relevant to the STMP1, DPYS and DCSTAMP genes are available online at https://research-repository.uwa.edu.au/.

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Author Contributions

B.M., K.Z., J.W. and S.W. conceived and designed the study. B.M., K.Z. and S.M. performed the patient recruitment and cell culture. B.M., S.B. and F.D. performed the data analysis. B.M., K.Z., J.X., S.B., J.T., N.P., F.D., J.W. and S.W. contributed to the interpretation of the data. B.M. and S.W. drafted the manuscript. B.M., K.Z., J.X., S.B., S.M., J.T., N.P., F.D., J.W. and S.W. contributed to revising the content of the manuscript.

Additional Information

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