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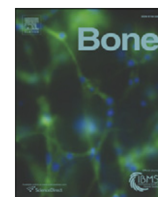

Original Full Length Article

Phospho1 deficiency transiently modifies bone architecture yet produces consistent modification in osteocyte differentiation and vascular porosity with ageing

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ABSTRACT

PHOSPHO1 is one of principal proteins involved in initiating bone matrix mineralisation. Recent studies have found that *Phospho1* KO mice (*Phospho1*-R74X) display multiple skeletal abnormalities with spontaneous fractures, bowed long bones, osteomalacia and scoliosis. These analyses have however been limited to young mice and it remains unclear whether the role of PHOSPHO1 is conserved in the mature murine skeleton where bone turnover is limited. In this study, we have used *ex-vivo* computerised tomography to examine the effect of *Phospho1* deletion on tibial bone architecture in mice at a range of ages (5, 7, 16 and 34 weeks of age) to establish whether its role is conserved during skeletal growth and maturation. Matrix mineralisation has also been reported to influence terminal osteoblast differentiation into osteocytes and we have also explored whether hypomineralised bones in *Phospho1* KO mice exhibit modified osteocyte lacunar and vascular porosity. Our data reveal that *Phospho1* deficiency generates age-related defects in trabecular architecture and compromised cortical microarchitecture with greater porosity accompanied by marked alterations in osteocyte shape, significant increases in osteocytic lacuna and vessel number. Our in vitro studies examining the behaviour of osteoblast derived from *Phospho1* KO and wild-type mice reveal reduced levels of matrix mineralisation and modified osteocytogenic programming in cells deficient in PHOSPHO1. Together our data suggest that deficiency in PHOSPHO1 exerts modifications in bone architecture that are transient and depend upon age, yet produces consistent modification in lacunar and vascular porosity. It is possible that the inhibitory role of PHOSPHO1 on osteocyte differentiation leads to these age-related changes in bone architecture. It is also intriguing to note that this apparent acceleration in osteocyte differentiation evident in the hypomineralised bones of *Phospho1* KO mice suggests an uncoupling of the interplay between osteocytogenesis and biomineralisation. Further studies are required to dissect the molecular processes underlying the regulatory influences exerted by PHOSPHO1 on the skeleton with ageing.

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1. Introduction

Bone formation involves a cascade of events leading to the deposition of mineral (biomineralisation), critical to skeletal maintenance throughout life. Mineralisation occurs by a series a complex physico-chemical and biochemical processes that facilitate the deposition of a solid hydroxyapatite (HA) phase [1]. Biomineralisation can be considered a two-step process, which involves de novo induction of mineral formation within the protective enclave of the lumen of osteoblast and chondrocyte matrix vesicles (MVs) followed by propagation of induced mineral into the extravesicular matrix [2,3]. These formation and propagation steps of HA deposition are carefully regulated by a balance of mineralisation promoters and inhibitors.

The recognised local inhibitors include inorganic pyrophosphate (PP_i) and organic non-collagenous proteins or peptides of the extracellular matrix (ECM) such as osteopontin [4–6]. Bone mineralisation is also dependent on a tight local balance between extracellular

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(e) levels of P*i* and PP_i and when ePP_i is deficient or in excess, the skeleton is either over- or under-mineralised, respectively [7,8]. The complex interplay between PP_i formation, transport and degradation directly controls the eP*i*/PP_i balance and thereby the propagation of HA out with the confines of the MV.

Current evidence suggests that there are several principal proteins involved in regulating bone mineralisation, which include tissue-nonspecific alkaline phosphatase (TNAP), an ectoenzyme expressed on the surface of chondrocytes, osteoblasts and their shed MVs [9]. TNAP hydrolysis maintains ePP_i levels at physiological concentrations which also yields P_i for HA formation within the ECM [10]. Nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) also regulates mineralisation by generating PP_i ectoplasmically from nucleoside triphosphate substrates [11,12] whereas the multiple-pass transmembrane protein ANK achieves this by mediating intracellular to extracellular channelling of PP_i [13,14]. Mouse models with NPP1 or ANK mutations show decreased levels of PP_i and bone hypermineralisation [14,15]. PHOSPHO1 (phosphatase, orphan 1) which directly regulates PP*i* availability, has also now been identified.

TNAP deficiency in humans results in hypophosphatasia (HPP) and is linked to increased plasma PP_i levels due to impaired pyrophosphatase function. Similarly, mice deficient in TNAP function ($Alpl^{-/-}$) are born with normally calcified skeletons but by postnatal day 6 skeletal hypomineralisation becomes apparent and worsens with age until their early demise by postnatal day 20 [7,16]. The failure of bones to calcify after birth appears to result from a block in HA propagation in the ECM, beyond the confines of the MV membrane [17,18], as a consequence of accumulated ePP_i levels due to lack of TNAP's pyrophosphatase activity [19–21] and concomitant pyrophosphate-induced increase in osteoblast production of osteopontin [22,23]. Importantly, electron microscopy has revealed that MVs from $Alpl^{-/-}$ mice and from patients with hypophosphatasia possess the ability to initiate HA formation within the sheltered interior of the MV [24,25]. These findings suggest that alternative mechanisms may regulate the intravesicular initiation of mineral formation. One candidate is PHOSPHO1, a soluble cytosolic phosphatase and a member of the haloacid dehalogenase (HAD) superfamily of hydrolases [26].

PHOSPHO1 was first identified in the chick where it is expressed at 120-fold higher levels in mineralising than non-mineralising tissues [27]. It is active in osteoblast and chondrocyte MVs and has specificity for phosphoethanolamine (PEA) and phosphocholine (PCho) [28,29]. The reduced ability of the chick wing and leg long bones to mineralize in the presence of the PHOSPHO1 inhibitor, lansoprazole, provided initial confirmation of the pivotal functional role of PHOSPHO1 in skeletal mineralisation [30]. More recently, PHOSPHO1 deficient mice, *Phospho1*-R74X (*Phospho1* KO) were found to show elevated ePP_i levels and to display multiple skeletal abnormalities, including spontaneous fractures, bowed long bones, osteomalacia and scoliosis in early life [31]. These pathological changes were clearly evident at 1 month of age in *Phospho1* KO mice, and this effect is thought to become progressively worse with age. Furthermore, tibiae from *Phospho1* KO mice are more ductile and did not fracture during 3-point bending but deformed plastically [32,33], likely due to a reduced elastic modulus and hardness [32,33].

Previous micro-computed tomography (μCT) analysis of 1-month-old *Phospho1* KO showed increased trabecular number and decreased trabecular space but no significant difference in BV/TV ratio compared to WT mice, along with a significant reduction in cortical mineral density in both femur and tibia [31]. Together, these findings suggest that PHOSPHO1 serves a critical role in bone mineralisation during development and growth. This, we have hypothesised, is related to its capacity to scavenge P_i from both PEA and PCho in order to generate the P_i concentration needed to establish a P_i/PP_i ratio permissive for the initial formation of HA crystal inside the MVs [3,29].

To date, analyses of the *Phospho1* KO phenotype have been limited to young mice which are characterised by active modelling of the skeleton and as PHOSPHO1 has been implicated in the initiation of bone mineralisation, it is unclear whether this role is conserved in later life in the mature murine skeleton where bone turnover is limited [31,32]. Furthermore, the level of mineralisation and the properties of the bone matrix are associated with bone strength and stiffness [34–36]. We therefore sought to determine how *Phospho1* contributes to tibial surface strain and stiffness using digital image correlation as reported previously [37]. Since matrix mineralisation has also been reported to influence terminal osteoblast differentiation into osteocytes [38,39] and angiogenesis [40–46], it is also important to determine whether the osteoblast-to-osteocyte transition and vascular porosity [47–51] are impaired in PHOSPHO1 deficient mice as this may have profound effects on skeletal architecture and biomechanical properties due to an impaired ability of the skeleton to respond appropriately to mechanical loading [52,53]. In this study, we have therefore used high resolution CT to examine the effect of *Phospho1* deletion on tibial bone architecture in mice at a range of ages to establish whether its role is conserved during growth and maturation of the skeleton. Furthermore, we have also explored whether the hypomineralised bones in these mice exhibit modified osteocytic and vascular content.

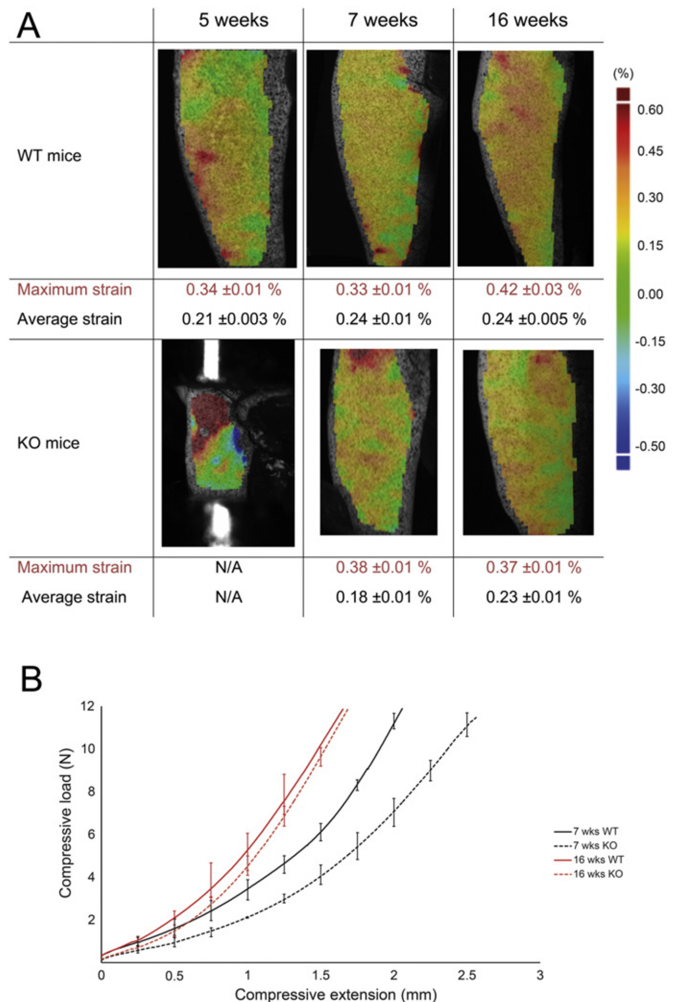

Fig. 1. A) Longitudinal strain map on the medial side of the bone surface of WT and *Phospho1* KO tibia at 5, 7 and 16 weeks of age with maximum and average values obtained following 12 N compressive load. B) Loading displacement for 7 and 16 week old *Phospho1* KO and WT mice.

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