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The functional co-operativity of tissue-nonspecific alkaline phosphatase (TNAP) and PHOSPHO1 during initiation of skeletal mineralization.

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ABSTRACT

Phosphatases are recognized to have important functions in the initiation of skeletal mineralization. Tissue-nonspecific alkaline phosphatase (TNAP) and PHOSPHO1 are indispensable for bone and cartilage mineralization but their functional relationship in the mineralization process remains unclear. In this study, we have used osteoblast and *ex-vivo* metatarsal cultures to obtain biochemical evidence for co-operativity and cross-talk between PHOSPHO1 and TNAP in the initiation of mineralization. Clones 14 and 24 of the MC3T3-E1 cell line were used in the initial studies. Clone 14 cells expressed high levels of PHOSPHO1 and low levels of TNAP and in the presence of β -glycerol phosphate (β GP) or phosphocholine (P-Cho) as substrates and they mineralized their matrix strongly. In contrast clone 24 cells expressed high levels of TNAP and low levels of PHOSPHO1 and mineralized their matrix poorly. Lentiviral *Phospho1* overexpression in clone 24 cells resulted in higher PHOSPHO1 and TNAP protein expression and increased levels of matrix mineralization. To uncouple the roles of PHOSPHO1 and TNAP in promoting matrix mineralization we used PHOSPHO1 (MLS-0263839) and TNAP (MLS-0038949) specific inhibitors, which individually reduced mineralization levels of *Phospho1* overexpressing C24 cells, whereas the simultaneous addition of both inhibitors essentially abolished matrix mineralization (85%; $P < 0.001$). Using metatarsals from E15 mice as a physiological *ex vivo* model of mineralization, the response to both TNAP and PHOSPHO1 inhibitors appeared to be substrate dependent. Nevertheless, in the presence of β GP, mineralization was reduced by the TNAP inhibitor alone and almost completely eliminated by the co-incubation of both inhibitors. These data suggest critical non-redundant roles for PHOSPHO1 and TNAP during the initiation of osteoblast and chondrocyte mineralization.

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1. Introduction

Tissue-nonspecific alkaline phosphatase (TNAP) is the isozyme of the alkaline phosphatase family whose activity is linked to the promotion of matrix mineralization in bone and cartilage [1]. Indeed, both a P_i -generating function, as well as the ability to hydrolyze a mineralization inhibitor were proposed for TNAP since the discovery of this enzyme in bone [2]. A primary inhibitor of ECM mineralization is extracellular inorganic pyrophosphate (PP_i)

[3], produced ectoplasmically by the enzymatic action of nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) on extracellular ATP [4]. Intracellularly produced PP_i is also transported to the extracellular milieu by the channeling function of the ankylosis protein (ANK) [5]. TNAP activity is crucial for restricting the concentration of extracellular PP_i to maintain a P_i/PP_i ratio permissive for normal bone mineralization [6,7]. Indeed, lack of TNAP function (*Alpl*^{-/-}) leads to the soft bones condition known as hypophosphatasia (HPP), caused by accumulation of extracellular PP_i that blocks the propagation of HA onto the ECM [8,9]. However, chondrocyte- and osteoblast-derived matrix vesicles (MV) derived from both HPP patients and *Alpl*^{-/-} mice retain the ability to initiate intravesicular mineral formation and contain HA crystals [10,11] demonstrating that TNAP is not essential for the initiation of MV mediated ECM mineralization.

Instead, PHOSPHO1, a member of the haloacid dehalogenase superfamily [12,13] highly expressed in mineralizing cartilage,

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bone and dentin [14–16], appears to be involved in the initiation of MV-mediated mineralization. PHOSPHO1 shows high phosphohydrolase activity towards phosphoethanolamine (P-Etn) and phosphocholine (P-Cho) and is active inside chondrocyte- and osteoblast derived MVs where it may have a role scavenging P_i from MV membrane phospholipids to favor intra-vesicular HA deposition [17,18]. Small molecule compounds that inhibit PHOSPHO1 activity in *Alpl*^{-/-} MVs cause a significant decrease in MV-mediated calcification *in vitro* [15] and the absence of PHOSPHO1 results in a lower accumulation of mineral, which leads to a more deformable bone [19]. Therefore, *Phospho1*^{-/-} mice show skeletal abnormalities that include decreased bone mineral density, spontaneous fractures, osteomalacia and scoliosis. However, lack of PHOSPHO1 does not prevent the intravesicular deposition of mineral, although the double ablation of *Phospho1* and *Alpl* lead to complete lack of skeletal mineralization [20]. Here, we have used osteoblast and *ex-vivo* metatarsal cultures to obtain biochemical evidence for co-operativity and cross-talk between PHOSPHO1 and TNAP in the initiation of mineralization.

2. Methods

2.1. Animals

Phospho1-R74X null mutant (*Phospho1*^{-/-}) mice were generated by N-ethyl-N-nitrosourea mutagenesis in a C3HeB/FeJ (Stock No. 000658, Jackson Laboratories, Bar Harbor, ME, USA) background, then bred to C57BL/6 mice to segregate other possible undesired mutations [20]. All animal experiments were approved by 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.

2.2. Cell culture

Previously characterized clones 14 and 24 of the MC3T3-E1 cell line [21]; a generous gift of Prof Renny Franceschi, Michigan, USA) were cultured in maintenance medium (α -MEM (Invitrogen, Paisley, UK) containing 10% (v/v) fetal bovine serum (FBS, (Invitrogen) and 0.05 mg/ml gentamycin (Invitrogen)) at 37 °C in 5% CO₂.

2.3. Lentiviral vectors and cell infection

Mouse *Phospho1* sequence was amplified from mouse primary osteoblast cDNA adding a FLAG tag sequence to the 5' end and cloned into a commercially available pLVX vector (Clontech Mountain View, CA, USA). An empty vector was used as control. For lentivirus packaging, a T25 tissue culture flask was seeded with 1.6×10^6 HEK293T cells in 6mls medium (DMEM, 10% FBS, 1% NEAA; Invitrogen), incubated for 24 h and transfected when 70–90% confluent. The transfection mix was set up in 145 μ l Opti-Mem (Invitrogen) containing 2 μ g psPAX2, 1 μ g of VSV-G and 1.5 μ g of the desired pLVX plasmid and 17 μ l of Fugene HD (Roche, East Sussex, UK). The transfection mix was incubated for 15 min at room temperature prior to adding to the cells. The transfected cells were incubated at 37 °C in 5% CO₂ overnight and the medium was collected 24 and 48 h post transfection to concentrate and titrate the virus. MC3T3-E1 clones were plated at 2×10^5 cells per T25 flask and transduced the next day with the desired lentivirus at 2 virus particles per cell plated. Selection was done by antibiotic selection with puromycin (Invitrogen) at a final concentration 2 μ g/ml.

2.4. Expression and preparation of test enzymes

Recombinant human PHOSPHO1 and TNAP protein was produced and purified as previously described [17]. Enzyme reactions were initiated by the addition of P-Cho and allowed to proceed for 60 min at room temperature and pH 7.3 as previously described [22].

2.5. Mineralization cultures

Cells were plated at 2.5×10^4 cells per well in 12 well plates and cultured in maintenance medium for two days before changing to mineralization medium (maintenance medium + 50 μ g/ml ascorbic acid and 5 mM β -glycerol phosphate (β GP; Sigma) or 3 mM P-Cho (Sigma) as phosphate donors. PHOSPHO1 and TNAP inhibitors, MLS-0263839 and MLS-0038949 (both 30 μ M) were added where indicated. This concentration was derived from our previous dose response experiments and showed maximum enzyme inhibition and no cellular toxicity [22]. Medium was changed every 3–4 days for up to 21 days. Cells were fixed for alizarin red staining or lysed for protein or mRNA extraction.

2.6. Alizarin red staining for matrix mineralization

Cells were fixed with 4% paraformaldehyde for 30 min at RT, prior to staining with 2% alizarin red, pH 4.2. After image capture, the cells were destained with 10% cetylpyridium chloride and the optical density determined at 570 nm [23].

2.7. Western Blotting

Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH. 8.0, 135 mM NaCl, 10% glycerol, 1% IGEPAL, 0.1% SDS, 0.5% Na deoxycholate, 2 mM EDTA; Invitrogen) containing “complete” protease inhibitor cocktail according to manufacturer's instructions (Roche) and protein concentration determined using the standard DC assay (Bio-Rad, Hemel Hempstead, UK). Proteins (8 μ g) were run in a 10% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Nebraska, USA) for 1 h and probed overnight at 4 °C with anti-PHOSPHO1 (recombinant Fab, AbD Serotec, Martinsried/Planegg, Germany) anti-TNAP (R&D, Abingdon, UK) and anti β -actin (Cell signaling technology, Hitchin, UK) antibodies diluted 1:1000, 1:500 and 1:1000 respectively in Odyssey blocking buffer. After washing in PBS the membranes were incubated with goat anti-Human (800CW), goat anti-Rat (800CW) and goat anti-Rabbit (680 RD) for 50 min (LI-COR Biosciences, 1:1250 dilution in Odyssey blocking buffer). The resulting blots were subsequently washed in PBS and visualized using the LI-COR Odyssey infrared scanner and software (LI-COR biosciences) with a scan resolution of 169 μ m.

2.8. Analysis of gene expression using quantitative RT-PCR

RNA was extracted from cells using RNeasy total RNA (Qiagen Ltd., Crawley, 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 by NanoDrop (Fischer Scientific, Loughborough, UK). RNA was reverse transcribed and the PCR reaction undertaken as described previously [24]. All genes were analyzed with the SYBR green detection method using the Stratagene Mx3000P real-time QPCR system (Stratagene, CA, USA). Each sample was assayed in triplicate. All gene expression data were normalized against the house keeping gene, *Atp5b* (Primer Design, Southampton, UK). The control values are expressed as 1 to indicate a precise fold change value for each gene

of interest. Primers for *Phospho1* (Forward; TTCTCATTTCCGGATGC-CAACA, Reverse; TGAGGATGCCGGCGGAATAA) and *Alpl* (Forward 5' GGG ACG AAT CTC AGG GTA CA3', Reverse 5' AGT AAC TGG GGT CTC TCT CTT T3'). Data were analysed by the delta delta Ct method.

2.9. Metatarsal culture

Embryonic metatarsal organ cultures provide a well-established *ex vivo* model of endochondral bone growth [25,26]. In addition, 15-day-old fetal metatarsal bones (E15) are comprised of early proliferating chondrocytes with no evidence of a mineralized core and are therefore an informative physiological model to investigate the effects of TNAP and PHOSPHO1 on the initiation of bone mineralization [23,27]. Metatarsals from E15 WT and *Phospho1*^{-/-} mice were cultured as previously described [23] and PHOSPHO1 (30 μ M) and TNAP (30 μ M) inhibitors were added where indicated. Metatarsals were maintained in medium for at least 7 days (no medium change) and the length of the mineralization zone and of the whole bone was measured as previously described [23]. Bone mineralization status of each culture was assessed by calculating the ratio of the length of the mineralized zone over the total length of the bone.

2.10. Statistics

SigmaStat v 11.0 was used for statistical analysis. Data (mean \pm SEM) comparison was conducted by regular t-tests or ANOVA unless the data was not normally distributed, in which case a suitable non-parametric test was selected.

3. Results

3.1. Effect of substrate choice on *in vitro* osteoblast ECM mineralization

Although β GP can be hydrolyzed by PHOSPHO1 to release Pi it has been previously reported to be a poor substrate for PHOSPHO1 in comparison to TNAP [17,22]. We therefore first determined the potential of P-Cho and β GP as phosphate donors for ECM mineralization of MC3T3-E1 clones 14 and 24. These clones were chosen because of their reported differences in TNAP expression and mineralization ability [21]. The supplementation of clone 14 cultures by β GP or P-Cho promoted mineralization from day 7 onwards (Fig. 1A and B). It is possible that during the process of mineralization and the destruction of MVs, PHOSPHO1 is released into the extracellular milieu and is able to generate Pi from the added substrates. NPP1 can also act as an efficient phosphatase in the absence of TNAP and this may be another potential source of Pi for mineral formation [28]. Matrix mineralization of clone 24 cells in

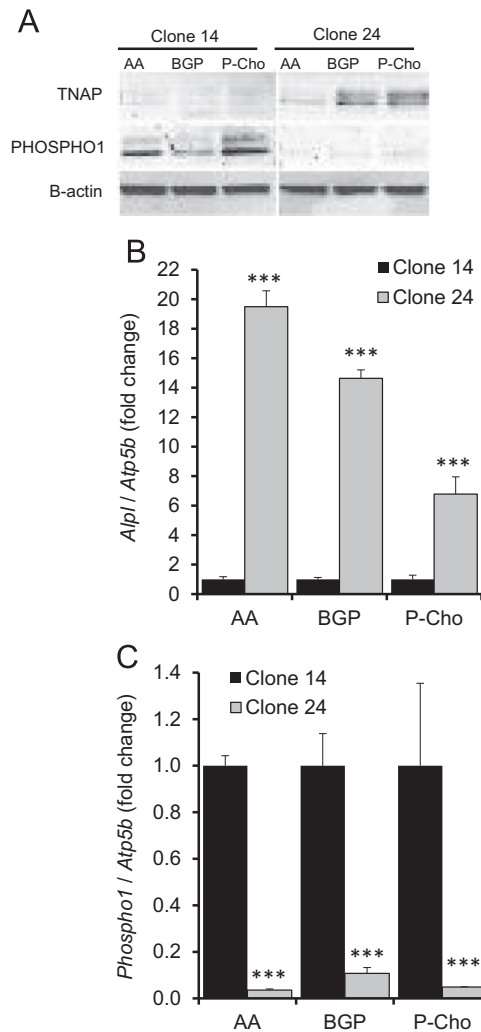


Fig. 2. TNAP and PHOSPHO1 expression in MC3T3 clones 14 and 24. MC3T3s were cultured in the presence of 50 μ g/ml Ascorbic Acid (AA) with 5 mM β GP or 3 mM P-Cho as substrates and analysed for (A) TNAP and PHOSPHO1 protein expression (B) *Alpl* gene expression and (C) and *Phospho1* gene expression. $n=3$. *** $P < 0.001$.

the presence of β GP also was noted from day 7 onwards but, as expected, was less than their clone 14 counterparts. Mineralization in the presence of P-Cho was also less and its initiation was delayed till day 14 of culture (Fig. 1A and B). Whilst clone 24 mineralizes its matrix poorly it had greater TNAP expression than the more highly mineralizing clone 14 (Fig. 2A and B). In contrast, PHOSPHO1 expression by clone 14 was higher than that of clone 24 at both the gene and protein level (Fig. 2A and C). These data

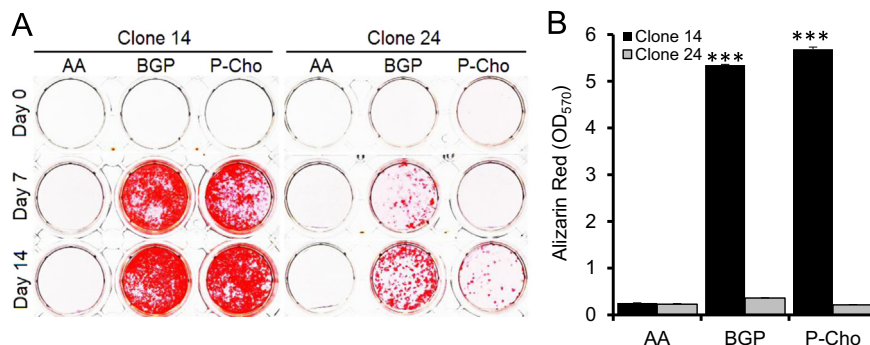


Fig. 1. *In vitro* substrate mineralization assay on MC3T3 clones 14 and 24. MC3T3s were cultured in the presence of 50 μ g/ml Ascorbic Acid (AA) with 5 mM β GP or 3 mM P-Cho as substrates and (A) stained with alizarin red and (B) quantified after leaching. $n=3$. *** $P < 0.001$.

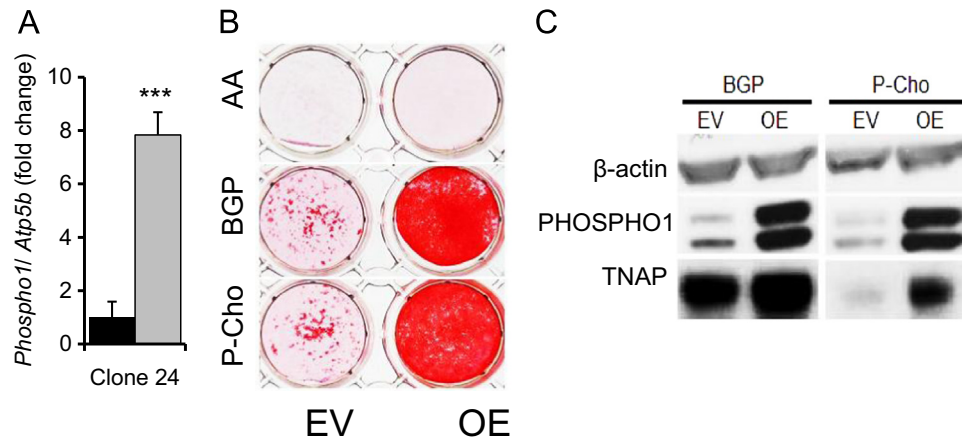


Fig. 3. Overexpression of *Phospho1* in MC3T3 clone 24 cells. (A) *Phospho1* expression levels in cells transfected with empty vector (black bars) or *Phospho1* overexpression vector (gray bars). (B) Mineralization after 14 days in cells transfected with empty vector (EV) or *Phospho1* overexpression vector (OE) and cultured with 50 $\mu\text{g/ml}$ of AA and 5 mM βGP or 3 mM P-Cho. (C) Western blotting of TNAP and PHOSPHO1 protein expression in EV or OE cells cultured with 50 $\mu\text{g/ml}$ of AA and 5 mM βGP or 3 mM P-Cho. $n=3$. *** $P < 0.001$.

revealed that P-Cho, which can be cleaved by both TNAP and PHOSPHO1 (Supplemental Fig. 1), was a suitable phosphate substrate for the promotion of ECM mineralization and that poor ECM mineralization of MC3T3-E1 clone 24 cells was associated with very low PHOSPHO1 expression.

3.2. *Phospho1* overexpression induces ECM mineralization

To directly assess the co-operativity and cross-talk between PHOSPHO1 and TNAP expression in ECM mineralization we overexpressed *Phospho1* in clone 24 cells which had low *Phospho1* expression and mineralized their ECM poorly. This resulted in an 8-fold increase in *Phospho1* expression in cells maintained under non-mineralizing conditions (Fig. 3A). When cultured in the presence of βGP or P-Cho, *Phospho1* overexpression resulted in higher PHOSPHO1 and TNAP protein expression and increased levels of matrix mineralization (Fig. 3B and C). The increased TNAP expression by βGP (Fig. 3C) has been noted previously [29]. Despite increased TNAP expression it was, in the absence of higher

PHOSPHO1 expression, unable to promote matrix mineralization possibly implicating the necessity for PHOSPHO1 in mineral initiation.

3.3. Joint PHOSPHO1 and TNAP activity is required for MC3T3 matrix mineralization

TNAP (MLS-0038949) and PHOSPHO1 (MLS-0263839) inhibitors were tested on *Phospho1* overexpressing clone 24 cells in the presence of 3 mM P-Cho to avoid interference from βGP induced TNAP expression (Fig. 3C). At 30 μM no cell toxicity was observed and this is in agreement with our previous reports [22]. *Phospho1* overexpression induced a 2.8 fold increase ($P < 0.01$) in alizarin red staining, which was reduced (72%; $P < 0.01$) to control (empty vector transduced) levels after treatment with MLS-0263839. The PHOSPHO1 inhibitor did not however alter ECM mineralization of control cells possibly reflecting their low level of endogenous PHOSPHO1 expression (Fig. 4A). The TNAP inhibitor also reduced matrix mineralization of both control (75%; $P < 0.05$)

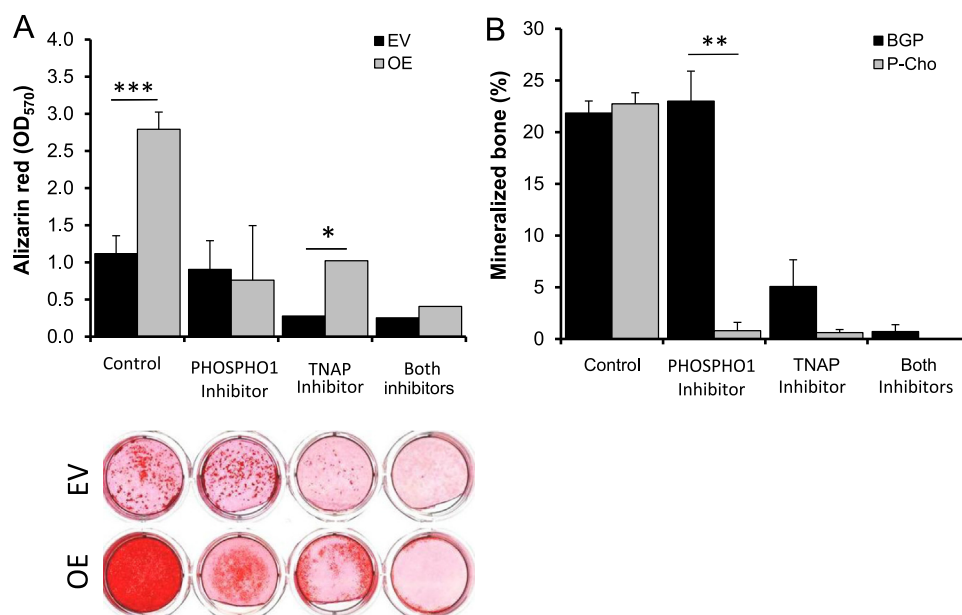


Fig. 4. Inhibition of osteoblast and metatarsal matrix mineralization. (A) Clone 14 cells and (B) metatarsals show reduced mineralization in the presence of PHOSPHO1 and TNAP inhibitors alone and in combination. $n=4$. * $P < 0.05$, *** $P < 0.001$.

and *Phospho1* overexpressing (63%; $P < 0.01$) cells, possibly reflecting the inhibition of both basal (control) and *Phospho1*-induced TNAP activity. The simultaneous addition of both inhibitors essentially abolished ECM mineralization in *Phospho1* overexpressing cells (85%; $P < 0.001$) (Fig. 4A).

3.4. Joint PHOSPHO1 and TNAP activity is required for metatarsal mineralization

Next we used metatarsals from WT E15 mice, in the presence or absence of MLS-0038949 and MLS-0263839 (Fig. 4B), to probe the co-operativity of PHOSPHO1 and TNAP in a physiological *ex vivo* model of mineralization. The inhibitors did not affect metatarsal linear growth indicating a lack of toxicity at the concentrations used (data not shown). Mineralization of metatarsals cultured in the presence of β GP was unaffected by the addition of the PHOSPHO1 inhibitors whereas TNAP inhibitors reduced mineralization by 73% ($P < 0.001$). Conversely, mineralization was almost completely inhibited by either inhibitor when P-Cho was the substrate. Also, in the presence of both inhibitors when using β GP as substrate, mineralization is almost completely eliminated (Fig. 4B). These experiments were repeated 4 times and similar results were obtained in all occasions.

4. Discussion

PHOSPHO1 is now recognized to be essential for the initiation of skeletal mineralization. Its genetic ablation in mice results in osteoidosis and decreased BMD which are likely to explain the observed spontaneous fractures, bowed long bones, osteomalacia and scoliosis in early life [19,20]. PHOSPHO1 is present and active inside chondrocyte- and osteoblast-derived MVs through its ability to scavenge P_i from MV membrane phospholipids [17]. Furthermore, whilst small molecule compounds that inhibit PHOSPHO1 activity decreased the ability of chick chondrocyte micro-mass cultures and isolated MVs to mineralize this was to a lesser extent than similarly treated MVs extracted from cultured *Alpl*^{-/-} osteoblasts [15,30]. This is a revealing observation and suggests that TNAP's P_i generating ability can at least, in part, compensate for the lack of PHOSPHO1 function in driving the mineralization process. Moreover, the complete ablation of PHOSPHO1 function only leads to a decrease in the calcification ability of MVs and not to a complete lack of calcification [20]. Conversely, chondrocyte- and osteoblast-derived MVs in hypophosphatasia patients and *Alpl*^{-/-} mice retain the ability to initiate intra-vesicular mineral formation and contain HA crystals and this, we speculate, is through the actions of PHOSPHO1 [11,31]. Both scenarios are consistent with our recent observations in mice with a double ablation of PHOSPHO1 and TNAP function where there is complete lack of MV and skeletal mineralization due to a complete lack of P_i generation from intra- and extra-vesicular sources [20]. This current biochemical study was designed to extend these observations and examine the functional interplay of both phosphatases in the regulation of ECM mineralization.

We first exploited MC3T3-E1 clones 14 and 24 because of their reported differences in TNAP expression and mineralization ability [21]. In this original report mineralizing clone 14 did not express *Alpl* but was able to induce *in vivo* osteogenesis whereas the poorly-mineralizing clone 24 cells expressed *Alpl* but failed to promote *in vivo* osteogenesis [21]. A possible explanation for the differing mineralization abilities of the clones are the differences in matrix gene expression between the two clones. Previous reports have shown that clone 14 cells express high levels of bone sialoprotein (BSP), osteocalcin and Runx2 compared with clone 24 cells [21]. BSP shows a tempo-spatial pattern of expression that

closely parallels early mineral formation and is a potent nucleator of HA crystal formation under conditions where osteopontin is inactive or inhibitory [32]. We confirmed that clone 14 cells mineralize their ECM strongly despite having very low expression levels of TNAP but we now extend these observations by showing that these cells express high levels of PHOSPHO1 which may provide sufficient intra-vesicular P_i for mineralization to be initiated. The importance of PHOSPHO1 to the mineralization process was further underscored by poor PHOSPHO1 expression in clone 24 cells which mineralized their ECM less well. Deletion of PHOSPHO1 would suppress intra-vesicular generation of P_i but would leave extra-vesicular P_i generation via TNAP activity and influx via P_i transporters unaffected [20,31]. Therefore mineralization in the absence of PHOSPHO1 is a slower process. This observation is consistent with the reduced mineralization noted in the skeletons of *Phospho1*^{-/-} mice [20].

Although it is recognized that the inactivation of PHOSPHO1 in the developing chick limb or its genetic ablation in mice results in an osteoidosis and osteomalacia [20,30] we further exploited clone 24 cells to show that *Phospho1* overexpression was sufficient to promote robust matrix mineralization in this poorly mineralizing cell line. These data provide persuasive evidence for the importance of this phosphatase to the mineralization process. Interestingly, when cultured in the presence of β GP or P-Cho, *Phospho1* overexpression resulted in higher TNAP protein expression; an observation consistent with our previous reports which noted that *Phospho1*^{-/-} mice exhibit reduced TNAP expression and activity [20]. Although the mechanism(s) by which the ablation of PHOSPHO1 expression results in low TNAP expression is unclear, the transgenic overexpression of TNAP does not correct the bone osteomalacia of *Phospho1*^{-/-} mice despite normalization of their plasma PP_i levels [20]. This indicates that the elevation of serum PP_i in the *Phospho1*^{-/-} mouse (as a result of reduced TNAP) is not the cause of the hypomineralized phenotype. Indeed, our more recent studies have implicated a role for PP_i induced osteopontin expression in the hypomineralization phenotype of the *Phospho1*^{-/-} mouse [33].

More definitive data on the function of PHOSPHO1 and TNAP in promoting matrix mineralization was provided by the use of previously validated PHOSPHO1 (MLS-0263839) and TNAP (MLS-0038949) inhibitors [22,34]. Both inhibitors when used individually reduced mineralization levels of the *Phospho1* overexpressing C24 cells to control (empty vector transduced) levels. Only when both inhibitors were used in combination did we note an almost complete inhibition of all mineralization. These observations suggest non-redundant actions of PHOSPHO1 and TNAP on the mineralization process and this supports our *in vivo* observations where the ablation of either PHOSPHO1 or TNAP results in a hypomineralized skeleton whereas the double ablation of both phosphatases leads to the complete absence of skeletal mineralization [21]. These data suggest that both PHOSPHO1 and TNAP are required for the formation of a fully mineralized matrix. Using the more physiological metatarsal mineralization model, the response to both TNAP and PHOSPHO1 inhibitors appeared to be substrate dependent. We know that β GP is a poor substrate for PHOSPHO1 and therefore normal metatarsal mineralization in the presence of β GP and MLS-0263839 was as expected [17]. Unexpectedly, in the presence of P-Cho, a substrate for both TNAP and PHOSPHO1, mineralization was almost completely inhibited when either TNAP or PHOSPHO1 activity was inhibited alone and it is unclear why residual TNAP (in the presence of PHOSPHO1 inhibitor) or PHOSPHO1 (in the presence of TNAP inhibitor) was not able to maintain limited mineralization. Nevertheless, in the presence of β GP, the reduction in mineralization by the TNAP inhibitor and the almost complete elimination of mineralization by both inhibitors is again supportive of our previous observations that

both phosphatases are required for full matrix mineralization. These data highlight the important non-redundant role of PHOSPHO1 and TNAP in the control of ECM mineralization. It is possible that the initiation of skeletal mineralization involves the action of PHOSPHO1 inside MVs together with transporter mediated influx of extra-vesicular Pi, produced by TNAP activity [31]. This consequence of events, whilst attractive, needs to be examined *in vivo* using the judicious use of genetic mouse models.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.09.013>.

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