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Identification of novel regulators of osteoblast matrix mineralization by time series transcriptional profiling

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Short title: Microarray analysis of novel regulators of osteoblast mineralization

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Abstract

Bone mineralization is a carefully orchestrated process, regulated by a number of promoters and inhibitors that function to ensure effective hydroxyapatite formation. Here we sought to identify new regulators of this process through a time series microarray analysis of mineralising primary osteoblast cultures over a 27 day culture period. To our knowledge this the first microarray study investigating murine calvarial osteoblasts cultured under conditions that permit both physiological extracellular matrix mineralization through the formation of discrete nodules and the terminal differentiation of osteoblasts into osteocytes. RT-qPCR was used to validate and expand the microarray findings. We demonstrate the significant up-regulation of >13,000 genes during the osteoblast mineralization process, the highest-ranked differentially expressed genes of which were dominated by members of the PPAR- γ signalling pathway, namely *Adipoq*, *Cd36* and *Fabp4*. We also identify *Cilp*, *Phex*, *Trb3*, *Sox11*, *Psat1* and *Ptgs2* as novel regulators of matrix mineralization. Further studies examining the precise function of the identified genes and their interactions will advance our understanding of the mechanisms underpinning biomineralization.

Introduction

During endochondral bone formation, chondrocytes and osteoblasts mineralize their extracellular matrix (ECM) by promoting the initial formation of crystalline hydroxyapatite (HA) in the sheltered interior of membrane-limited matrix vesicles (MVs) [2]. This is followed by the modulation of matrix composition to further promote propagation of HA outside of the MVs [Anderson et al., 1990]. This biphasic mineralization process is critically dependent on a regulated balance of a number of factors which include calcium and inorganic phosphate (P_i), ECM proteins, and the presence of mineralization inhibitors such as inorganic pyrophosphate (PP_i), matrix gla protein, and osteopontin [Meyer, 1984; Sodek et al, 2000; Murshed et al, 2004; Johnson et al, 2000]. Three osteoblast molecules have been identified as affecting the controlled deposition of HA by regulating the extracellular levels of PP_i , and in turn, of osteopontin: tissue-nonspecific alkaline phosphatase (TNAP), ectonucleotide pyrophosphatase/phosphodiesterases-1 (NPP1) and the ankylosis protein (ANK) (Johnson et al, 2000; Johnson et al, 2003; Hessle et al, 2002; Harmey et al, 2004; Harmey et al, 2006).

TNAP is responsible for the generation of P_i from its substrate PP_i for HA crystallisation (Fallon et al., 1980) and the restriction of PP_i , to maintain a P_i/PP_i ratio permissive for normal bone mineralization (Harmey et al, 2004; Addison et al., 2007). NPP1 inhibits HA precipitation by its PP_i -generating properties within the ECM whereas ANK functions as a transmembrane PP_i -channeling protein, allowing PP_i molecules to pass through the plasma membrane from the cytoplasm to ECM ((Johnson et al., 1999; Numberg et al., 2001). The bone specific phosphatase PHOSPHO1 is also essential for the accumulation of P_i within MVs and bone mineralization.[13-18] Indeed, we have found that PHOSPHO1 and TNAP have non-redundant functions in regulating HA formation and mineralization; genetic ablation of either produces a hypomineralised, functionally-impaired skeleton and spontaneous fracture, and simultaneous ablation, a complete lack of all skeletal mineralisation (yadav et al; huesa et al)

Further mechanisms unrelated to the supply and hydrolysis of PP_i also exist to control matrix mineralization. Extracellular nucleotides adenosine triphosphate (ATP), and uridine triphosphate (UTP) signal via the $P2Y_2$ receptor to inhibit bone mineralization (Orriss et al, 2007), and that the ATP-sensitive $P2X_1$ and $P2X_7$ receptors also negatively regulate bone mineralization (Orriss et al, 2012). Recent progress has also identified roles for key non collagenous proteins in the regulation of HA crystal initiation and propagation, including bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1) and matrix extracellular phosphoglycoprotein (MEPE).[30] Furthermore, the formation of multicellular networks permeating the mineralized ECM by osteocytes may play pivotal mechano-modulatory roles in directing bone formation and mineralization (Burger and Klein-Nulend, 1999).

Despite these recent advances in our knowledge, the full mechanisms underpinning osteoblast matrix mineralization have yet to be fully elucidated. In the present study we have exploited a microarray approach to identify novel genes that are differentially expressed during the mineralization process. To our knowledge this the first microarray study investigating murine calvarial osteoblasts cultured under conditions that permit both physiological ECM mineralization through the formation of discrete nodules and terminal differentiation of osteoblasts into osteocytes. The identification of novel differentially expressed genes during the process of bone mineralization may open up new potential avenues for the therapeutic

treatment of clinical mineralization disorders including osteoporosis, osteopetrosis and vascular calcification.

Materials and methods

Ethics statement

All animal experiments were approved by The Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office (UK) guidelines for the care and use of laboratory animals.

Primary osteoblast isolation

Primary mouse calvarial osteoblasts were obtained from 3-day old C57Bl/6 mice by sequential enzyme digestion of excised calvarial bones using a four-step process [1 mg/ml collagenase type II in Hanks' balanced salt solution (HBSS) for 10 min; 1 mg/ml collagenase type II in HBSS for 30 min; 4 mM ethylenediaminetetraacetic acid (EDTA) for 10 min; 1 mg/ml collagenase type II in HBSS for 30 min]. The first digest was discarded and the cells subsequently obtained were resuspended in growth medium consisting of α -MEM (Invitrogen, Paisley, UK) supplemented with 10% FCS (Invitrogen) and 1% gentamycin (Invitrogen). Cells were cultured for 4 days in a humidified atmosphere (95% air/5% CO₂, 37°C) in T75 tissue culture flasks (Greiner Bio-One, GmbH, Frickenhausen, Baden-Württemberg, Germany) until confluent.

Primary osteoblast culture

Osteoblasts were seeded at a density of 1.5×10^4 cells/cm² in ???????. At confluency (day 0), growth medium was supplemented with 2.5mM β GP and 50 μ g/ml ascorbic acid for up to 28 days to induce matrix calcification. Incubation was at 37°C in a humidified atmosphere of 95% air/5% CO₂ and the medium was changed every second/third day. Previous studies have shown that during this 28 day culture period, murine calvarial osteoblastcultures display increasing ECM mineralization associated with elevated TNAP activity and the up-regulation of osteocyte markers, DMP1, E11 and sclerostin.[31, 32] This *in vitro* model mimics the full osteoblast differentiation cascade observed *in vivo* and is therefore appropriate for the identification of potential novel regulators of matrix mineralization.

cDNA microarray hybridisation

For microarray analysis, RNA was extracted from cells at days 0, 9 and 27 of culture using RNeasy mini kit (Qiagen Ltd, Crawley, West Sussex, UK), according to the manufacturer's instructions. For each sample, total RNA content was assessed by absorbance at 260 nm and purity by A260/A280 ratios. The quality of each sample was considered suitable by a ratio of greater than 1.9. Samples were sent to Ark Genomics (The Roslin Institute, University of Edinburgh, UK) where target preparation and hybridisation to the Affymetrix Murine 430 2.0 GeneChip was carried out following the standard Ark Genomics protocols (<http://www.ark-genomics.org/protocols>). This GeneChip contains ~39,000 full-length mouse genes and EST clusters from the UniGene database. The data obtained from these hybridisations can be viewed on the EMBL-EBI Array Express repository ([http://www.ebi.ac.uk/microarray-as/aer/?#ae-main\[0\]](http://www.ebi.ac.uk/microarray-as/aer/?#ae-main[0])) (accession no. [E-MEXP-1244](http://www.ebi.ac.uk/microarray-as/aer/?#ae-main[0])).

Microarray data analysis

Normalisation across all arrays was achieved using the robust multi-array average (RMA) expression measure which results in expression measures (summarised intensities) in log base 2.[34] Comparisons were undertaken using linear modelling. Subsequently, empirical Bayesian analysis was applied (including vertical (within a given comparison) p value adjustment for multiple testing, which controls for false discovery rate) for which the Bioconductor package limma was used [33]. An overview of the underlying biological changes occurring within each comparison can be obtained by functional enrichment analysis. This was performed from two perspectives, namely KEGG pathway membership and Gene Ontology (GO) terms. The level of statistical significance for functional analysis was chosen to be the most stringent level at which 1% of the array features were, on average, significant. For the current dataset, adjusted $p < 0.0001$ was appropriate for the comparisons performed. Significant genes (adjusted $p < 0.0001$) from each comparison were analysed for enrichment of KEGG pathways and GO terms using separate hypergeometric tests. Enrichment ($p < 0.001$) was assessed for up-regulated and down-regulated pathways and genes separately.

Detection of mineralization

Calcium deposition was evaluated by alizarin red staining (Zhu et al, 2011 Staines et al, 2012). Cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 5 min at 4°C, stained with 2% alizarin red (pH 4.2) for 10 min at room temperature and rinsed with distilled water.

Analysis of gene expression using quantitative RT-PCR

Quantitative PCR (qRT-PCR) was used to confirm changes in the expression of selected genes. RNA was extracted at days 0, 7, 14, 21 and 28 of culture using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. For each sample, total RNA content was assessed by absorbance at 260nm and purity by A260/A280 ratios. RNA was reverse transcribed and the PCR reaction undertaken as described previously.[35, zhu et al 2011; macrae et al 2009] All genes were analysed with the SYBR green detection method using the Stratagene Mx3000P real-time qPCR system (Stratagene, CA, USA). Each PCR was run in triplicate. All gene expression data were normalised against *Gapdh* and the control values expressed as 1 to indicate a precise fold change value for each gene of interest. All primers were purchased from Qiagen (sequences unknown), with the exception of *Gapdh* (sequence unknown) and *Phex* (forward: CTAACCACCCACTCCCACTT, reverse: CCAATAGACTCCAAACCTGAAGA) which were purchased from Primer Design (Southampton, UK).

Results

Total RNA isolated from primary osteoblasts at 0, 9 and 27 days of culture was hybridised to Affymetrix Murine 430 2.0 Array. Analysis in Bioconductor limma software demonstrated that following normalisation, 13,812 genes were up-regulated and 17,403 genes down-regulated following 9 days of culture ($n=4$, >1.5 fold change). 14,834 genes were up-regulated and 14,072 down-regulated at day 27 of culture, in comparison to day 0 ($n=4$, >1.5 fold change). The top twenty up-regulated and down-regulated genes at day 9 and day 27 are listed in Table 1 and Table 2 respectively.

Transcripts significantly up-regulated during mineralization at both day 9 and day 27 compared to day 0 included key members of the peroxisome proliferator-activated receptor gamma (PPAR- γ) signaling pathway, namely adiponectin (*Adipoq*), cluster of differentiation 36 (*Cd36*), and fatty acid binding protein 4 (*Fabp4*). Cartilage intermediate layer protein (*Cilp*) and phosphate-regulating neutral endopeptidase (*Phex*) were also markedly up-regulated (Table 1). Conversely, transcripts significantly down-regulated included Tribbles 3 (*Trib3*), phosphoserine aminotransferase 1 (*Psat1*) and sex determining region Y-box (*Sox11*) (Table 2). Functional enrichment analysis identified the significant up-regulation of the PPAR- γ signalling pathway (Figure 1).

To confirm and extend these data, further gene expression studies were undertaken in calvarial osteoblasts cultured for 28 days under mineralising conditions, and sampled at regular intervals. Calvarial osteoblasts at confluency had negligible amounts of alizarin red staining (calcium deposition) (Fig. 2a). Further culture in mineralising conditions for an additional 7, 14, 21 and 28 d resulted in the formation of discrete nodules, as detected by increased alizarin red staining. In addition, significant increases in mRNA expression of key genes associated with osteoblast mineralization, tissue non-specific alkaline phosphatase (*Alpl*) (X fold change; P<0.001) and osteocalcin (X fold change; P<0.001), were observed at day 7, with increased expression maintained throughout the culture period (Fig. 2b and c respectively). These data confirm the formation of calcified matrix in calvarial osteoblasts over the 28-day time course examined.

To validate the microarray expression data, the eight mineralization-responsive genes listed above were selected for analysis of gene expression by qRT-PCR. Analysis at day 7 revealed significant increases in the mRNA expression of *Adipoq* (x fold change; P<0.001), *Cd36* (x fold change; P<0.001), *Fabp4* (x fold change; P<0.001), *Cilp* (X fold change; P<0.001) and *Phex* (x fold change; P<0.001). Increased expression of these genes was maintained throughout the culture period (Fig. 3). The induction of these genes therefore paralleled the expression patterns obtained by microarray analysis. Similarly, a significant down-regulation of *Trib3* (x fold change; P<0.001), *Psat1* (x fold change; P<0.001) and *Sox11* (x fold change; P<0.001) was detected by qRT-PCR analysis at day 7. Reduced expression of these genes was also observed throughout the culture period (Fig. 4). Together our data have identified eight genes differentially expressed during the process of calvarial osteoblast mineralization under conditions that permit both physiological ECM mineralization and terminal differentiation into osteocytes.

Discussion

Whilst numerous *in vivo* and *in vitro* studies have examined the mechanisms of osteoblast differentiation and mineralization, few have incorporated the transition from osteoblasts to osteocytes. Osteocytes are non-proliferative, terminally differentiated cells in the osteoblast lineage, and the most abundant cell type in bone. Recent studies have indicated that osteocytes function as a mechanosensor in the bone, coordinate bone homeostasis, and secrete phosphate regulating molecules (Bonewald, 2011). To our knowledge this is the first microarray study to investigate murine calvarial osteoblasts that terminally differentiate into osteocytes.

β GP is an exogenous phosphate source which acts as a substrate for ALP. Therefore cells treated with β GP dictate when the P_i is released through their differentiation and expression of ALP. Previous studies have demonstrated that widespread dystrophic deposition of mineral occurs in calvarial osteoblasts cultured with high concentrations (10mM) of β GP (Orriss et al,

2007). Therefore in the present study, murine calvarial osteoblasts were cultured with 2.5mM β GP, and reproducibly formed abundant, discrete nodules with characteristic trabecular morphology; alizarin red staining revealed that mineralization was exclusively confined to defined regions of these matrix structures. This is the first study to undertake a microarray analysis of mineralising murine calvarial osteoblasts treated with physiological levels of β GP.

Despite recent advances in our knowledge, the full mechanisms underpinning osteoblast matrix mineralization have yet to be fully elucidated. This study has clearly demonstrated differential expression of novel genes in mineralising primary calvarial osteoblast cultures. Furthermore, many of these genes have not been reported in previous osteoblast mineralization microarray studies (Doi et al., 2001). These marked differences are likely to be directly attributable to the culture conditions in the present study that enabled the physiological mineralization of the ECM and the terminal differentiation of osteoblasts into osteocytes *in vitro*.

Microarray analysis, functional enrichment and qRT-PCR validation studies together identified the up-regulation of *Adipoq*, *Cd36* and *Fabp4* during *in vitro* osteoblast mineralization, key genes involved in the Peroxisome Proliferator-Activated Receptor Gamma (PPAR- γ) signalling pathway and adipogenesis. *Cd36*, also known as fatty acid translocase (FAT), is a long chain fatty acid transporter which is present at the plasma membrane and activates the PPARs [36]. *Fabp4* and *Adipoq* act downstream of PPAR- γ , and have both previously been shown to be involved in adipocyte differentiation (Fig. 3). *Fabp4* is a member of the FABP family of which nine isoforms have been identified which have been localised to mature adipocytes and adipose tissue.[47] Similarly, *Adipoq* is strongly expressed by adipocytes and is known to stimulate the differentiation of osteoblasts through the induction of prostaglandin-endoperoxide synthase 2 and bone morphogenetic protein 2 (BMP2) in mesenchymal cells.[48]

Previous microarray analysis of mesenchymal lineage markers in mouse calvaria cell cultures have indicated that adipocyte-associated genes are transcriptionally induced together with osteoblast-associated genes during osteoblast development (Garcia et al., 2002). Similarly, data has shown that adipocytes emerge along with osteogenic potential in a fraction of fetal rat calvaria cells treated with BRL-49653, a synthetic ligand for PPAR γ (Hasegawa et al., 2008). The demonstration that a subset of osteoblasts maintain capacity for adipogenic fate selection, even at relatively mature developmental stages implies an unexpected plasticity with important implications in normal and pathological bone development (Yoshiko et al., 2010). The molecular basis by which this subset of osteogenic cells acquires high endogenous expression of adipogenic transcription factors, and the functional significance of this acquisition, remains to be determined.

This study has also identified the up-regulation of *Phex* during *in vitro* osteoblast mineralization. *Phex* gene homology with members of the M13 membrane-bound zinc metalloendopeptidase family. It has been proposed that a mutation in the human *PHEX* gene is directly responsible for the phenotypic changes in patients with X-linked hypophosphatemia (Holm et al., 1997). Investigation of murine tissues and cell cultures has revealed that *Phex* is predominantly expressed in bones and teeth (Du et al, 1996; Ruchon et al., 1998). Within bone, *Phex* expression is limited to cells of the osteoblast lineage - osteoblasts, and osteocytes (Ruchon et al, 1998; Thompson et al., 2002).

PHEX has not been reported to have any direct effects on mineralization, however it is able to rescue the inhibitory effects of the MEPE-ASARM peptide by preventing its release and by proteolytically cleaving the ASARM peptide deeming it inactive.[35, 50, 51] Furthermore, it is well established that the increased expression of ASARM peptides are responsible for the mineralization defect observed in the *Hyp* mouse model, a model for XLH caused by an inactivating mutation in *Phex*. [52] The increased expression of *Phex* observed here is therefore indicative of the emerging evidence for a critical role of the ASARM peptides in matrix mineralization.

Our data also demonstrate an increased expression of *Cilp* during osteoblast mineralization. CILP is a key ECM component, expressed predominantly in the articular cartilage.[53] Studies in chondrocytes have demonstrated that both transforming growth factor beta (TGF β) and insulin-like growth factor 1 (IGF1) induce *Cilp* expression [56]. Whilst *Cilp* has been implicated in numerous diseases of cartilage, to our knowledge this the first report of CILP expression within osteoblasts. This data, together with previous studies showing that CILP may generate extracellular PP_i, [56], highlight the requirement for further investigations into the role of CILP in controlling bone mineralization, potentially through modulation of P_i/PP_i balance.

The results presented here have also identified the down-regulation of *Sox11* during osteoblast differentiation and matrix mineralization. *Sox11* is a transcription factor which is expressed during embryonic development and tissue remodelling.[57] Whilst this is the first report of altered *Sox11* expression in osteoblasts, previous microarray studies have shown that *Sox11* is differentially expressed during the initiation of chondrogenesis (Cameron et al, 2009). Additionally, *Sox11* null mice display craniofacial and skeletal malformations, and delayed endochondral ossification.[58] Further studies are therefore required to elucidate the mechanisms through which *Sox11* modulates chondrocyte and osteoblast dynamics.

The *Psat1* gene codes for a phosphoserine aminotransferase which is involved in cell proliferation *in vitro* [59]. To our knowledge, this is the first demonstration of reduced expression of *Psat1* during osteoblast ECM mineralization. However, a recent study analysing the genes involved in mineralization during differentiation of human periodontal ligament stem cells, also noted a significant down-regulation of *Psat1* expression (Choi et al, 2011). Together these data suggest a key role for *Psat1* in regulating biomineralization across a range of tissue types.

The present study also provides evidence for the down-regulation of *Trib3*. In fact this was the most highly ranked down-regulated gene following both 9 and 27 days of culture. This homolog of the Tribbles family plays a key role in the regulation of nuclear factor of activated B cells (NF-kappaB) activation, which is known to inhibit osteoblastic bone formation.[60] Furthermore, TRIB3 stabilises the expression of SMAD, the major BMP signalling transcription factor, through its dissociation from the BMP II receptor and its promotion of the subsequent degradation of Smad ubiquitin regulatory factor 1. Therefore through this mechanism, TRIB3 has the ability to promote osteogenic differentiation and mineralization.[61] In addition, the BMP signalling pathway has been shown in numerous studies to interact with the Wnt signalling pathway to potentiate its effects on osteoblast differentiation.[62] Sclerostin, a marker of mature deeply embedded osteocytes exerts negative effects of on osteoblastogenesis and bone formation through activation of the Wnt signalling pathway and BMP antagonism [63]. It is therefore plausible that the decreased

expression of *Trib3* observed in the present study reflects the terminal differentiation of the osteoblast into the osteocyte.[64]

In conclusion, this study exemplifies that the use of array technologies represents a powerful method for the discovery of genes involved in bone mineralization. These results will enable future investigations into the precise mechanisms underpinning osteogenic differentiation. Furthermore, our data will inform future investigations into the molecular mechanisms surrounding diseases of biomineralization such as osteomalacia, vascular calcification and osteoporosis.

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