Role of ATP and ATPase Activity in Mineralization

Aya A. Bennagi

Faculty of Dentistry, McGill University

Montreal, Quebec, Canada

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“Allah loves, when one of you is doing something, that he / she does it in the most excellent manner” Prophet Mohamed peace upon him

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ABSTRACT

Physiologic mineralization, i.e., deposition of hydroxyapatite (HA) crystals into type I collagen extracellular matrix, is a carefully orchestrated process controlled by molecular promoters and inhibitors of inorganic phosphate homeostasis. Mineralization is initiated upon local increased levels of inorganic phosphate (Pi) which can be generated from phosphate containing molecules via hydrolysis. The main enzyme capable of elevating extracellular Pi levels is tissue-nonspecific alkaline phosphatase (TNAP). Mutation or absence of TNAP gene results in significant decrease, but not complete lack of matrix mineralization, as seen in patients with rickets, osteomalacia as well as TNAP null mice models. This indicates that supplementary mechanisms to generate Pi and nucleate HA exist.

In this thesis we have examined the role of adenosine triphosphate (ATP) and ATPase activity in mineralization of MC3T3-E1 osteoblasts cultures. Bone formation environment are enriched with ATP and ATPase activity. ATPase activities are involved in local Pi and calcium (Ca^{2+}) homeostasis that are critical for HA formation. ATP is also a potent endogenous source of phosphate. It is externalized by osteoblasts upon several stimuli including mechanical stimulation. Phosphatases with ATPase activity are required to hydrolyze ATP and elevate local Pi concentration. Further, ATP hydrolysis generates energy essential to power different ions transporter mechanisms. Pi and Ca^{2+} transportation and extrusion at site of matrix mineralization are regulated by ATPase actions. Sodium-potassium ATPase pump generates membranous sodium gradients critical to power both Pi transporter (PiT1) and Ca^{2+} extruder (NCX).

In this thesis, we have investigated the effect of ectoATPase inhibitor ARL67156 in matrix mineralization of MC3T3-E1 cultures. Cells were cultured for 12 days and mineralization was induced by adding either β-glycerophosphate (βGP), sodium monophosphate (SMP), or ATP. In addition, we have observed ATP release from βGP- and SMP-mediated mineralizing cultures. We have found that inhibition of ATPase activity in MC3T3-E1 cultures resulted in complete absence of mineralization without altering levels of type I collagen scaffold. Importantly, all cultures were affected including SMP-treated cultures that do not require Pi generation mechanism. ARL67156 neither affect

TNAP activity nor inorganic pyrophosphate levels. Also, extracellular Pi levels remain high in presence of this inhibitor. We have recorded an altered levels of sodium ions when cells treated with ectoATPase inhibitor that suggests an effect on sodium-potassium ATPase pump. We concluded that even in presence of high concentration of free Pi, ATPase activities are essentially required for local ion homeostasis and HA formation. Furthermore, we have recognized that MC3T3-E1 cells behave differently in presence of either βGP or SMP. ATP released from cells was increased progressively when cells treated with βGP. To the contrary, adding SMP to MC3T3-E1 cultures lowered ATP amounts released in the media. This might indicate that the endogenous ATP release is upregulated during Pi generation mechanism.

RÉSUMÉ

La minéralisation physiologique se traduit par le dépôt des cristaux d'hydroxyapatite (HA) dans les fibres de collagène type I de la matrice extracellulaire. Ce processus parfaitement contrôlé par des promoteurs et des inhibiteurs de l'homéostasie du phosphate inorganique (Pi) est initié par une augmentation locale de Pi provenant de l'hydrolyse de molécules contenant du phosphate. La principale enzyme responsable de cette augmentation de Pi extracellulaire est la TNAP (tissue-nonspecific alkaline phosphatase). La mutation ou l'absence du gène codant pour cette enzyme résulte en une diminution significative de la minéralisation (mais toujours existante). Ces défauts de minéralisation sont retrouvés aussi bien chez les patients atteints de rachitisme ou d'ostéomalacie que chez les souris déficients en TNAP. Le fait que la minéralisation même fortement réduite persiste toujours indique qu'il doit exister d'autres mécanismes pour générer du Pi et de l'HA.

Dans ce travail, nous avons étudié le rôle de l'adénosine triphosphate (ATP) et de l'activité ATPase dans la minéralisation des cultures d'ostéoblastes MC3T3-E1. L'environnement de la formation de l'os est enrichi avec l'ATP et l'activité ATPase. Des activités enzymatiques ATPase sont impliquées dans les processus locaux d'homéostasie de Pi et de calcium, processus déterminant dans la formation de l'HA. L'ATP est aussi une source endogène potentielle de phosphate externalisé par les ostéoblastes par plusieurs stimuli pouvant être mécaniques. Les phosphatases sont requises pour hydrolyser l'ATP et augmenter la concentration locale de Pi. De plus, cette hydrolyse génère l'énergie essentielle pour les mécanismes de transport d'ions. Le transport Pi et Ca^{2+} et leur extrusion sur le site de minéralisation sont régulés par les activités ATPase. La pompe sodium-potassium génère des gradients de sodium membranaires essentiels pour permettre le transport de Pi (PiT1) et l'extrusion de Ca^{2+} (NCX).

Dans cette thèse, nous avons étudié l'effet de l'inhibiteur ectoATPase ARL67156 sur la minéralisation matricielle dans les cultures de MC3T3-E1. Les cellules sont mises en culture 12 jours et la minéralisation est induite par l'addition de β-glycerophosphate (βGP), de sodium monophosphate (SMP), ou d'ATP. Nous avons observé un relargage

d'ATP dans les cultures utilisant βGP et SMP. L'inhibition de l'activité ATPase dans les cultures MC3T3-E1 entrainait une absence totale de minéralisation sans altération du réseau de collagène type I. Par ailleurs, toutes les cultures ont été affectées même celles qui ne nécessitaient pas la génération de Pi (SMP). ARL67156 n'affecte ni l'activité de la TNAP ni la quantité de pyrophosphate inorganique. De plus, les niveaux de Pi extracellulaire restent élevés en présence de cet inhibiteur. Nous avons enregistré des niveaux altérés de sodium lorsque les cellules ont été traitées avec l'inhibiteur de l'ectoATPase ; ceci suggère un effet sur la pompe sodium-potassium ATPase. Nous pouvons conclure que même en présence d'une forte concentration de Pi libre, les activités ATPase sont essentiellement requises pour l'homéostasie locale des ions et pour la formation de l'HA. De plus, les cellules MC3T3-E1 se comportent différemment en présence de βGP ou de SMP. Le relargage de l'ATP est augmenté lorsque les cellules sont traitées avec βGP. A l'opposé, l'addition de SMP aux cultures MC3T3-E1 réduit le niveau d'ATP dans le milieu. Ceci pourrait indiquer que le relargage d'ATP endogène est régulé positivement lors du mécanisme de génération de Pi.

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ABBREVIATIONS

AA	Ascorbic acid
ALP	Alkaline phosphatase
ANK	Progressive ankylosis gene
ATP	Adenosine triphosphate
βGP	β-glycerophosphate
COL I	Collagen type I
DMP-1	Dentin matrix protein-1
ECM	Extracellular Matrix
ENPP1	Ectonuceotide pyrophosphatase phosphodiesterase
ePi	extracellular inorganic phosphate
ePPi	extracellular inorganic pyrophosphate
FGF23	Fibroblasts growth factor 23
HA	Hydroxyapatite
MVs	Matrix vesicles
MEPE	Matrix extracellular phosphoglycoprotein
Na-K ATPase	Sodium-potassium ATPase
NCX	Sodium calcium exchanger
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline

PHOSPH1	Phospho orphan 1
PHEX	Phosphate regulating gene with homolog, X-linked
PiT1	Inorganic phosphate transporter 1
PMCA	Plasma membrane calcium ATPase
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor-kappa B
RANKL	RANK-ligand
SMP	Sodium monophosphate
TNAP	Tissue nonspecific alkaline phosphatase

1. Literature review

1.1 Osteogenesis, bone growth and remodelling

1.1.1 Skeleton

Bone is a specialized connective tissue and Skeleton is made of a framework of 270 distinct pieces of bones at birth. The continuous bone adaptation, fusion and remodelling after birth reduce total number of bones in adult skeleton to 206 bones [1].

Human skeleton is a dynamic robust organ that serves a variety of functions. It levers muscle actions, provides strength and support for soft tissues and permits movement and locomotion. Internal delicate organs, such as brain, spinal cord, lungs and heart are enclosed and protected inside bony compartments. In addition, bone is considered as an endocrine organ that is actively involved in essential biological functions. For example, osteocalcin is a bone derived hormone that regulates glucose metabolism, male fertility, brain development as well as cognitive functions [2]. Further, bone regulates mineral ion homeostasis and acts as a major reservoir of Ca^{2+}, cytokines and growth factors. In addition, bone marrow is the primary site of hematopoiesis [3, 4].

Human bones have a wide range of shapes, however, it is classified basically into two main groups: axial and appendicular. Axial group includes skull, vertebrae, sternum and ribs. Appendicular bones define upper and lower limbs [5]. A closer look to osseous tissue reveals two components, cortical (hard outer shell) and trabecular (spongy inner compartment). The outer shell is the primary site of levering muscle actions. Trabecular bone is considered as the metabolically active part. It has an extensive surface areas

with high vascularity designed to be ideal place of hematopoiesis and ions homeostasis [5].

Bone is formed via two distinguished mechanisms, endochondral and intramembranous. The former requires a cartilaginous templates that degraded and replaced later by bone. It occurs during embryonic development as well as postnatal growth. Long bones, short bones, and irregular bones are formed by endochondral ossification [6]. Meanwhile, intramembranous bones are formed in the absence of cartilage guide [4]. It is characterized by ossification centers of condensed mesenchymal cells that directly transformed into osteoblasts and start bone formation [7]. Flat bones such as cranial vault, mandible and clavicles are examples of bones formed by intramembranous osteogenesis [5].

To maintain bone integrity, skeleton is continuously remodeled. Bone remodeling is a dynamic active process where bone resorption is balanced by bone formation. This carefully controlled process allows bone growth, healing and adaptation to different stimuli. For instance, calcium homeostasis is secured via physiologic bone remodeling [8].

1.1.2 Bone cells: osteoblasts, osteocytes and osteoclasts

The internal boney architecture contains mainly three specialized cells; osteoblasts, osteocytes and osteoclasts. These cells are continuously under the influence of local and systemic factors to maintain bone mass and integrity via balanced bone formation and remodeling.

Osteoblasts are cuboidal bone-forming cells derived from mesenchymal stem cells (MSCs). These pluripotent cells are regulated by several transcription factors, hormones and growth factors to be differentiated into adipocytes, chondrocytes, fibroblasts and osteoblasts (Fig. 1) [8, 9]. The transcription factor Cbfa-1/RUNX-2 is absolutely required for osteoblasts differentiation and expression of osteoblast-specific genes such as osteocalcin [9, 10]. At sites of bone formation, osteoblasts secrete about 0.5 μm of organic matrix (osteoid) per day which is mainly composed of type I collagen (85-90%) and of 10-15% noncollagenous proteins, proteoglycan and tissue-nonspecific alkaline phosphatase (TNAP) [9]. Their active lives last about 100 days, afterward osteoblasts are either trapped into the osteoid and become osteocytes, or they pass through programed cell death, apoptosis, or become a quiescent bone lining cells on the bone surface, periosteum and endosteum [11]. Defective osteoblast activity is associated with several bone pathologies, including osteoporosis, Paget's disease and osteoarthritis [9].

Osteocytes are osteoblasts that become embedded within the mineralized osteoid and undergo terminal differentiation. They represent 90-95% of total bone cells [12]. Osteocytogenesis is remarked by specific bone markers, most importantly upregulation of matrix extracellular phosphoglycoprotein (MEPE) and downregulation of bone markers such as TNAP and type I collagen [12]. Osteocytes are in communication with each other via an extensive network of canaliculi. These canaliculi can detect changes in interstitial fluid pressure or hormonal levels and play a vital role in regulation of bone metabolism, turnover and mechanosensing [3]. It is suggested that osteocytes is the

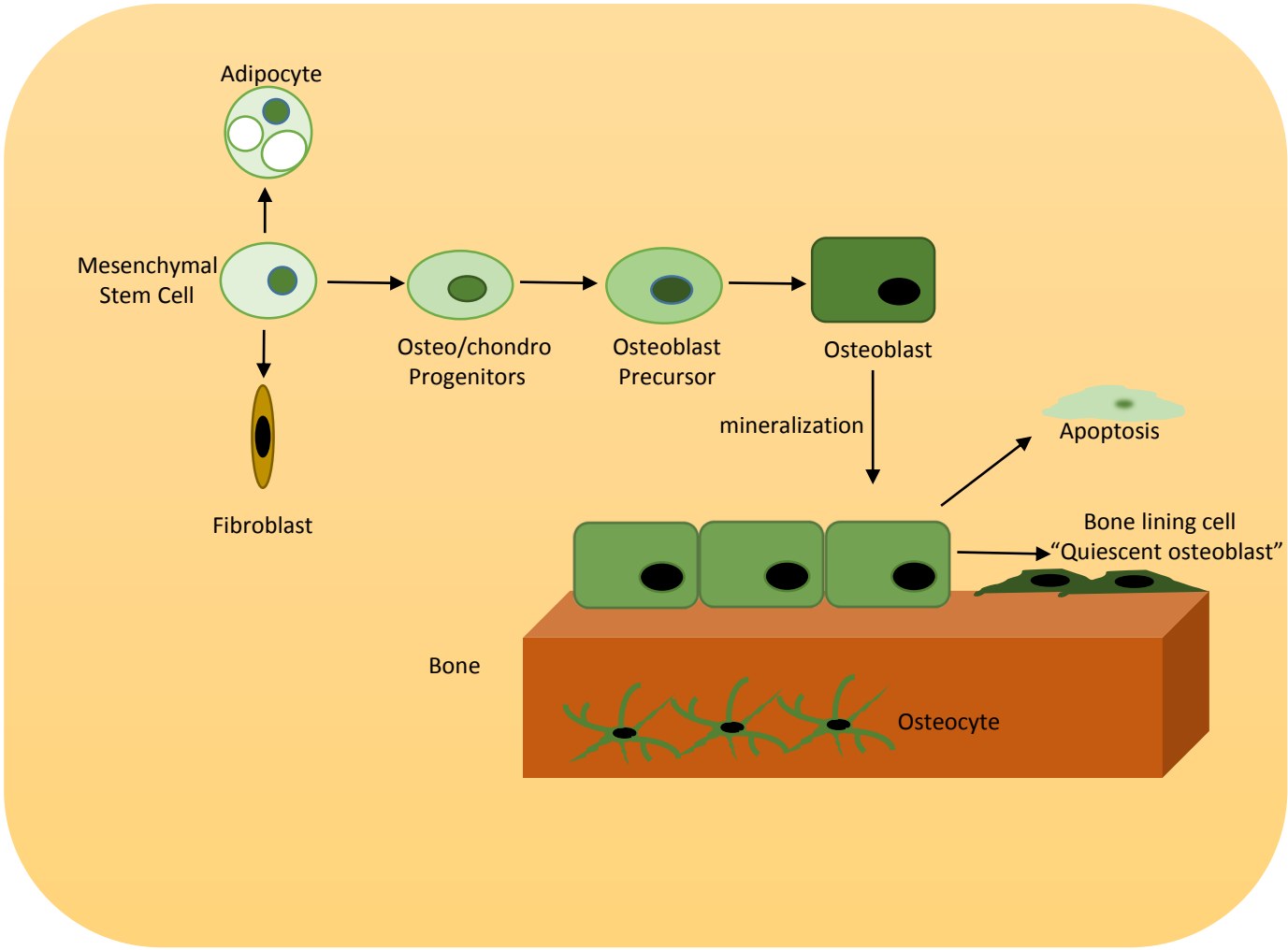

Figure 1. Differentiation of mesenchymal stem cells into osteoblasts. Osteoblasts derived from mesenchymal stem cells which are able to differentiate into several cell types including chondrocytes and adipocytes. Mature osteoblasts start mineralization in the proper environment. Afterwards, osteoblasts can become bone lining cells, differentiate into osteocytes or undergo programmed cell death.

orchestrator of bone formation. It sends signals to osteoblasts and osteoclasts to regulate bone formation and resorption, respectively [13].

Osteoclasts are bone-resorbing cells originated from monocyte/macrophage lineage. Osteoclasts are multinucleated cells that are present in low numbers in adult bone. Osteoclasts have the ability to resorb bone and thus remove it quickly. Osteoclasts are characterized by ruffled border which is highly convoluted membrane polarized toward resorption site. The acidic environment in the resorption pit (pH 4) results in demineralization of bone followed by degradation of proteinaceous matrix mainly by cathepsin K [3]. Osteoclast differentiation and maturation is induced upon RANKL-RANK interaction. RANKL is an osteoclast activator factor expressed by osteoblasts and received by RANK (osteoclast receptor) to initiate bone resorption [3, 9]. Impaired activity of RANKL and RANK results in osteopetrosis, an insufficient bone resorption and excessive bone mass [8]. On the other hand, osteoporosis was linked to mutation or defective osteoprotegerin (OPG), an inhibitor of RANKL-RANK interaction [9, 14].

1.1.3 Bone extracellular matrix

Osteoblasts synthesize and secrete a unique three-dimensional extracellular matrix (ECM) that is essential as a scaffold for later mineral deposition. About 25% of mature mineralized ECM is organic component [15]. The major component of organic matrix is type I collagen (85-90%), also traces of type III and V collagens are present in bone ECM [3, 16]. Type I collagen is secreted as propeptide (procollagen) that is processed intra and extracellulary to form collagen fibrils. Each collagen molecule comprises of three chains, two α1 and one α2 that coil around each other to form triple

helix. Mature type I collagen helices are cross-linked via lysyl oxidase activity and assembled as three-dimensional fibrils with holes and overlap zones providing spaces for HA deposition [11, 17].

Osteoblasts secrete noncollagenous proteins include osteocalcin, osteonectin, TNAP, fibronectin, sialoprotein, and osteopontin. Bone also contains proteoglycans [9]. Noncollagenous proteins regulate several physiological functions such as cell-matrix interaction, cell adhesion and signalling [18]. Moreover, some of these non-collagenous proteins have an influence on the mineralization process [18]. Osteopontin, a highly phosphorylated sialoprotein, has mineral-binding domain that allows its binding to mineral [19]. It is suggested that osteopontin is an essential regulator and stabilizer of HA crystal growth within type I collagen fibrils [19].

Mutations or defects in bone matrix proteins results in wide range of serious life-threatening conditions. For instance, type I collagen mutations lead to different forms of osteogenesis imperfect, whereas abnormal collagen III is detected in Ehlers-Danlos syndrome. Further, tumor-induced osteomalacia is linked to defective bone sialoprotein [3].

1.2 Hydroxyapatite formation and role of matrix vesicles

Mineralization of extracellular matrix is a multifactorial process which is still incompletely understood. Osteoblasts regulate mineral deposition, yet, detailed sequence of the process is not established. Bone is composed of 50-70% minerals mostly HA [$Ca_{10}(PO_4)_6(OH)_2$] with traces of magnesium, carbonate and acid phosphate [3].These insoluble acid resistant crystals have a length ranged between 30 to 70 nm and deposited

in the space of triple helical fibrils of type I collagen (hole region) under the influence of several local factors [12, 20]. Both Ca^{2+}-ions and phosphate (Pi) must accumulate in specific concentrations to commence the deposition of HA crystals, however, it is speculated that the initiation of crystal formation needs macromolecular “nucleators”, most probably bone sialoproteins [9]. Regardless of the elevated levels of Pi and Ca^{2+}, the co-expression of type 1 collagen and TNAP in hard-tissues forming cells are essentially required to initiate mineralization [21].

Generally, there are two suggested mechanisms of matrix mineralization. First theory based on direct deposition of Ca^{2+}-ions and Pi in the “hole regions” of collagen fibrils under the influence of noncollagenous proteins. Other literatures support matrix vesicles (MV)-dependent mineralization However, recent studies suggesting that both mechanisms are concurrently involved [22]. However, the role of MVs in mineralization is yet controversial. MVs are suggested to be the nucleation site of HA crystals. These membrane-bound vesicles, with diameter 30-1000 nm, have been recognized in osteoid, pre-calcifying zone in cartilage and into pre-dentine [23]. The evidence of mineralizing MVs inside human aorta indicates that vesicles are even involved in pathological mineralization [24].

Based on electron microscopy reports, insoluble Ca^{2+}/Pi crystals are mobilized from intracellular compartment, pinched off cell membrane within vesicles and released into ECM [20]. Accordingly, these organelles are derived from the cell membrane and enriched with acidic phospholipids and proteolipids [20]. Further, MVs are equipped with

cell membrane enzymes essential for Ca^{2+}, Pi and pyrophosphate (PPi) homeostasis, such as plasma membrane calcium ATPase (PMCA), sodium-potassium ATPase (Na^{+}-K^{+} ATPase), phospho orphan 1 (PHOSPHO1), ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1), and TNAP [23, 25, 26]. It is believed that these ideal internal microenvironment are required for Ca^{2+} and Pi to reach essential concentrations to precipitate as crystalline calcium phosphate salts [3]. Once crystals inside MVs started to enlarge they rupture vesicle's membrane and enter the ECM. Crystal growth continues in ECM in high phosphate concentration [22].

1.3 Phosphate homeostasis and mineralization

Inorganic phosphate (Pi) is an essential element for normal mammal cell functions. It is required for cell signalling, DNA and phospholipid synthesis as well as production of energy. Moreover, Pi is an indispensable ion for hard tissue formation [27]. Abnormal Pi levels are linked to cardiovascular diseases, cancers, soft tissue mineralization and poor quality of bone and teeth [28]. In order to perform all these functions, Pi must be translocated between cells, extracellular fluids and systemic circulation [29].

1.3.1 Systemic regulation of inorganic phosphate homeostasis

A physiological serum Pi levels are maintained via coordinated actions of several hormones and growth factors working mainly on the gut, bone, kidney and parathyroid glands [30]. Active form of vitamin D3 ($1,25(OH)_2$ D3), parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) are considered the major regulators of serum Pi concentration (Fig. 2). Serum Pi levels are maintained via balanced dietary Pi absorption

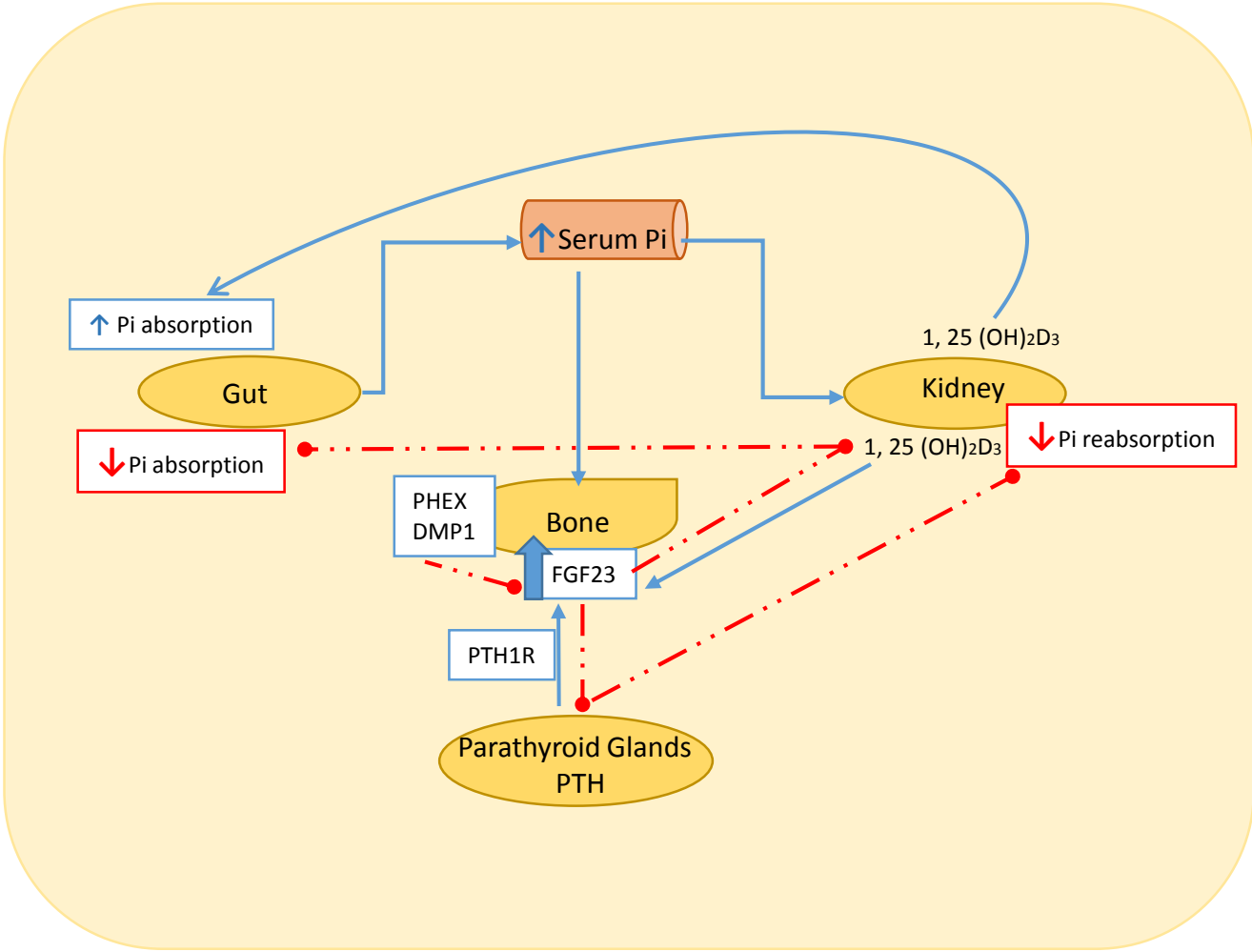

Figure 2. Systemic regulation of phosphate homeostasis. Inorganic phosphate is absorbed from diet, stored in bones and excreted via kidneys. Low serum Pi levels induce vitamin D release from the kidney that stimulates intestinal absorption of dietary Pi. In turn, higher serum Pi trigger synthesis and secretion of FGF23 from bone that leads to inhibitory effect on PTH and subsequently decrease renal Pi reabsorption. FGF23 also downregulates excretion of 1, 25 (OH)2D3 and Pi absorption from the gut. FGF23 is upregulated by high levels of vitamin D and serum Pi. FGF23 is down regulated by PHEX and DMP1. To conclude, FGF23 decrease serum Pi concentration via reducing intestinal Pi absorption and renal Pi reabsorption.

from the small intestine and renal Pi reabsorption. About 30% of dietary Pi is absorbed in intestine under the control of $1,25(OH)_2D3$. Further, the transportation of Pi from the urine back to the circulation is mainly done via type II and III sodium-dependent phosphate cotransporters under the control of PTH and FGF23 [28, 30, 31]. Both PTH and FGF23 are upregulated by increased levels of serum Pi and their net effect is to inhibit renal Pi reabsorption and decrease serum Pi levels [31].

PTH is known as a regulator of serum calcium “calciostat”. Parathyroid cell membranes have a calcium-sensor receptor (CaR) which are sensitive to serum Ca^{2+} levels [31]. Beside regulation of Ca^{2+} homeostasis, PTH activates signalling pathways in kidney that lead to internalization of sodium-dependent phosphate cotransporters and wasting of renal Pi [32]. Moreover, PTH stimulates receptors on osteoblasts and osteocytes to increase expression of FGF23 [33]. FGF23 is synthesized and secreted mainly by osteocytes. It is a phosphaturic factor that inhibits renal Pi reabsorption and reduce serum Pi levels [31]. Increased levels of FGF23 stimulate osteoblasts and osteocytes to secrete PHEX and DMP1 that simultaneously supress FGF23 secretion and increase serum Pi levels [12]. Recently, more scientific works emphasize the role of FGF23 in Pi homeostasis when autosomal dominant hypophosphatemic rickets (ADHR) is linked to mutant FGF23 [31]. However, both high and low serum FGF23 concentrations are linked to pathological conditions, hypophosphatemia and hyperphosphatemia [31]. It is known that $1,25(OH)_2D3$ activates dietary Pi absorption and indirectly increases Pi renal reabsorption via inhibiting PTH synthesis and secretion [31, 34]. Other hormones,

mainly insulin, are believed to play an important role in Pi homeostasis, however, little is known about the mechanism.

Importantly, high serum Pi levels are reported as a switcher of mineralization process in cardiovascular system, however, the exact mechanism is not yet known [10]. In vitro studies have correlated elevated serum Pi levels to phenotypic changes in hard tissue-forming cells, including osteoblasts, chondrocytes, cementoblasts, odontoblasts as well as vascular smooth muscle cells [28].

1.3.2 Local regulation of inorganic phosphate and pyrophosphate balance (ePi /ePPi)

Although serum Pi levels affect mineralization, several observations indicated that Pi levels in bone ECM do not only depend on its systemic levels [35]. At site of mineralization, local regulatory factors modulate the Pi homeostasis include TNAP, osteopontin as well as the extracellular balance between Pi and inorganic pyrophosphate (PPi). Importantly, ePi/ePPi ratio is crucial determinant for HA growth and propagation.

PPi is a potent mineralization inhibitor composed of two inorganic phosphate molecules joined by a hydrolyzable high-energy ester bond [12]. It is present in intra- and extracellular compartments as well as body fluids including plasma [26]. PPi is a by-product of many intracellular metabolic reactions. It can be generated extracellulary by hydrolysis of ATP by ENPP1, also intracellular PPi (iPPi) can reach extracellular milieu via ANK, a multi-pass transmembrane protein facilitates extrusion of iPPi to extracellular milieu [12, 18]. TNAP is upregulated upon high levels of extracellular PPi (ePPi). It exerts

pyrophosphatase activity to break ePPi into two Pi, elevating Pi to counteract high ePPi levels.

ePPi has the ability to adsorb to hydroxyapatite crystals and impede further crystal growth and propagation as seen in hypomineralization conditions [18]. Also, ePPi stimulates OPN expression by osteoblasts that results in inhibition of mineralization. Moreover, accumulation of ePPi is linked to downregulation of TNAP activity [18]. Higher levels of ePi are counterbalanced by generation and translocation of iPPi via ENPP1 and ANK [36]. Deficiency of ePPi due to either mutation of ENPP1 or ANK leads to pathologic mineralization of soft tissues or imbalanced bone formation [37].

Imbalanced relationship of ePi/ePPi results in different bone and teeth conditions, such as hypophosphatemia, osteomalacia and rickets. During development of teeth, excessive ePPi causes abnormal cementum formation, defective roots and premature loss of teeth. While teeth with extremely thick cementum is reported in ank null mice [38].

1.4 Phosphatases and their effects on mineralization

As outlined above, mineralization occurs in high Pi conditions. Phosphatases such as TNAP, PHOSPHO1 and ENPP1 release Pi from inorganic and organic sources. The main inorganic source of Pi is PPi, however, it is not fully understood which organic phospho-molecules can provide phosphate for mineralization. Indeed, the following phospho-containing molecules have been indicated to provide Pi for biomineralization: adenosine 5′-triphosphate (ATP), pyridoxal-5′-phosphate (PLP), phosphoethanolamine (PEA) and phosphocholin (PC) [39].

1.4.1 Tissue-nonspecific alkaline phosphatase (TNAP)

TNAP is an isoenzyme of alkaline phosphatase family, one of the important phosphate-regulating genes encoded by *ALPL* gene in humans and *Alpl* gene in mice [22]. It is expressed at high levels by hard tissues-forming cells such as osteoblasts, odontoblasts, fibroblasts in periodontal ligaments as well as detected on MVs [40]. This critical enzyme is recognized as membrane-bound protein or soluble protein within the ECM [3]. TNAP has a major biological functions in hard tissue formation. It has an essential pyrophosphatase as well as ATPase activity (Fig. 3) [22]. It can generate Pi locally via hydrolysis of substrates such as ATP, PEA, PLP and PPi. However, amount of Pi released from PPi cleavage by TNAP is in micromolar range and cannot alone promote mineralization [36]. In addition, TNAP has a promoter effect on mineralization via dephosphorylation of osteopontin, an extracellular glycoprotein with high mineral affinity that interfere with crystal propagation [18]. Also, TNAP hydrolyzes AMP into adenosine that stimulates P1 receptors on osteoblasts [41]. Mutations in TNAP genes result in accumulation of ePPi that interferes with propagation of mineralized crystals onto ECM, as seen in rickets, osteomalacia and hypophosphatasia [22, 42].

Hypophosphatasia is a mineralization disorder characterized by impaired TNAP activity. It has variable phenotypes ranging from hypomineralization and pathologic fracture to severe impaired mineralization shown in stillborn fetus [43]. Low plasma concentration of TNAP is regularly used as a biomarker of hypophosphatasia, however, failed systemic treatments to replete TNAP indicate that enzyme is locally defected [43].

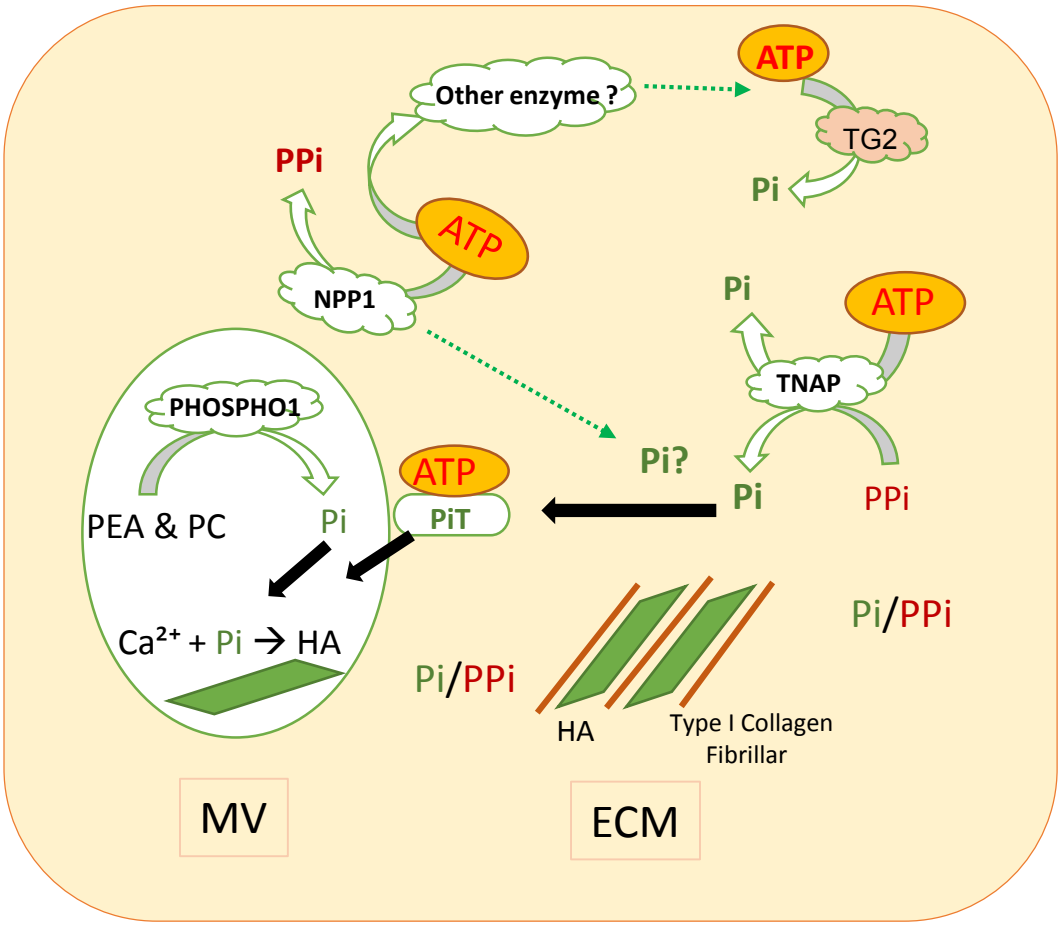

Figure 3. Illustration of suggested roles of phosphatases in mineralization. PHOSPHO1 is active inside MVs, having high intravesicular affinity to phosphoethanolamine (PEA) and phosphocholine (PC), PHOSPHO1 generates iPi. Also, ePi is transported via Pit1 to increase iPi levels to initiate HA formation. HA breaks MV membrane and spread onto extracellular collagenous matrix. Extracellulary, both TNAP and ENPP1 regulate propagation of HA. ePi/ePPi levels are regulated carefully via TNAP and ENPP1. TNAP has pyrophosphatase and ATPase activities, both elevate ePi levels and reducing ePPi to promote mineralization. ENPP1 is responsible for the production of ePPi. However, an ATPase backup mechanism of ENPP1 is suggested. Other extracellular enzymes with ATPase activity, such as TG2, might be involved in ePi generation form Pi-containing molecules.

Figure is adopted originally from Millán, J.L., *The role of phosphatases in the initiation of skeletal mineralization.* Calcified tissue international, 2013. **93**(4): p. 299-306.

Importantly, TNAP knockout mice (*Alpl*-/-) apparently have normal skeleton at birth, but show postnatal signs of defective mineralization due to inability of HA crystals to grow beyond the confines of MVs. Such evidence indicates that TNAP is not the only factor that can initiate mineralization and that other molecules(s) and/or mechanism(s) are involved in this process [22, 42].

1.4.2 Phospho orphan 1 (PHOSPHO1)

Phospho orphan 1 **(**PHOSPHO1) is a cytosolic enzyme expressed at 100-fold higher in mineralizing tissues than non-mineralizing ones [44]. This soluble phosphatase was first described in chicken with high phosphohydrolase activity inside MVs toward PEA and PC [45]. Therefore, it is suggested that PHOSPHO1 is responsible for initial iPi generation and HA formation inside MVs during endochondral ossification [12, 22]. Nevertheless, data indicate that Pi influx into MVs augment iPi levels essential for initiation of mineralization (Fig. 3) [22]. TNAP and PHOSPHO1 were found to be coexpressed throughout the stages of limb development in the chick [22]. Correlatively, defective PHOSPHO1 has indirect effect on ePPi concentration via lowering TNAP activity [12]. Yet, overexpression of TNAP does not overcome PHOSPHO1 deficiency indicating that both phosphatases are working on different pathways [45].

Impaired activity of PHOSPHO1 results in abnormalities in the endochondral growth plate centers, defective secondary ossification centers, decreased bone mineral density, osteomalacia, scoliosis, and spontaneous fractures [12]. Interestingly, and in despite of normal systemic Pi levels, double knockout mice (*Phospho1*-/-;*Alpl*-/-) showed complete absence of mineralization and perinatal lethality [22]. Recent study

demonstrated that inhibition of PHOSPHO1 prevents mineralization of vascular smooth muscle cells [45] which emphasizes the role of PHOSPHO1 in both physiologic and pathologic mineralization.

1.4.3 Nucleotide pyrophosphatase phosphodiesterase 1 (NPP1)

NPP1 is previously known as plasma cell membrane glycoprotein-1 (PC-1). It is a plasma membrane-bound protein that highly expressed on the surface of osteoblasts, chondrocytes and their MVs [21, 46]. It is known as a primary supplier of iPPi and ePPi using ATP as a substrate (Fig. 3) [36]. Thus, NPP1 indirectly regulates purinergic receptor signalling via ATP scavenging [25]. NPP1 is encoded by *ENPP1* gene (in humans) and associated with ePPi regulation. *Enpp1*-/- and/or *Ank*-/- mice are characterized by soft tissue mineralization including vascular calcification, due to low levels of the mineralization inhibitor, ePPi [25, 42]. Moreover, deficiencies in ENPP1 are linked to generalized infantile arterial calcification, a genetic disease characterized by arterial fibrosis, mineralization and stenosis due to lack of ePPi [47]. Phenotype of *Enpp1*-/-, tiptoe walking, is rescued with deletion of TNAP in this system which showed a partial normalization of mineralization indicating that an alternate mechanism is available for mineralization [42]. Recent interesting paper suggested that NPP1 have backup phosphatase activity on both PPi and ATP. This can explain why *Alpl*-/- mice developed mineralized skeleton for the first 6 days of life [22, 48].

1.5 ATP role in mineralization

Extracellular nucleotides are ubiquitous molecules with broad spectrum of biological functions. ATP and other nucleotides are formed intracellularly and released via coordinated pathways, exocytosis, upon mechanical stimulation or cell rupture (Fig. 4) [49]. Moreover, ATP exocytosis is also stimulated by acute transient hypoxia and 1α,25$(OH)_2$ D3 [50, 51]. Intracellularly, ATP is a well-known metabolite essential as a source of energy [52]. Extracellulary, it has crucial roles in wide array of cellular activities especially as a messenger molecule for cell-to-cell communication [53]. Once released, nucleotides and their metabolites work in autocrine / paracrine manner as signalling molecules activating purinergic P2 receptors and exerting variety of physiochemical responses [53]. Purinergic P2 receptors are present in almost all mammalian tissues and subdivided into P2X ligand-gated ion channels and P2Y G-protein-coupled receptors [51].

In bone biology, ecto-nucleotides possess significant roles in functions of osteoblasts and osteoclasts via purinergic signalling [54, 55]. Data showed that, in differentiation-dependent process, osteoblasts express at least seven different P2 receptors subtypes (P2X2, P2X5, P2X7, P2Y1, P2Y2, P2Y4, and P2Y6) [43, 54]. In addition to expression of ATP receptors, osteoblasts release ATP constitutively and, in turn, ATP influence osteoblasts gene expression and proliferation [53, 56].

Accumulating data indicate that ATP has vital functions in hard tissue formation. It has been demonstrated that higher amount of ATP is released in hypertrophic zone than the reverse zone in mineralizing cartilage [26]. At low concentrations, range of 0.1-1.0 μM, ATP and ADP have an osteolytic effect mediated via purinergic signalling in osteoclast (P2Y1 receptors on osteoclasts) and inhibit osteoblast mineralization [43, 57].

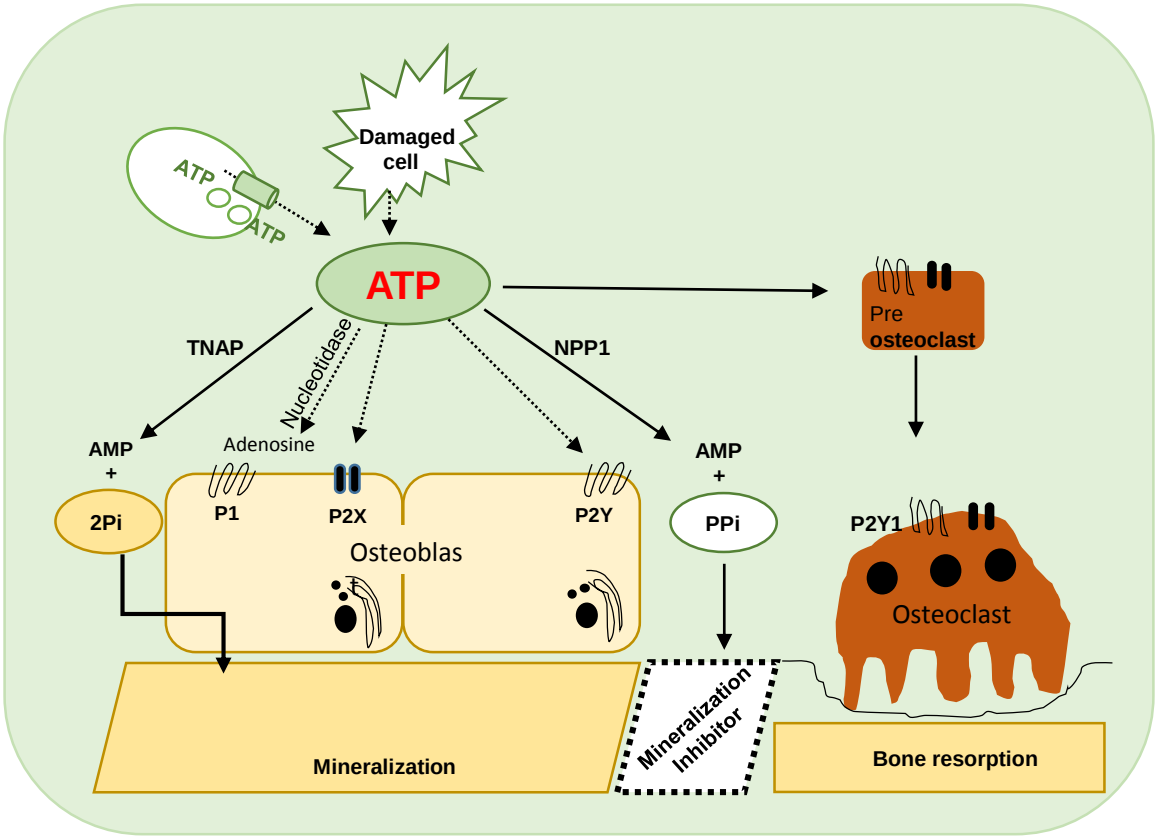

Figure 4. ATP and ATP receptors in osteoblasts and osteoclasts. ATP is released via different pathway. Upon release, ATP is able to activate P2X and P2Y receptors. Also, ATP is rapidly hydrolyzed by extracellular nucleotidases that result in in either ePi to promote mineralization or ePPi to inhibit it. Also, ATP is cleaved to adenosine which is P1 receptor agonist. ATP is also has an osteolytic effect via activation of P2Y1 receptors on preosteoclasts and osteoclasts.

On the other hand, ATP at higher concentration (4-5 mM) is a potent donor of Pi [39]. ATP can be hydrolyzed by either specific ATPase or TNAP [26]. Generally, phosphatases with ATPase activity such as TNAP, NPP1 and transglutaminase 2 can cause ATP hydrolysis and release of Pi [58]. In cell culture systems, and in comparison to β-glycerophosphate, ATP-mediated mineralization produces HA crystals more similar to human bone crystals [57]. In contrary, hydrolysis of ATP by NPP1 results in formation of ePPi, mineralization inhibitor. Therefore, ATP could inhibit mineralization via two pathways, ePPi generation and mediating osteolytic activity (Fig. 4) [53]. Interestingly, data shows that deletion of ENPP1 or ANK with TNAP results in corrected ePPi levels and improved mineralization indicating the availability of ePi from other sources, most likely ATP [36].

1.6 Role of ATPase pumps in ion homeostasis and mineralization

Cells transport numerous vital molecules across plasma cell membrane. Ions and molecules movement across the cell membranes is crucial for cell viability and numerous biochemical functions. Even though ATPase powered pumps are present in almost all cell types, the unique distribution in osteoblast indicates a specific and important roles in biomineralization (Fig. 5).

1.6.1 Sodium-potassium ATPase (Na+-K+ pump) and role of membranous sodium gradients on mineralization

Sodium-potassium ATPase (Na+-K+ pump) was first discovered in 1950(s) by Jens Christian Skou [59]. It belongs to P-type superfamily of ATPase pumps and presents

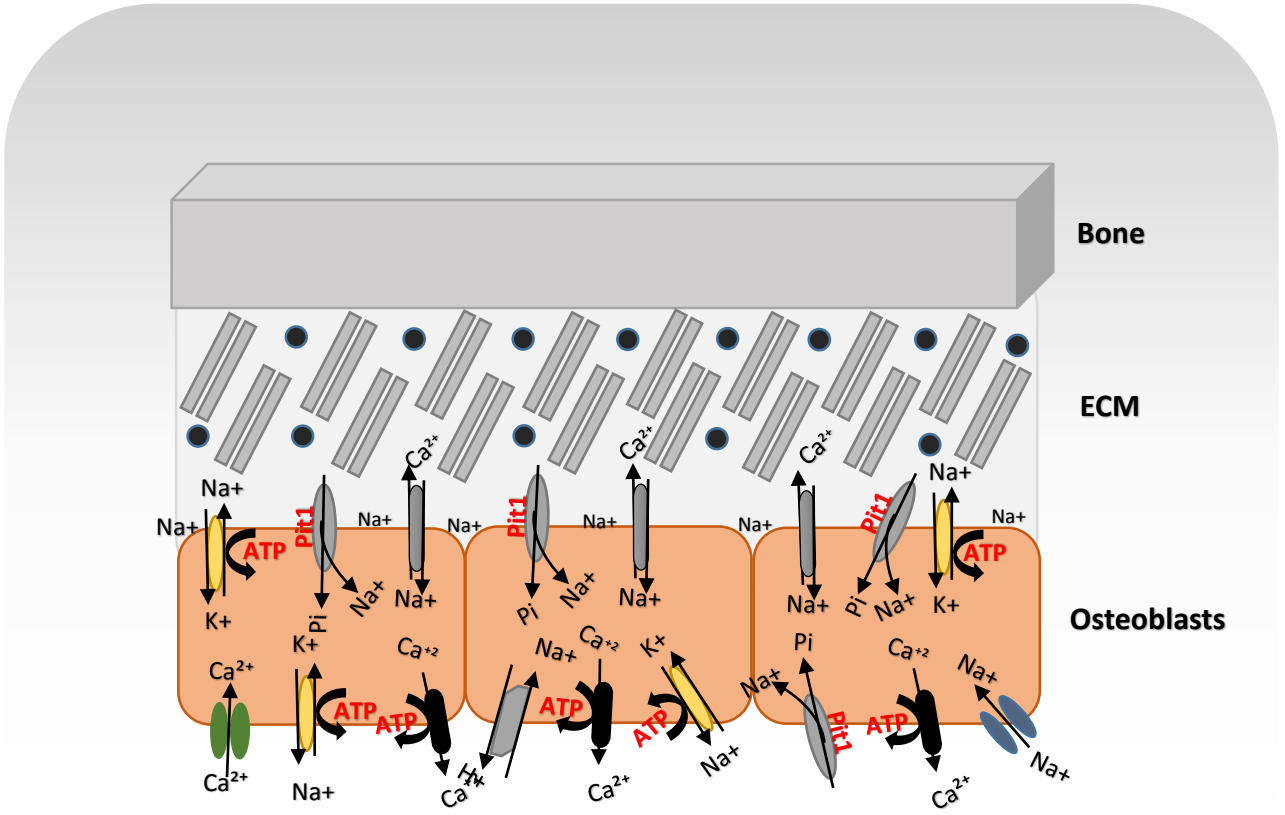

Figure 5. Ion homeostasis in osteoblast and role of ATPase pumps. Osteoblasts are polarized cells with unique distribution of ATPase pumps and transporters. Na^+-K^+ATPase pump extrudes Na^+ and creates Na^+membranous gradients essential to transport other molecules. In contrast to Ca^{2+} ATPase pump, sodium calcium exchangers NCX are distributed along plasma membrane facing mineralizing ECM and actively deliver Ca^{2+} into osteoid. Continuous Ca^{2+} ions influx occurs via voltage-gated channels. Pit1 is cotransporters of Pi and Na^+, it is the main cellular entry of ePi. Na^+ influx also occurs via sodium channels and Na^+-H^+ exchanger.

in all mammalian cells. This protein is composed of 2 domains, α and β. Currently four α and three β isoforms are identified [60]. β-Subunit is recognized as the active part of the enzyme. It contains the binding sites for a cation, nucleotides as well as the phosphorylation site [60]. Also, there is an insulin binding site. It is believed that insulin is playing a regulatory role on Na^+-K^+ pump. Data show that physiological levels of insulin lead to an increase in K^+ influx and bone formation [61]. In osteoblasts, the predominant isoform of Na^+-K^+ ATPase pump is α1 subunit with a higher affinity for Na^+ than other cations suggesting a momentous role in the activity of the cell. Hydrolysis of ATP generates the energy necessary to power this pump. The activated pump extrudes three Na^+ molecules for two K^+-ions [60]. This mechanism is essential for cell viability and various physiologic activities. Generally, moving Na^+-cation against its concentration maintains physiologic osmolality and cell volume. In addition, this pump plays a major role in keeping cell resting potential in different tissues. Moreover, extrusion of Na^+ outside cells is crucial to create membranous electrochemical gradient essential for secondary carrier mechanisms [60]. Na^+ concentrations appear to play a pivotal role in general mineral ion homeostasis. Pi, Ca^{2+}-ions as well as protons are essential for biochemical activities of bone cells. Their movement across osteoblast and osteoclast membranes is dependent on Na^+ electrochemical gradient. Lower intercellular Na^+ level is crucial to activate secondary transport carrier where Na^+ influx into the cell carrying other molecules. Studies showed that Na^+ induces Ca^{2+} efflux from osteoblasts and odontoblasts in dose-dependent manner [62].

1.6.2 Calcium translocation

In eukaryotes, Ca^{2+} is an essential ion for physiologic activities and metabolism [63]. Beside the well-known significant signalling role, Ca^{2+} is a vital molecule in neurotransmission, muscle contraction, blood clotting as well as a fundamental ion in biomineralization [64]. Ca^{2+} is absorbed in the small intestine under the influence of Ca^{2+} concentration and calciferol (vitamin D active form) and excreted via kidney, biliary and salivary glands [14]. To maintain cell viability, the continuous Ca^{2+} influx, mainly through voltage-gated channels, must be counteracted to maintain very low (50-200 nM) cytosolic Ca^{2+} levels [62]. Moreover, Ca^{2+} extrusion creates a membranous gradients crucial for Ca^{2+} to work as signalling molecules. In biomineralization, osteoblasts and odontoblasts actively deliver Ca^{2+} into bone matrix via two different mechanisms [62, 64, 65]. The main extrusion mechanisms for Ca^{2+} across the plasma membrane are plasma membrane calcium ATPase pump (PMCA) and sodium calcium exchanger (NCX) [49, 66].

1.6.2.1 Plasma membrane calcium ATPase pumps (PMCA)

Plasma membrane calcium ATPase pump (PMCA) was first discovered in erythrocytes by Schatzmannn in 1966 [49]. Later, it was identified in different species and tissues, such as human and rat kidney, placenta as well as osteoblasts [67]. Four different genes encode basic tissue-specific expression isoforms, *PMCA1* to *PMCA4*, and more diversity is created via alternative splicing [49, 68]. While PMCA1 and PMCA4 are ubiquitously present in most tissues, PMCA2 and PMCA3 are more confined to specific cells [63]. The expression rate and activities of this pump are influenced by several factors, for example, Ca^{2+} levels and Ca^{2+} regulating hormones as $1,25(OH)_2D3$ [63,

67]. In addition, both calmodulin and acidic phospholipids activate this transport protein and increase its Ca^{2+} affinity [63]. Although this protein is characterized by low transport capacity for Ca^{2+}, it has the highest affinity for it and considered as the fine tuner for intracellular Ca^{2+} levels [63].

In bone mineralization, Ca^{2+} provision to osteoid is regulated by osteoblasts [65]. Osteoblasts express at least two PMCA isoforms, PMCA1 and PMCA2 [60, 69].This ATP-powered pump extrudes one Ca^{2+}-ion per one ATP molecule hydrolysis [70]. Although the longstanding reputation of PMCA as the major delivery mechanism of Ca^{2+} into the bone ECM, growing volume of data suggest an indirect role of this pump in mineralization [69]. It was demonstrated that osteoblasts have specific spatial distribution of PMCA along cell membrane, distal to the bone matrix, and distinctly absent from the secretory side of the cells [65]. Moreover, PMCA is down-regulated during active mineral deposition phase [69]. Further, the activity of PMCA in osteoblasts is significantly lower than that in other tissues [71].Taken together, these findings support an indirect role of PMCA in mineralization , primarily maintaining physiological levels of cytosolic Ca^{2+} [60, 69].

1.6.2.2 Sodium calcium exchanger (NCX)

Sodium calcium exchanger (NCX) is a Na^{+} gradient-driven antiporter that first described in late 1960(s) [72] and in 1980, Krieger and Tashjian described it in bone organ culture [69]. Three basic isoforms were identified in mammalian cells, NCX1, NCX2 and NCX3 [66]. High levels of NCX1 are detected in excitable cells, such as neurons and heart cells [66]. During osteoblasts differentiation, NCX3 is the predominant expressed

isoform, with low levels of NCX1 [66, 69]. NCX is not an enzyme, hence it is difficult to measure its activity. Currently, the best description is available for NCX in heart and kidney cells [63]. This exchanger extrudes one Ca^{2+} ion for three Na^{+} ion influx, however, the movement can be reversed depending on membrane potential and ions concentrations [63, 66]. Membranous Na^{+} gradient maintained by Na^{+}-K^{+} ATPase is necessary to power this exchanger [65]. Because of its high Ca^{2+} capacity, NCX plays a powerful role in regulating Ca^{2+} homeostasis in different tissues [63]. NCX1 is abundantly expressed in the basolateral membrane of distal nephrons in kidneys. It is involved in regulation of systemic Ca^{2+} levels via active reabsorption of Ca^{2+} [63]. The role of this exchanger in mineralization, as a major calcium efflux mechanism, is securing more attention. In contrast to PMCA, the localization of NCX along basement membrane of osteoblasts facing the osteoid as well as the steady expression rate of this exchanger during osteoblasts differentiation and mineralization phases indicate a crucial role in mineral deposition [69]. Further, Statin and Gay showed in 2001, in vitro, that inhibition of this transport protein interferes with the process of mineralization [16, 69].

1.6.3 Sodium-dependent inorganic phosphate transporter

Sodium-dependent phosphate cotransporters (NaPiT) are the primary mechanism for cellular Pi entry. In kidney and intestine, type I NaPiT and type II NaPiT are responsible for Pi intestinal absorption, Pi renal reabsorption and maintaining proper serum Pi [28, 73]. On the other hand, type III NaPiT are extensively expressed in mammalian tissues, such as brain, lung, heart, liver, blood cells, osteoblasts, chondrocytes, MVs, smooth muscle cells and others [27, 47]. Originally, type III NaPiT were identified as receptors for

retrovirus and sub classified into PiT1 and PiT2 [29, 74]. It is widely accepted that type III NaPiT is playing a major role in mineralizing tissues [22, 28]. In biomineralization, more reports emphasizing the importance of PiT1 as a major Pi transporters. This cotransporter is confined to mineralizing chondrocytes in cartilaginous tissues, supporting the role in matrix mineralization [75].

Osteoblasts express higher levels of PiT1 than PiT2 [74] and it was suggested that Pi transport is the driving force required for induction of mineralization [76]. Further, it was reported that PiT1 mRNA is upregulated during osteoblasts differentiation correlated with osteocalcin expression and beginning of mineralization [74, 77]. Data suggested that transglutaminase enzyme is one of the key regulator of PiT1 expression [47]. Moreover, insulin-like growth factor, bone morphogentic protein-2 (BMP-2) and parathyroid hormones upregulate PiT1 mRNA expression and Pi transport in osteoblasts [73, 78]. PTH has a selective stimulatory effect on Pi transport. It has an indirect influence on NaPiT via mechanism involve cAMP production [76, 79]. In vitro studies demonstrated that cellular Pi entry is exclusively Na^+-dependent process. Pi transporters utilize inwardly directed electrochemical gradient of Na^+ ions created by Na^+-K^+ ATPase pump [29]. Furthermore, MVs essentially have Pi transport operated by Na^+ gradients [79]. An observation showed that Pi accumulation inside MVs occurs several hours before accumulation of Ca^{2+} which emphasizes the importance of Pi as an initiator of mineralization [76]. In low Na^+ environment, only a trivial amounts of Pi can enter osteoblasts via diffusion means. Inhibition of Na^+-K^+ ATPase pump by ouabain markedly decreases Pi transportation confirming the relationship between transmembranous Na^+

gradients and Pi transport [35]. Also, dramatic reduction of Pi transport occurred in vitro when Na^+ in the extracellular environment was replaced by other molecule (choline) [79].

Studies suggested that high Pi levels stimulate smooth muscle cell phenotypic transition and mineralization via type III Na/Pi cotransporters. Moreover, it was documented that phosphonoformic acid is a competitive inhibitor of Na^+-dependent phosphate cotransporters PiT1 in bone and cartilage [75]. Similarly, data showed that phosphonoformic acid interfered with Pi uptake and mineralization in vascular smooth muscle cells [30, 75]. Taken together, Pi transportation via NaPiT is necessary for both physiologic and pathologic or mineralization.

Interestingly, a study showed that chondrocytes in Hyp mice, mural form of X-linked hypophosphatemia, have low expression of PiT1, low Pi uptake and less intracellular ATP synthesis [80].

1.7 Model of mineralization: MC3T3-E1 osteoblast cultures

MC3T3-E1 is a fetal calvarial preosteoblast cell line. This cell line is an excellent model for in vitro study of osteogenesis. It recapitulates osteoblastic differentiation steps well and is regularly used in vitro to study osteogenesis [81]. Cultured MC3T3-E1 cells undergo three distinctive phases of differentiation. First, proliferative stage results in a confluent monolayer, followed by differentiation phase characterized by expression of TNAP and later osteocalcin and bone matrix proteins such as bone sialoprotein [69]. Further, MC3T3-E1 cultures have shown to produce MVs [76] as well as high expression of Pi transporters during differentiation [38]. Treatment of MC3T3-E1 with ascorbic acid

is essential to induce type I collagen matrix deposition as well as others osteogenic proteins and markers. Ascorbic acid strongly stimulates expression of TNAP and osteocalcin mRNA [82, 83].

Adding phosphate source to MC3T3-E1 cultures permits mineralization and deposition of HA nodules within collagen fibrils. β-Glycerophosphate is routinely used as a phosphate source in MC3T3-E1 cells. TNAP is required for cleavage of βGP to release Pi for HA formation [84]. Moreover, ATP and sodium monophosphate are also proved as a Pi source in MC3T3-E1 cultures for mineralization [39].

2. Aim of the study

The aim of this thesis project was to investigate the effect of ATPase activity on mineralization of MC3T3-E1 cultures. We hypothesized that ATPase activity is essential to initiate the mineralization and functions via generating Pi for mineralization as well as via powering plasma membrane pumps that regulate ion-homeostasis in osteoblasts. ATPase activity was inhibited in MC3T3-E1 osteoblast cultures using a specific chemical inhibitor and effects on mineralization and mineralization determinants were examined.

3. Materials and methods

3.1. Cell culture

Experiments were performed using the mouse calvaria pre-osteoblasts, MC3T3-E1 subclone 14, which were a generous gift from Dr. Renny T Franceshi from University of Michigan, MI, USA. For all experiments, cells were seeded in tissue culture plates at density of 50,000 cells per cm^2. Cells were grown in alpha minimum essential medium (α-MEM, Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), 0.225 mM L-aspartic acid (Sigma) and 2 mM L-glutamine (Invitrogen). Cell cultures were maintained at 37C° and 5% CO_2 atmosphere in humidified incubator, treated every second day and all experimental end-point was at day 12. Starting from day 0, 24 hours after seeding, 50 µg/ml ascorbic acid (AA) (Sigma) was added to the growth media to induce cell differentiation and deposition of ECM components. From day 6 and onward, a source of phosphate ions was added to the culture media to initiate the mineralization (Fig. 6). Three different sources of Pi were used in this study, 10 mM βGP, 5 mM SMP and 5 mM ATP, all were obtained from Sigma Aldrich. In contrast to SMP, both βGP and ATP required enzymatic hydrolysis to release Pi (Fig. 7). To inhibit the mineralization in some cell cultures two chemical inhibitors were added to the culture media starting from day 6, altogether with Pi source. ectoATPase inhibitor at 100 µM concentration (ARL67156, purchased from Sigma Aldrich) and 30 µM TNAP inhibitor (MLS0038949, Calbiochem), which was used as a control**.** ARL67156 is an ATP analogue where phosphodiester bond (P-O-P) presents in ATP is substituted by phosphomethyl bond (P-C-P) that cannot be hydrolyzed by NTPDase (Fig. 8). TNAP

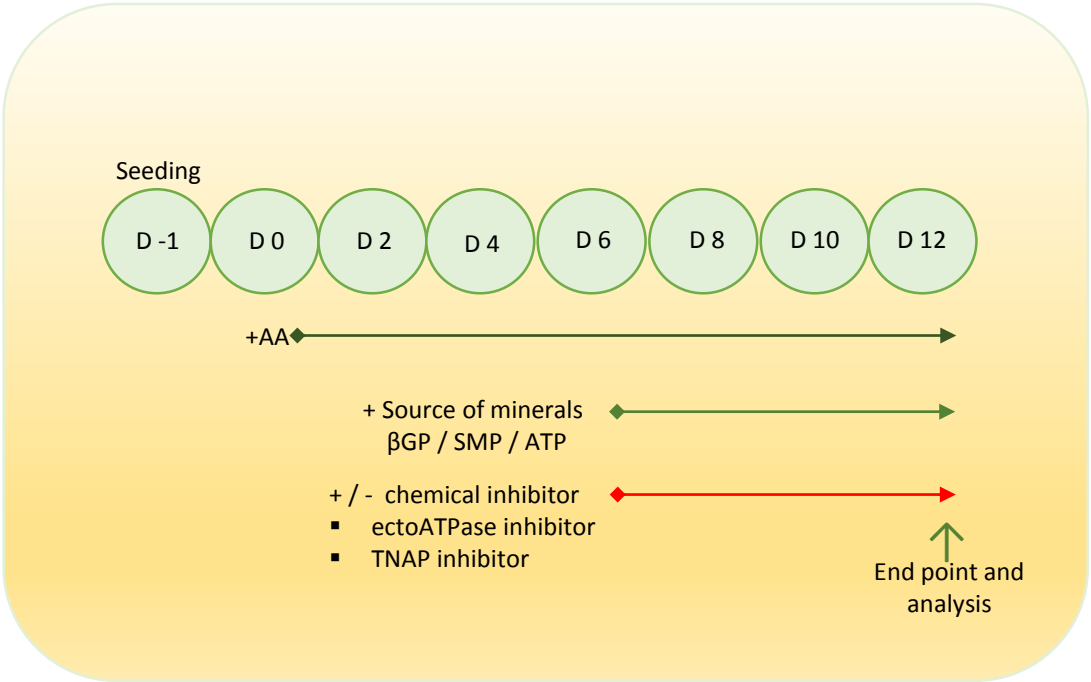

Figure 6. Experiment cell culture plan. MC3T3-E1 mouse calvarial osteoblast cells were cultured for 12 days. Cells were seeded in 35mm culture plates (day -1) at density 50,000 cells/cm² and grown in minimum essential media. Ascorbic acid (AA) was added after 24 hours (day 0) to induce differentiation and collagen I deposition, cells were treated every second day. From day 6 and onward three different sources of Pi were added (ATP, βGP or SMP) in presence or absence of mineralization inhibitors, TNAP inhibitor MLS-0038494 or ectoATPase inhibitor ARL67156. SMP is a positive control providing inorganic phosphate without the need for enzymatic cleavage. The end point of all experiments was day 12.

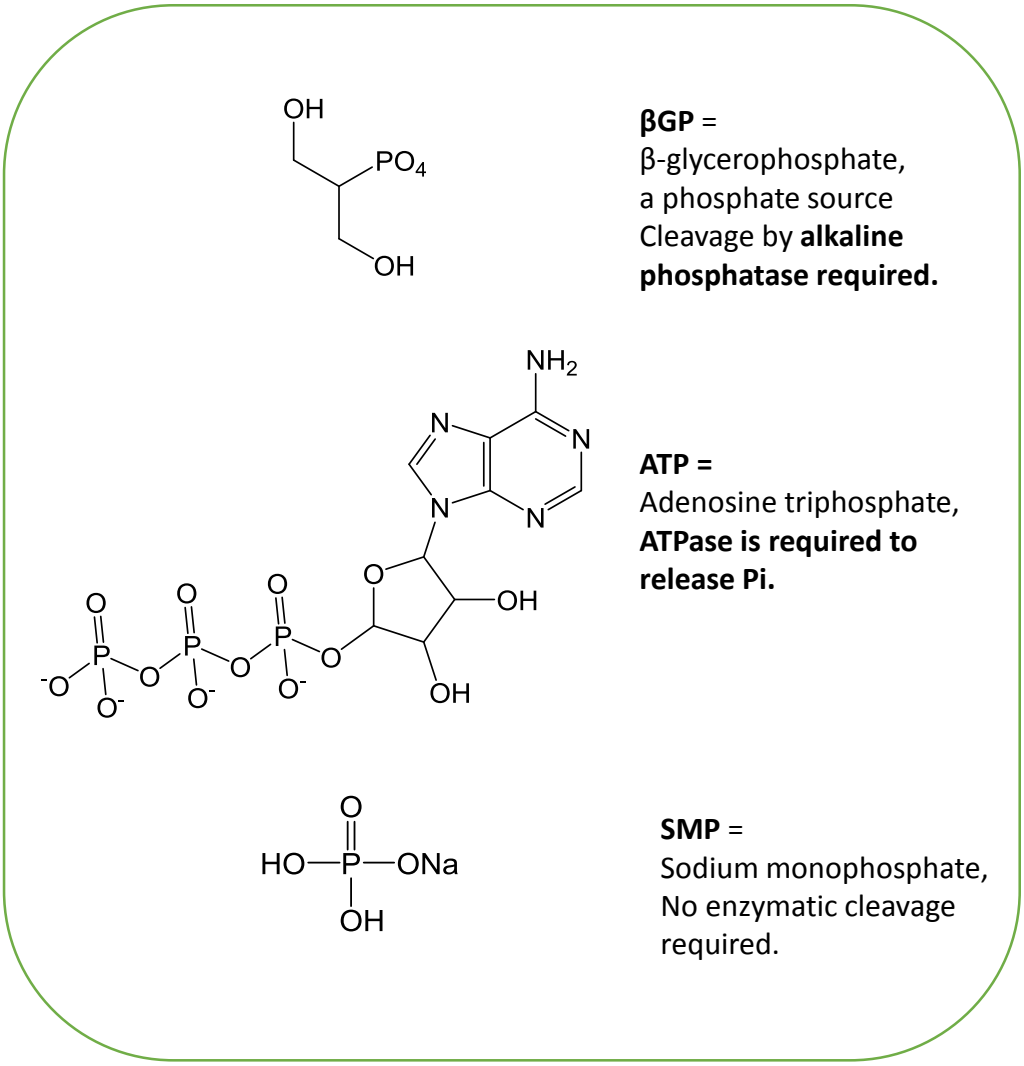

Figure 7. Molecular structures of βGP, ATP and SMP. βGP is regularly used, *in vitro*, as a source of organic Pi. It is hydrolyzed by tissue-nonspecific alkaline phosphatase to release Pi. ATP is a potent source of Pi. It is also require hydrolysis to release three Pi molecules. SMP also used *in vitro* to induce mineralization. It does not need cleavage.

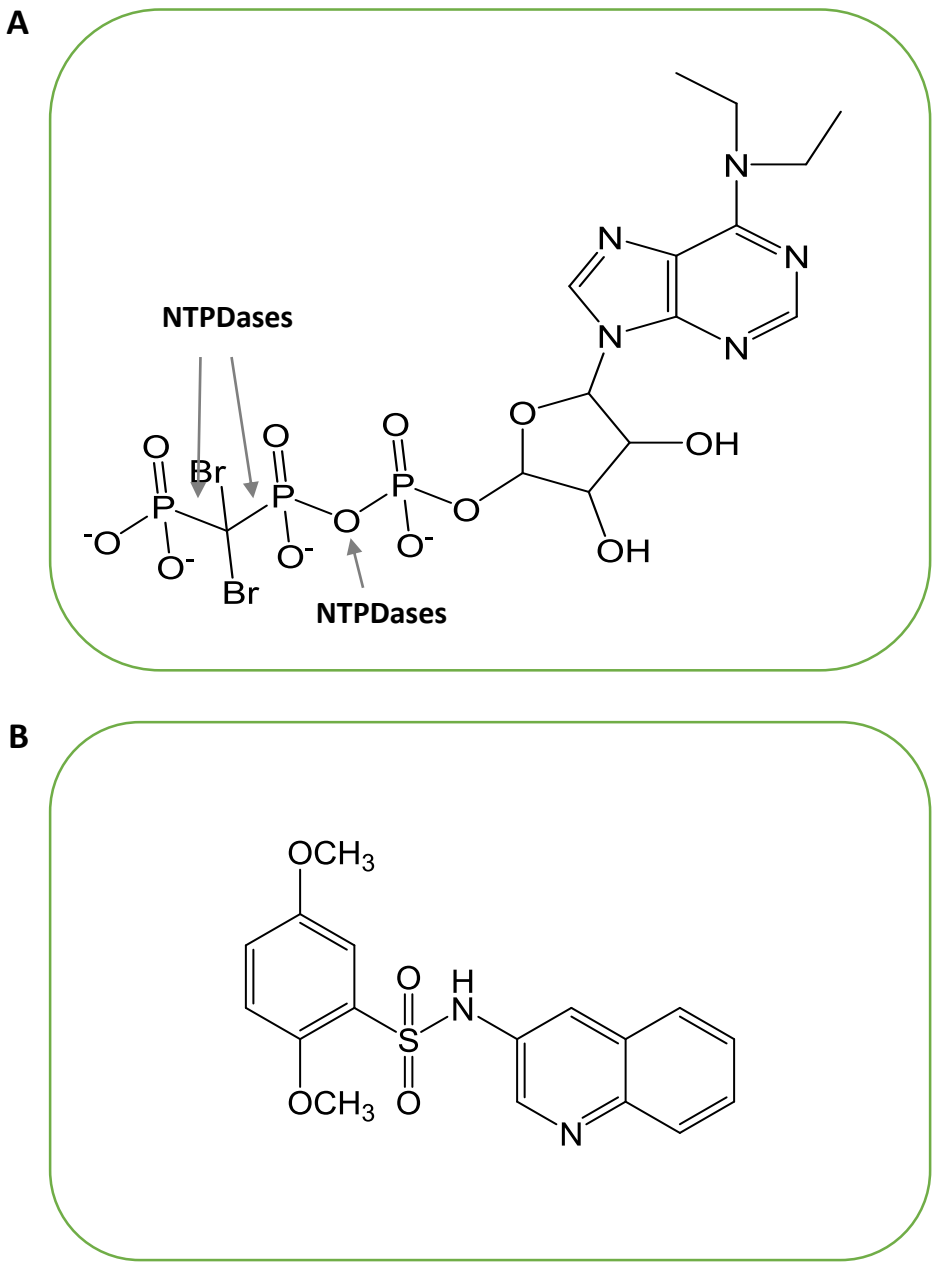

Figure 8. Molecular structure of ectoATPase inhibitor ARL67156 and TNAP inhibitor MLS-0038949. (A) ARL67156 is a structural analogue of ATP. It is non-hydrolyzable since the site of proposed cleavage by NTPDases is blocked by bromide (Br). NTPDase (nucleoside triphosphate diphosphohydrolyase). NPPS (nucleotide pyrophosphatase/phosphodiesterase). **(B) Chemical structure of TNAP inhibitor MLS-0038949**. It is a potent selective inhibitor to tissue-nonspecific alkaline phosphatase.

inhibitor MLS-0038949 is a potent and selective inhibitor for TNAP activity with no cross-inhibition for NPP1 and PHOSPHO1.

3.2. Visualization and quantification of mineralization

3.2.1 Von Kossa staining

Qualitative assessment of mineralization in osteoblast cultures was done on day 12 by Von Kossa staining using 5% silver nitrate (w/v). It is a precipitation reaction where silver nitrate interacts with Pi ions and turn to silver under light activation. To summarize the technique, cell layer was washed once using phosphate buffered saline (PBS), fixed by 95% ethanol and incubated for 15 minutes at 37°C. Then, cell layer was rehydrated gradually using ethanol consecutive concentrations of 80%, 50%, 20% and rinsed at the end twice with distilled water. Aqueous solution of silver nitrate was added and incubated in dark for one hour at 37°C. Cell/matrix layer was washed twice with distilled water and the plate was exposed to bright light for minimum of 30 minutes. Mineralization was visible as metallic silver precipitants.

3.2.2 Calcium and phosphate assays

Quantification of mineralization, in the presence or absence of the inhibitors, was performed for the cell/matrix layer and cell lysate. Cell/matrix layer were washed twice with PBS, insoluble Ca^{2+} deposits were dissolved in 0.5 M HCl. Samples were centrifuged and Ca^{2+} and Pi concentrations in the supernatant were measured spectrophotometrically using Ca^{2+} and Pi assay kits (Sekisui Diagnostics). To quantify the Ca^{2+} and Pi in the cell

lysates, RIPA buffer was used for extraction followed by sonication and centrifugation. The mineral levels assessed in the supernatant using the same kits.

3.3. Picrosirius staining

Picrosirius red method was used to stain type I collagen in the mineralizing MC3T3-E1 cultures. Cell layers were washed three times using PBS then fixed with Bouin's fluid (70% v/v saturated aqueous solution of picric acid, 9.25% v/v formaldehyde and 5% glacial acetic acid) for one hour at room temperature. Next, the cultures were washed twice and immersed in distilled water for 15 minutes, left to air-dry. Picrosirius solution, direct Red (sigma) 1 mg/ml in saturated aqueous picric acid, was added and incubated for 1 hour at room temperature with mild shaking. Unbound dye was dissolved and removed by washing twice with 0.01N HCl. At this stage all plates were photo documented.

3.4. Pyrophosphate assay

PPi amount was measured in the mineralizing cultures in media and in the cell lysates using EnzCheck® Pyrophosphate Assay kit (Molecular PROBES). Assay was performed following the manufacturer's instructions. The mechanism of the assay is based on principle described originally by Webb [85]. Each PPi molecule is cleaved by inorganic pyrophosphatase enzyme into two equivalent Pi. Then the Pi is detected by the conversion of 2-amino-6-mercapto-7-methylpurine ribonucleoside to 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase. Total Pi concentration was measured by spectrophotometer at 360 nm. For this assay the controls were samples

without the active component, inorganic pyrophosphatase enzyme. Kit is sensitive to PPi as little as 1 nanomole.

3.5. Sodium assay

Sodium Reagent set (BQ Kits) was used to quantify amount of Na^+ in MC3T3-E1 cultures. The Na^+ was assessed in cell lysates and media. The concept of this assay was first described by Maruna [86] and Trinder [87]. Fresh samples were used and reaction solutions were prepared following the manufacturer's instructions. Reaction mix develops a chromophore with absorbance inversely related to the Na^+ concentrations. Na^+ was measured at absorbance 550 nm spectrophotometer and data analysed based on the provided equation.

3.6 ATP assay

MC3T3 cells were seeded in 35 mm culture plates at density of 50,000 cells per cm^2. ATP was measured in conditioned (serum-free) media at day 0, day 4 and then every second day until day 12. At the selected time points, conditioned media were collected and assayed for ATP concentration using colorimetric assay (ATP Assay Kit, Abcam). The kit is able to measure as low as 1 µM of ATP. Kit was used according to manufacturer's instructions, standard curve was plotted and the trendline equation was used to calculate concentrations of ATP in the samples.

3.7 Cell viability assay

MTT assay (Thiazolyl Blue Tetrazolium Bromide, Sigma) was used to determine the effects of all different chemical compounds on the viability of MC3T3-E1 osteoblast cells. For this assay, cells were cultured in 96-well plates. On day 0, 4, 6, 10 and 12, the media were replaced by MTT aliquot in culture media (0.25 mg/ml). Culture plates were incubated in dark at 37°C/CO_2 for three hours. After incubation, MTT solution was removed and the precipitants were dissolved in 200 µl DMSO (dimethyl sulfoxide) per well. The ability of mitochondrial dehydrogenases to reduce yellow MTT solution and developing a purple product reflects the viability of the cells that is measured spectrophotometrically at 565 nm.

3.8. TNAP activity assay

TNAP activity was assessed in all cultures starting from day 0 and every 48 hours up to day 12. At the designated time intervals, cell/matrix layers were washed twice with PBS and lysed with 10 mM Tris pH 7.4 (containing 0.2% IGEPAL and 2 mM phenylmethanesulfonylfluoride protease inhibitor (PMSF)). Cell suspensions were sonicated, centrifuged and supernatants were used for the assay. TNAP activity was measured in triplicate using 1 mM p-nitrophenyl phosphate tablets as a substrate (SIGMAFAST™, Sigma). The substrates hydrolyzed by TNAP and converted into p-nitrophenol, a yellow end-product. Substrate solutions were prepared and incubated until color develops (15-45 minutes). The reaction was stopped using 0.5 M NaOH and the activity was determined by reading absorbance at 405nm. Serial dilution of TNAP (1 unit/10 µl, Sigma) was generated and used as a standard.

3.9. Protein extraction and Western Blotting

After 12 days of treatments, Cell/matrix layers were lysed in following buffer; 1% Triton 100X, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mM Tris, 150 mM sodium chloride, 0.5 M EDTA supplemented with 1 mM PMSF. The extracts were sonicated for one minute on ice and centrifuged at 10,000 rpm for 30 minutes at 4°C. Total protein concentrations in the supernatant were determined by Bichinonic Acid (BCA) protein assay (Fisher Scientific). Ten (10) µg protein from each sample was dissolved in SDS-loading buffer containing β-mercaptoethanol and boiled for 5 minutes. Electrophoretic separation of proteins was done using 10% SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membrane (PVDF) (Bio-Rad). Membranes were blocked for one hour at room temperature in 5% non-fat milk powder in Tris Buffered Saline-Tween 20. Incubation with rabbit anti-mouse HRP-conjugated OPN was done for one hour at room temperature. β-actin rabbit polyclonal antibody (Sigma) was used as a protein loading control. Bands were visualized using ECL Prime Western Blotting Detection Kit (GE healthcare) and chemiluminescence was detected using Hyperfilm ECL (GE Healthcare).

3.10. RNA isolation and RT-PCR analyses

On day 12 of culture, total RNA was extracted using TRIzol® reagent. Osteoblasts cell/matrix layer was extracted by adding 1 ml of TRIzol reagent per 35mm culture plate. RNA isolation protocol was followed and RNA precipitated as a pellet which washed and dissolved in DEPC water (75 % ethanol prepared in diethylpyrocarbonate treated distilled water).

One µg of total DNased-treated RNA was reverse-transcribed, denatured and amplified using the Superscript® III One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitorgen).

RNA was analyzed for gene expression of type 1 collagen (*Col1a1*), osterix (*Osx*), osteocalcin (*Ocn*) and osteopontin (*Opn*). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was analyzed as a housekeeping gene. Following the recommended PCR cycles, the amplified products were electrophoresed on 2% agarose gels, stained with ethidium bromide and bands were visualized by UV light illumination.

Primers were obtained from Invitrogen™, Life Technologies, prepared according to manufacturer's protocol. The sequences used are as the follows:

Gene name	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)
Col1a1	GAGGCATAAAGGGTCATCGTGG	CATTAGGCGCAGGAAGGTCAGC
Ocn	CTGGCCCTGGCTGCGCTCTGT	GGTCCTAAATAGTGATACCGTAGATGC
Osx	CCCTTCTCAAGCACCAATG	GCCTTGGGCTTATAGACATC
Opn	CTGCTAGTACACAAGCAGACA	CATGAGAAATTCGGAATTTCAG
Gapdh	TGGCAAAGTGGAGATTGTTG	TTCAGCTCTGGGATGACCT

3.11. Statistical analysis

Results were statistically analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Bonferroni's multiple comparison test. For the statistical significance, P-value was set as $p^* \leq 0.05$, $p^{**} \leq 0.01$, and $p^{***} \leq 0.001$. All experiments were done in duplicate or triplicate and error is presented as ± SEM.

4. Results

4.1 ATP-mediated mineralization is significantly affected by TNAP inhibitor indicating a major of TNAP in mineralization and ATP is a TNAP substrate

To examine the role of ATPase activity in mineralization, we first sought to investigate if TNAP can hydrolyze ATP for mineralization. For this we used two TNAP inhibitors, tetramisole hydrochloride (levamisole) and MLS-0038949, latter one which is a potent and uncompetitive selective inhibitor for p-nitrophenylphosphatase (pNPPase) and pyrophosphatase activities of TNAP with no cross-inhibition for NPP1 and PHOSPHO1 [37, 45]. MC3T3-E1 cells were cultured in growth media supplemented with AA and 4 mM ATP for 12 days. Both TNAP inhibitors were added to ATP-treated cultures starting from day 6 and media were replaced every second day. At the end of the experiment, Von Kossa and Picrosirius staining were done to visualize the effect of the inhibitors on mineralization and type I collagen deposition, respectively. Also, Ca^{2+} and Pi levels were quantified in the cell /matrix layers of the cultures. As seen in Figure 9A, ATP supplementation allowed normal mineral deposition. Tetramisole treatment did not abolish ATP-mediated mineralization, however, MLS-0038949 did eliminate all Von Kossa staining. Pi and Ca^{2+} levels were significantly reduced with TNAP inhibitor ML-0038949 (Figs. 9B, 9C). This suggests that ATP-mediated mineralization requires TNAP. Type I collagen deposition was not altered with either inhibitor treatments (Fig. 9A). As control experiment, we compared the effect of MLS-0038949 on βGP, ATP and SMP treated cultures. As seen in Von Kossa staining in Figure 10A**,** MLS-0038949 TNAP abolished ATP and βGP-mediated mineralization but did not interfere with mineralization in cultures

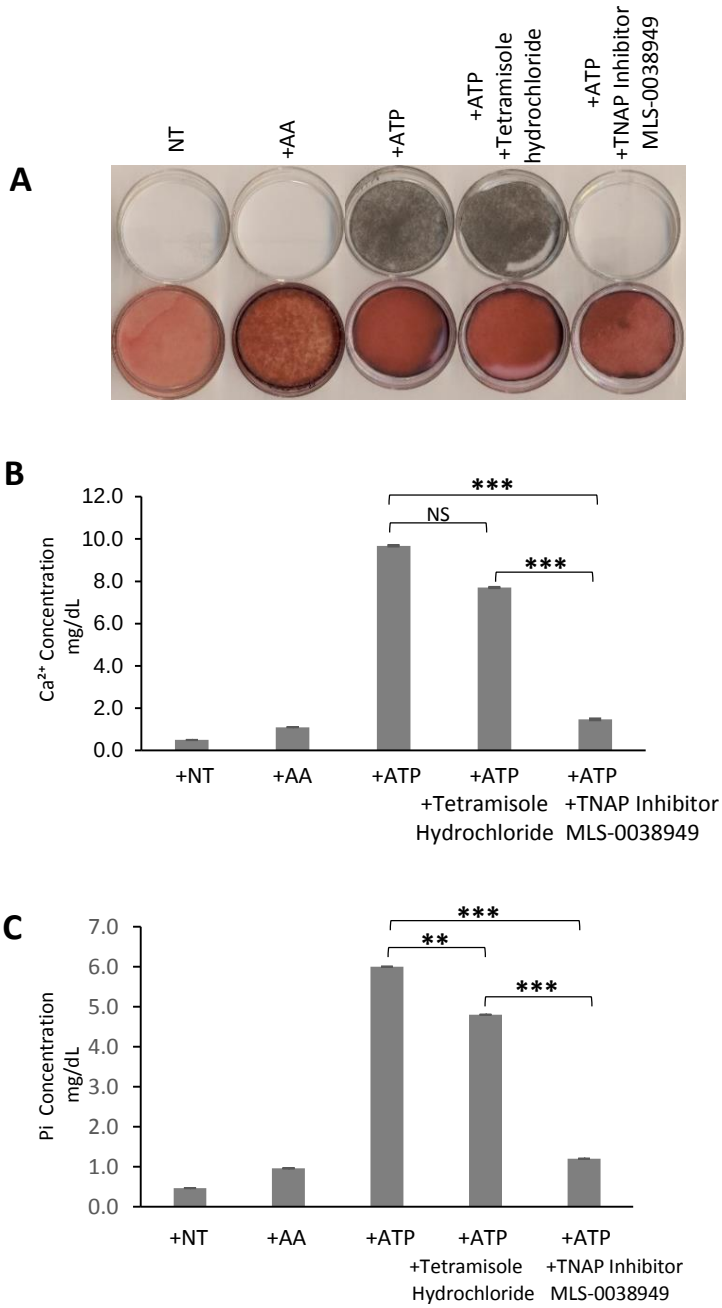

Figure 9 . TNAP Inhibitor MLS-0038949 does abolish ATP-mediated mineralization. MC3T3-E1 Cultures were treated with ATP as a source of inorganic phosphate starting from day 6. Tetramisole hydrochloride and MLS-0038949 were added and analysis was done on day12. **(A)** Von Kossa staining for the cultures demonstrate the lack of mineralization with TNAP inhibitor MLS-0038949. Picrosirius staining show comparable amounts of collagen I in presence and absence of the inhibitors indicating that inhibition of mineralization does not related to disrupted collagen I deposition. **(B** and **C)** Calcium and phosphate quantification from the cell/matrix layers indicate that MLS-0038949 inhibitor significantly interferes with the mineralization process in ATP-mediated mineralization. Tetramisole hydrochloride does not show inhibitory effect. NS (no significance), *** $P \leq 0.001$ ** $P \leq 0.01$ and error bars represent SEM.

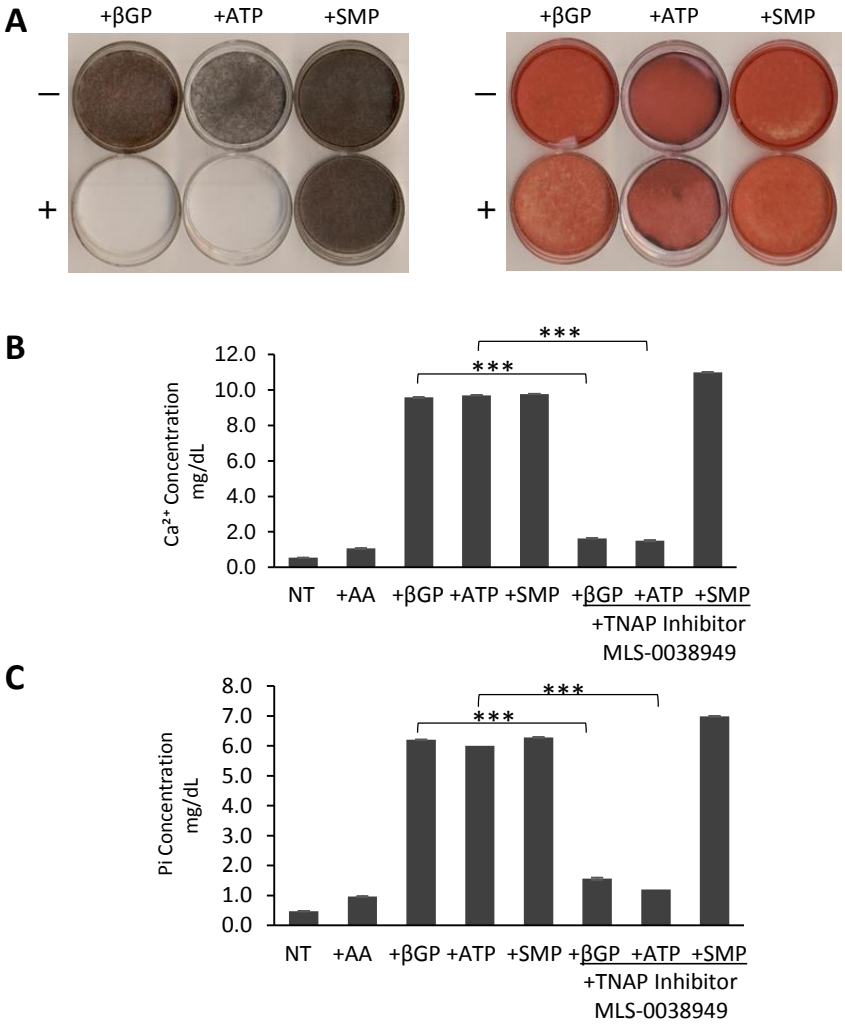

Figure 10 . TNAP Inhibitor MLS-0038949 interferes with mineralization process in MC3T3-E1 cultures treated with βGP or ATP. (A) Von Kossa staining for MC3T3-E1 cultures show that SMP-mediated mineralization is resistant to TNAP inhibitor MLS-038949 as expected since it does not require enzymatic cleavage. TNAP inhibitor MLS-0038949 abolished mineralization in βGP and ATP. Collagen I deposition was not disturbed in presence of the inhibitor, as seen in Picrosirius staining. **(B and C)** Analysis of calcium and phosphate from the cell/matrix layers mirror what is seen with Von Kossa staining. *** $P \leq 0.001$ and error bars represent SEM.

treated with SMP, as expected. Analysis of Ca^{2+} and Pi concentration in cell layers reflects Von Kossa results (Figs. 10B, 10C).

4.2 Inhibition of ATP hydrolysis prevents mineralization in MC3T3-E1 cultures

As we are interested in the role of ATPase activity in MC3T3-E1 cells, we examined the effect of ectoATPase inhibitor ARL67156 on mineralization. Cells were cultured as above, and Pi sources (βGP 10 mM, SMP 5 mM and ATP 4 mM) were added from day 6 to day 12 altogether with ectoATPase inhibitor (100 μM). Von Kossa staining demonstrated that inhibition of ATP hydrolysis by ectoATPase inhibitor ARL67156 results in significant lack of mineralization in all cultures with different Pi sources (Fig. 11A). Interestingly, mineralization was completely abrogated also in cultures supplemented with SMP which does not require phosphatase activity to generate Pi suggesting that ATPase inhibition interferes with more than Pi generation. Picrosirius staining of all cultures demonstrated that cultures deposited normal amounts of type I collagen confirming that normal mineralization scaffolds were available (Fig. 11A). Analysis of Ca^{2+} and Pi levels in the cell/matrix layers reflect the dramatic loss of mineral deposition in βGP and SMP mineralized cultures (Fig. 11B).

Osteoblasts differentiation was not altered as measured by expression of osteoblast markers (Fig. 12). We cultured MC3T3-E1 cells treated with βGP or SMP for 12 days in 35mm plates. ARL67156 and TNAP inhibitor MLS-0038949 were used too. On day 12, total RNA were extracted, samples and primers were prepared for RT-PCR. One μg of total RNA were processes for gene expression of type I collagen, osterix,

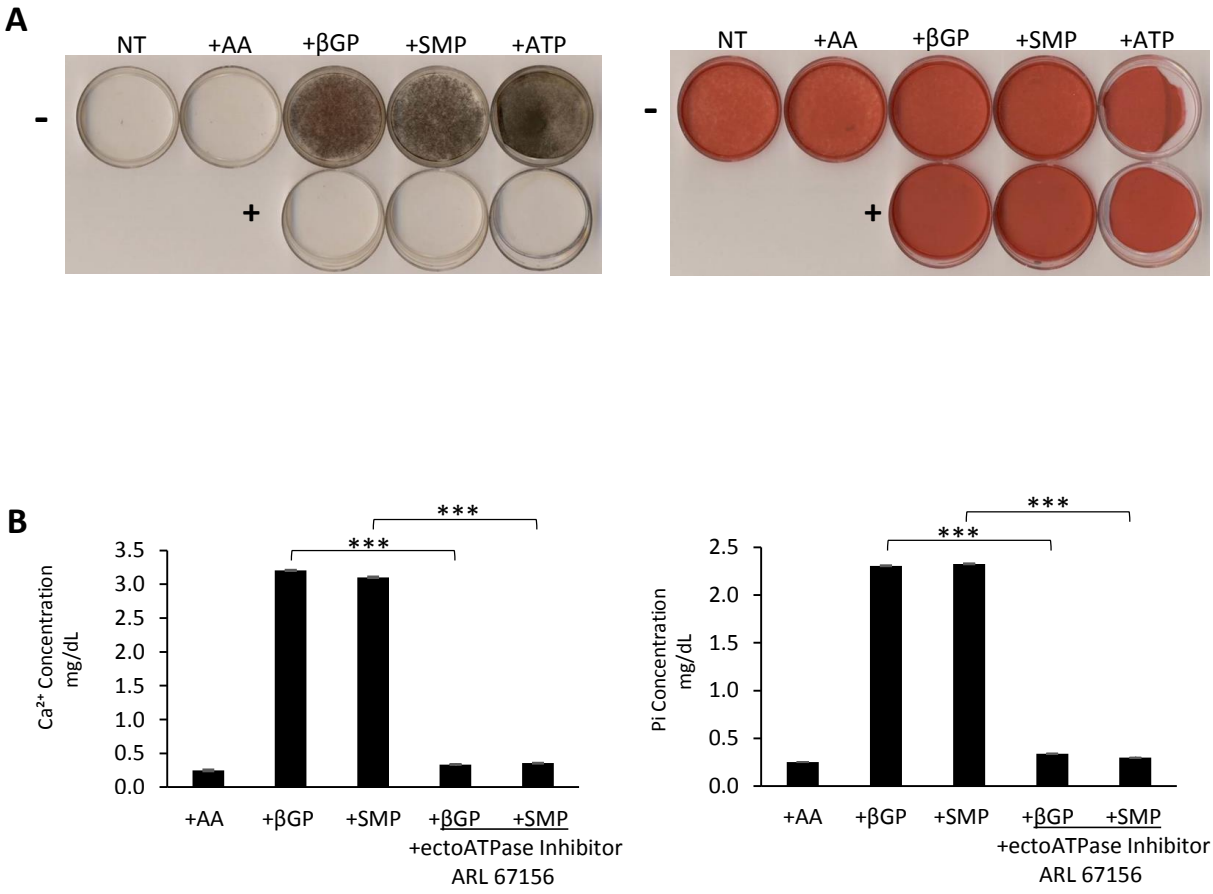

Figure 11 . Mineralization is thoroughly impaired with ectoATPase inhibitor ARL 67156 in βGP-, ATP- and SMP-mediated mineralization in MC3T3-E1 cultures. ARL67156 was added to the cultures from day 6, replaced every 48 hours and the analysis was done on day 12. **(A)** Von Kossa staining shows that the inhibitor completely abolished mineralization in all cultures. Collagen I deposition was not changed with this treatment. (**B)** Ca^{2+} and Pi analysis in the cell/matrix layer confirms the Von Kossa result. Mineralization was significantly interfered with ARL67156 including SMP treated ones that **does not** require Pi generation mechanism. *** P≤ 0.001 and error bars represent SEM.

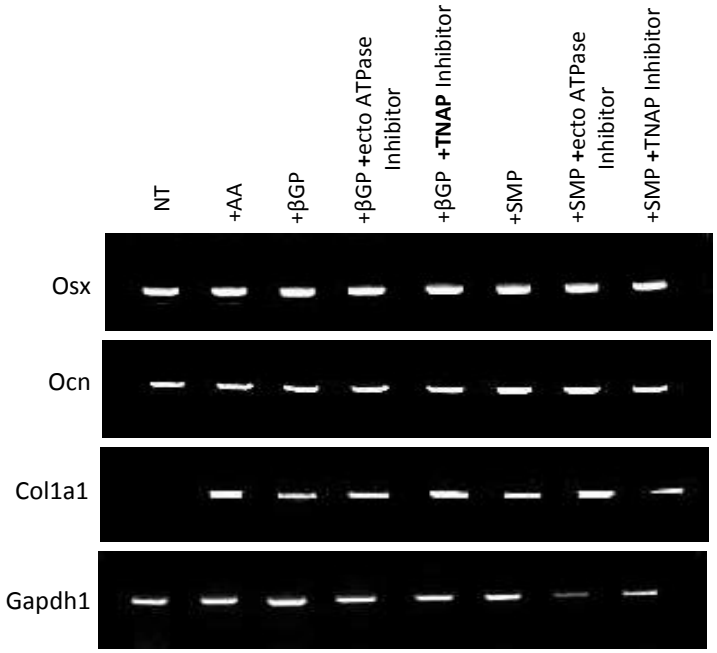

Figure 12. RT-PCR analysis of mRNA expression of *Col1a1* and osteoblast markers *Osx, Ocn* in MC3T3-E1 cultures. On day 12 total RNA was extracted from cultures treated with growth media only (NT), supplemented with ascorbic acid (AA) and with Pi sources (βGP and SMP) in presence and absence of mineralization inhibitors, ectoATPase and TNAP inhibitor. RT-PCR analysis indicates that mRNA expression of the osteoblast genes were not affected by the above chemical inhibitors. Gapgh1 expression was used as a baseline gene.

osteocalcin and osteopontin. Gapdh was analyzed as a housekeeping gene. Unaffected Gapdh levels in all cultures with different treatments indicates that treatments do not alter basal cell expression.

4.3 ATP is released to ECM in βGP-treated cultures

To begin investigation how ATPase activity may affect mineralization in βGP and SMP mineralized cultures, we hypothesized that osteoblasts likely generate their own extracellular ATP. Thus, we continued examining only these two modes of mineralization (βGP and SMP) and examined the ATP levels in osteoblast conditioned media of these cultures during mineralization phase at selected time points (day 0, day 4, day 6, day 8, day 10 and day 12). The complete growth media were replaced by serum-free media (conditioned media) 24 hours before media collection and ATP measurement. Interestingly, data showed that extracellular ATP concentration progressively increased in βGP-treated cultures starting from day 6, reaching maximum on day 12 (Fig. 13). On the other hand, ATP release was reduced after day 6 in SMP-mediated mineralization. This data suggest that ATP is released from cells during mineralization phase upon βGP treatment and affects mineralization of this particular scenario.

Next we asked if ATPase activity is essential to initiate mineralization and how the mineralization is affected if we block the ATP hydrolysis at specific days of mineralization phase. As seen in Figure 14, we designed an experiment that ectoATPase inhibitor was added to the cultures at selected days and the results were analyzed by Von Kossa staining on day 12. As shown in Figures 15A and 15B, blockage of ATP hydrolysis in βGP- and SMP-mediated cultures at day 6-8 did not interfere with mineral deposition.

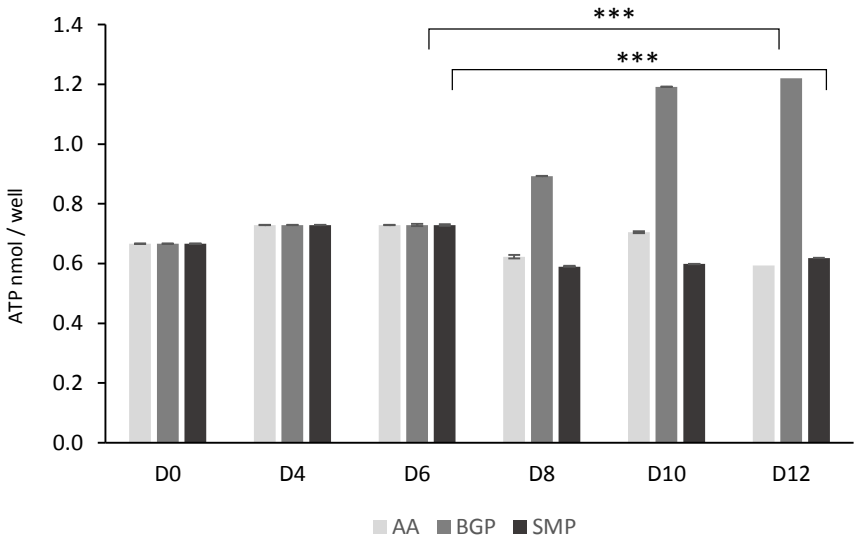

Figure 13. ATP levels were increased progressively in βGP-treated MC3T3-E1 cultures. MC3T3-E1 cultures were treated with AA supplemented media. From day 6, βGP or SMP were added. AA treated served as the control . At selected time points, ATP concentration in the conditioned media was measured and compared at day 6 and day 12. The graph demonstrates two different trends of ATP release. Two-Way ANOVA statistical analysis showed a significant increase of ATP levels synchronized with initiation of mineralization in the culture, peak at day 12. On the other hand, SMP-mediated mineralization showed a reversed pattern where ATP amounts fell significantly after day 6. *** $P \leq 0.001$ and error bars represent SEM

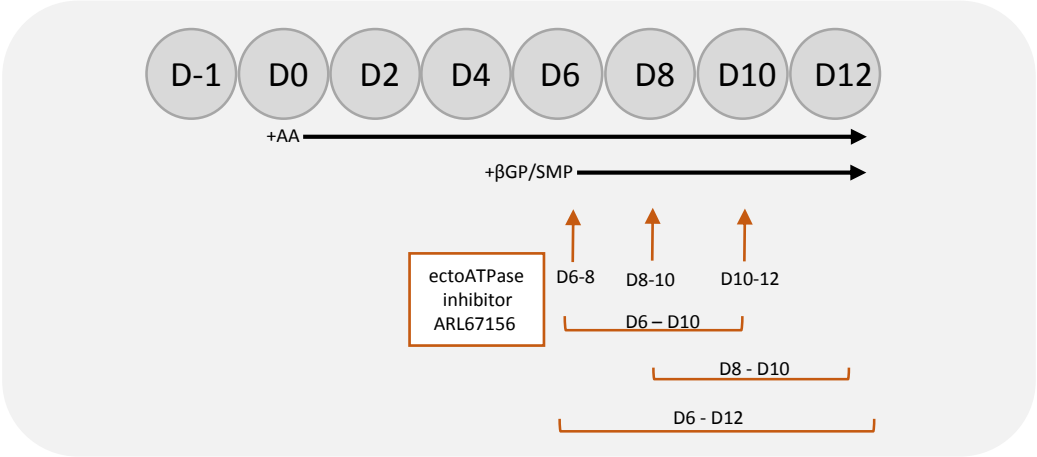

Figure 14. Experimental design of ectoATPase inhibition at selected time points. MC3T3-E1 were seeded in 35mm culture plates at density of 50,000cells per cm² . Ascorbic acid is added to the growth media starting from day 0. Source of phosphate, βGP and SMP, were added from day 6. ectoATPase inhibitor (100µM) was added to the mineralizing cultures at selected days. It was used at day 6-8, day 8-10 or day 10-12. In other cultures, the inhibitor was added for prolonged time; day 6-10 to 8-12 or day 8 to 10 and 6 to12. Von Kossa was done for all cultures at day 12.

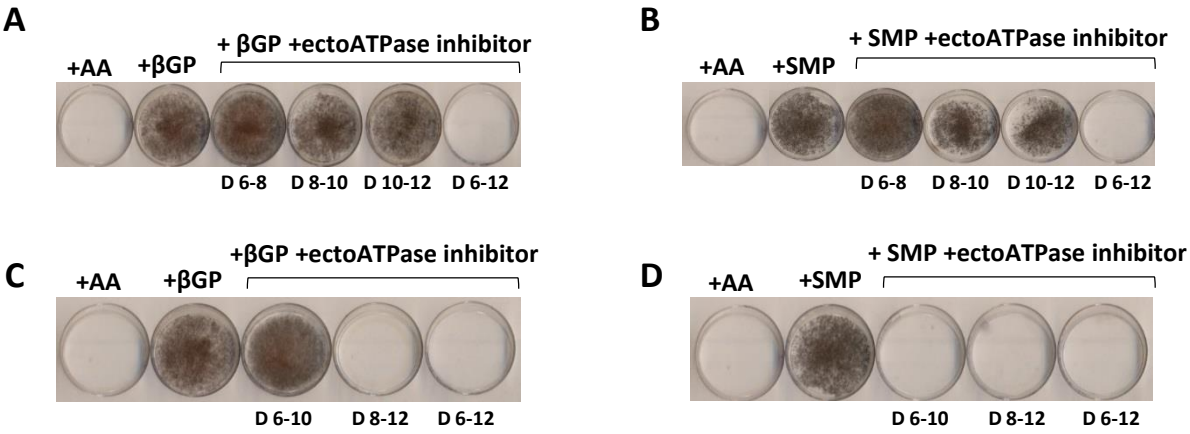

Figure 15. Von Kossa staining for MC3T3-E1 cultures treated with ectoATPase inhibitor at selected time points. **(A and B)** Blocking of ATP hydrolysis at day 6 to day 8 did not interfere with mineralization of βGP-and SMP-treated cultures. However, mineralization in both treatments were partially affected by adding ARL67156 at day 8 to 10 or day10 to 12. **(C** and **D)** Adding ectoATPase inhibitor at two subsequent treatment days ended by different results in βGP-and SMP-mediated mineralization. Comparing to βGP-treated cultures, mineralization with SMP appears to be more susceptible to inhibition by ectoATPase inhibitor.

However, mineralization was partially disturbed when the inhibitor was added at day 8-10 or 10-12. Inhibition of ATPase activity for longer time (days 8-12 or 6-12) showed a powerful blockage of mineralization especially with SMP-mediated mineralization (Figs. 15C, 15D).

4.4 Extracellular pyrophosphate concentrations and alkaline phosphatase activity in MC3T3-E1 cultures treated with ectoATPase inhibitor ARL67156

To understand the mechanisms of the complete inhibition of mineralization in all cultures, especially SMP-mediated one, we sought to measure Pi and Ca^{2+} levels at day 12 in complete media and cell lysates, and compare Pi levels in cultures treated with TNAP inhibitor as a control. As seen in Figure 16B and 17B**,** Pi levels in the media remained high in presence of ectoATPase inhibitor in both βGP- and SMP-cultures. However, and as expected, ePi levels dramatically decreased in βGP-treated media supplemented with TNAP inhibitor. Although high Pi levels in the media, iPi levels were dramatically reduced in cell lysates of MC3T3-E1cultures treated with ectoATPase inhibitor (Figs. 16C, 17C). Low levels of iPi in cell lysates reflected as lack of mineralization as seen in Von Kossa staining (Figs. 16A, 17A).

To determine whether ectoATPase inhibitor interferes with mineral deposition by increasing ePPi levels, we measured PPi indirectly using EnzCheck pyrophosphate kit. The assay contains pyrophosphatase enzyme that hydrolyzes PPi into two Pi and measures total Pi in the samples. Samples were taken from conditioned media and cell lysates. All samples were duplicated and assayed with and without the active component

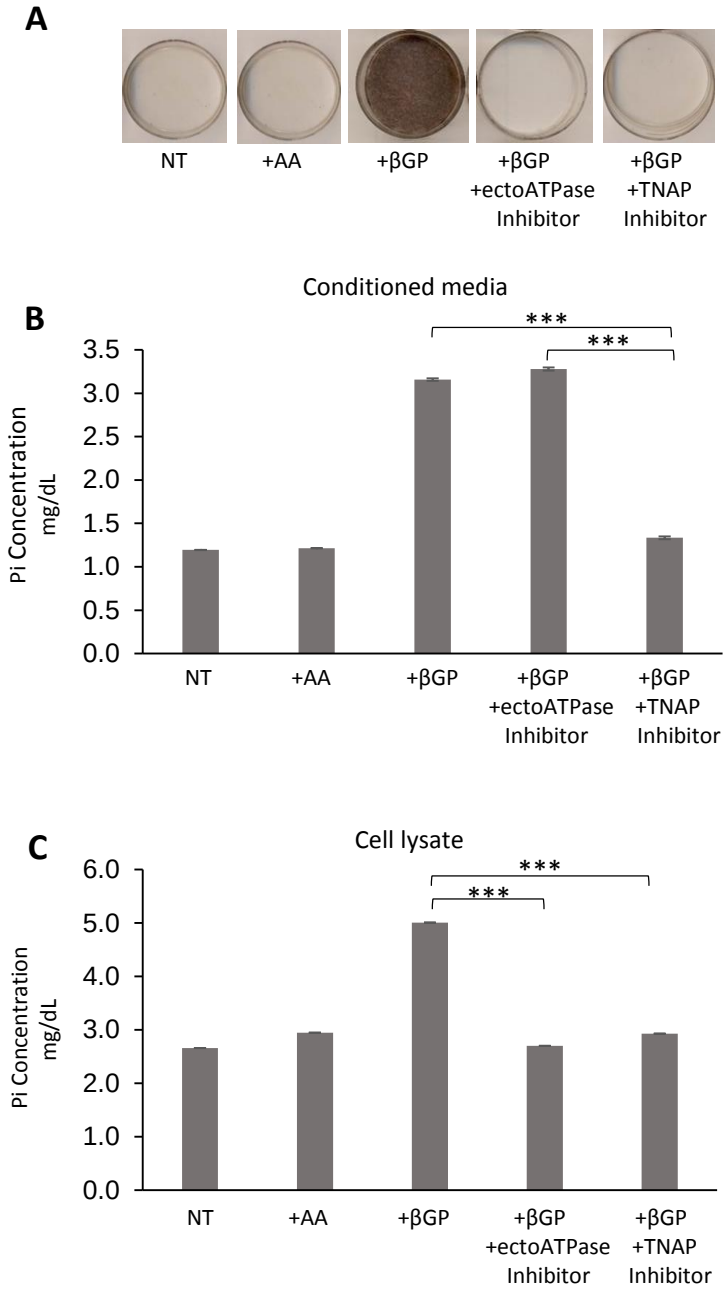

Figure 16. ectoATPase inhibitor ARL67156 does not affect inorganic phosphate concentrations in βGP-treated media. Analysis of Pi levels in βGP-mediated mineralization with ectoATPase inhibitor and TNAP inhibitor MLS-0038949 . **(A)** Von Kossa staining βGP-mediated mineralization in presence and absence of ectoATPase inhibitor and TNAP inhibitor, both inhibitors prevent mineral deposition. **(B)** In contrast to ectoATPase inhibitor, TNAP inhibitor significantly reduced Pi levels in conditioned media . **(C)** Pi levels in cell lysates. Although Pi level is high with ectoATPase inhibitor, Pi levels in cell lysates decreased significantly. *** $P \leq 0.001$ and error bars represent SEM.

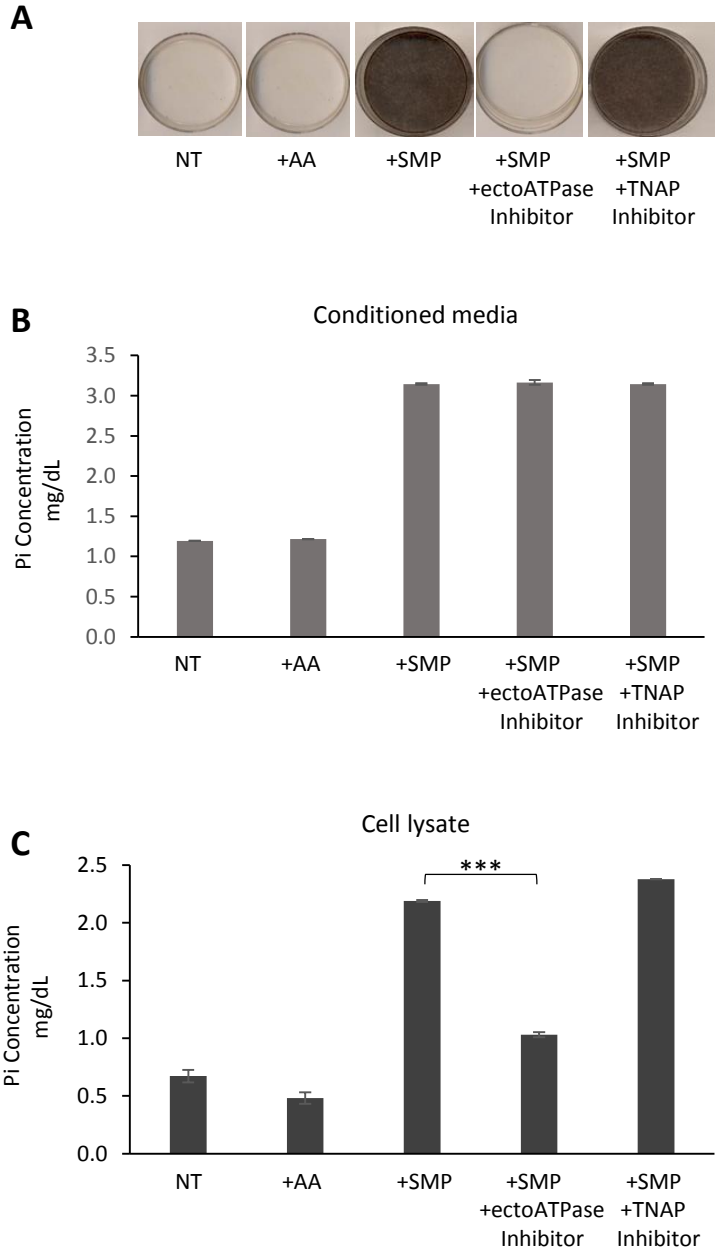

Figure 17. In SMP-treated cultures, intracellular inorganic phosphate levels were reduced only when cultures supplemented with ectoATPase inhibitor. (A) Von Kossa staining showed that ectoATPase inhibitor abolished mineralization in SMP-mediated mineralization. **(B)** analysis of Pi levels in the conditioned media showed high levels of Pi in presence of both inhibitors. **(C)** Pi levels in cell lysates were significantly decreased **in** SMP cultures treated with ectoATPase inhibitor**.** *** $P \leq 0.001$ and error bars represent SEM.

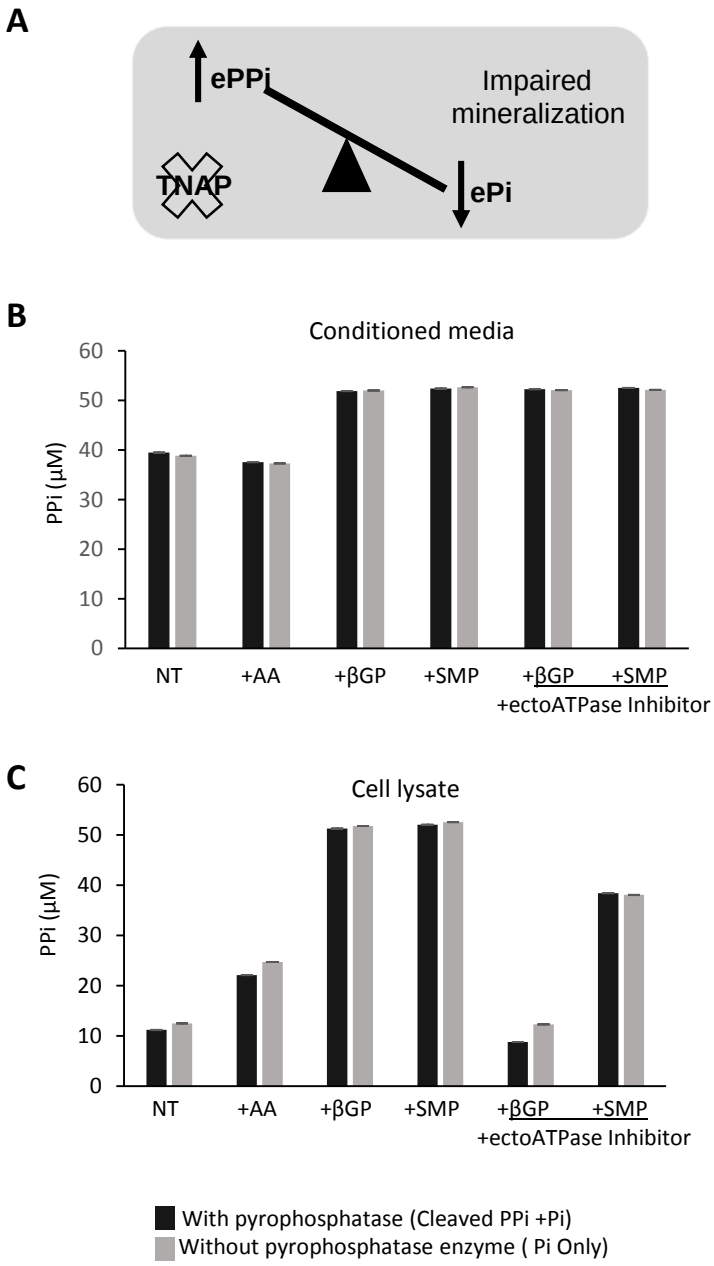

Figure 18. Extracellular pyrophosphate (ePPi) levels were not increased with ectoATPase inhibitor. (A) ePPi is a substrate of TNAP and a potent inhibitor of mineralization. Defective TNAP activity results in accumulation of ePPi and impaired mineralization**.** Levels of ePPi were investigated in conditioned media and cell lysates of MC3T3-E1 cultures treated with ectoATPase inhibitor. ePPi assay contain a pyrophosphatase enzyme that cleaves PPi and measures total Pi. Samples were duplicated, assayed with and without pyrophosphatase enzyme and results were compared. (B) Data show that ePPi levels in the conditioned media **are not increased** with ectoATPAse treatment. (**C**) PPi levels are neither increased in βGP nor SMP cultures treated with ectoATPase inhibitor. Error bars represent SEM.

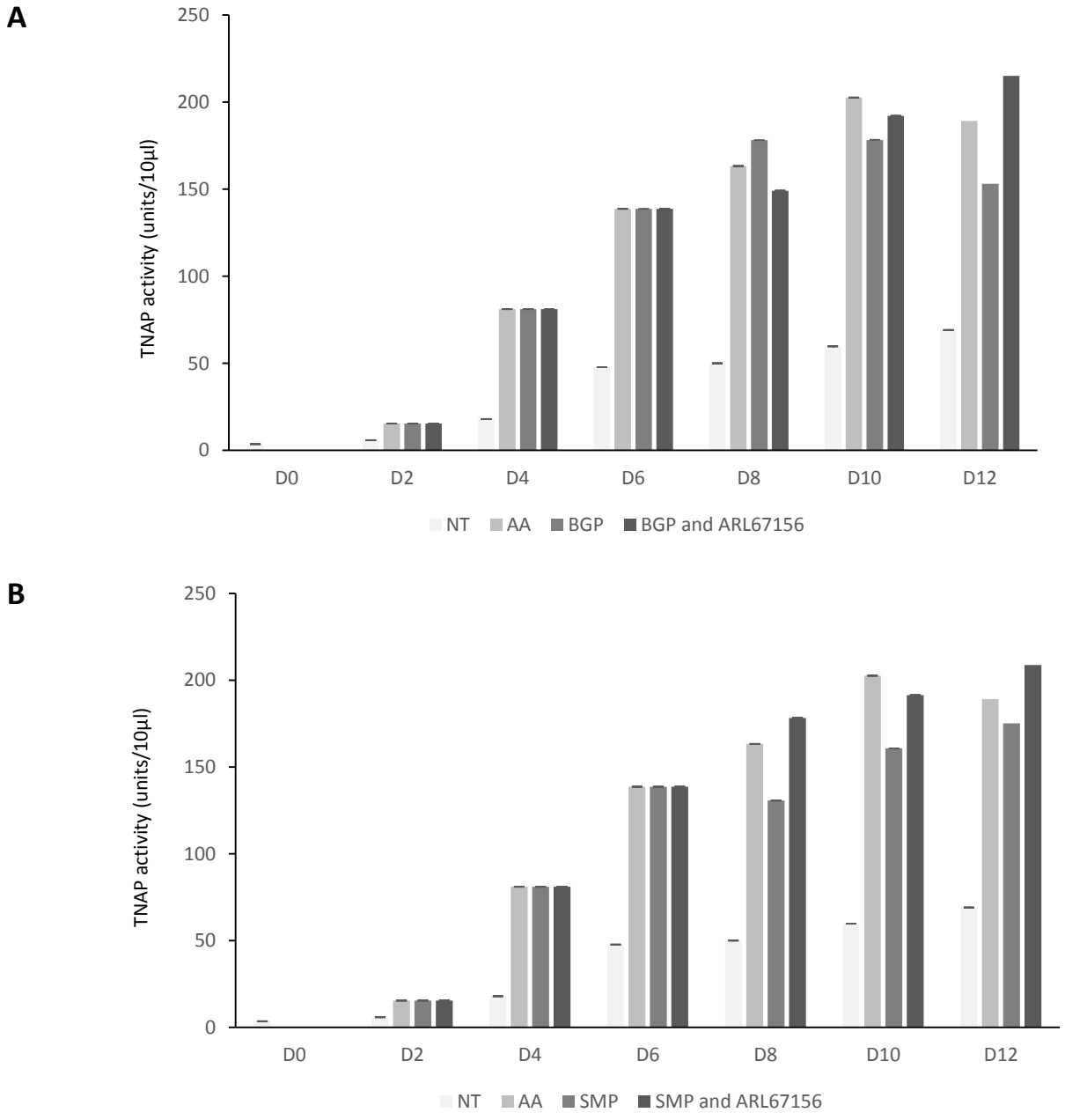

Figure 19. ectoATPase inhibitor does not affect TNAP activity in βGP- or SMP-treated MC3T3-E1 cultures. Cultures were supplemented with the indicated Pi sources and treated with ectoATPase inhibitor. Cell layers were harvested at the selected time points and TNAP activity analysed. Data showed that ectoATPase inhibitor does not affect TNAP activity in MC3T3-E1 cultures supplemented with βGP (A) or SMP (B). Error bars represent SEM.

(pyrophosphatase enzyme), results were plotted and analysed. As seen in Figure 18B, ePPi levels in the conditioned media were not increased with ectoATPase inhibitor in all treated cultures. Also, ePPi concentration in cell lysates were not altered in presence of ectoATPase inhibitor (Fig. 18C) and unlikely to be the cause of impaired mineralization. Further, our results showed that ectoATPase inhibitor did not affect TNAP activity in either βGP- or SMP-treated cultures (Fig. 19).

4.5 Sodium levels in MC3T3-E1 Cultures

Since the amounts of iPi in cell lysates were reduced dramatically with ectoATPase inhibitor, we questioned if this inhibitor interferes with Pi transport across osteoblasts. It is generally acknowledged that Na^+-K^+ ATPase is the primary mechanism creates transmembranous Na^+ gradients essential for secondary transport of different molecules including Pi and Ca^{2+} [35]. Thus, we investigated if Na^+ balance is altered in the cultures and measured the amount of Na^+ in conditioned media and cell lysates on day 12 in the presence and absence of ectoATPase inhibitor ARL67156 and compared it to TNAP inhibitor, as a control. Na^+ concentrations in the media were decreased in presence of both inhibitors (Fig. 20A). However, more significant reduction is seen in media supplemented with TNAP inhibitor. Interestingly, Na^+ levels in cell lysates of βGP cultures treated with ARL67156 decreased significantly while TNAP inhibitor MLS-0038949 did not have this effect (Fig. 20B). In SMP-mediated mineralization, Na^+ amounts in the media were significantly reduced in presence of TNAP inhibitor and ectoATPase inhibitor (Fig. 21A). Analysis of Na^+ levels in cell lysates showed that both inhibitors lowered

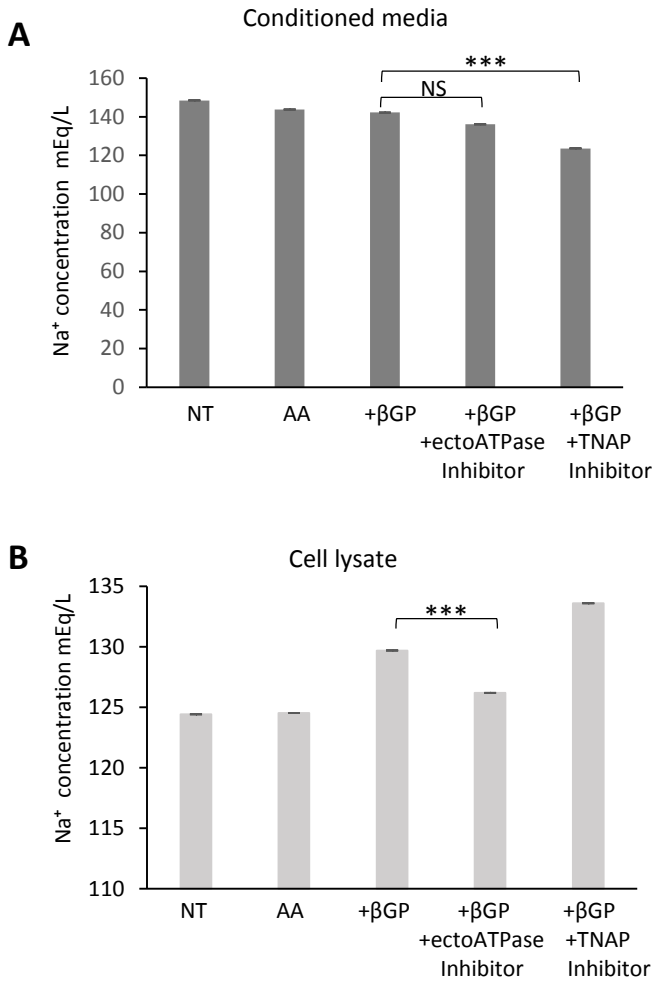

Figure 20. Sodium levels were decreased in cell lysates of βGP-mediated mineralization treated with ectoATPase inhibitor. MC3T3-E1 cells were cultured in media supplemented with βGP and treated with ectoATPase or TNAP inhibitor. On day 12, Sodium levels (Na^+) were measured in media and cell lysate . **(A)** Sodium concentrations in media were decreased significantly with TNAP inhibitor. **(B)** ectoATPase inhibitor significantly reduce amount of sodium in cell lysates. High Na^+ levels are maintained in the cell lysate of cultures treated with TNAP inhibitor. NS (no significance), *** $P \leq 0.001$ and error bars represent SEM.

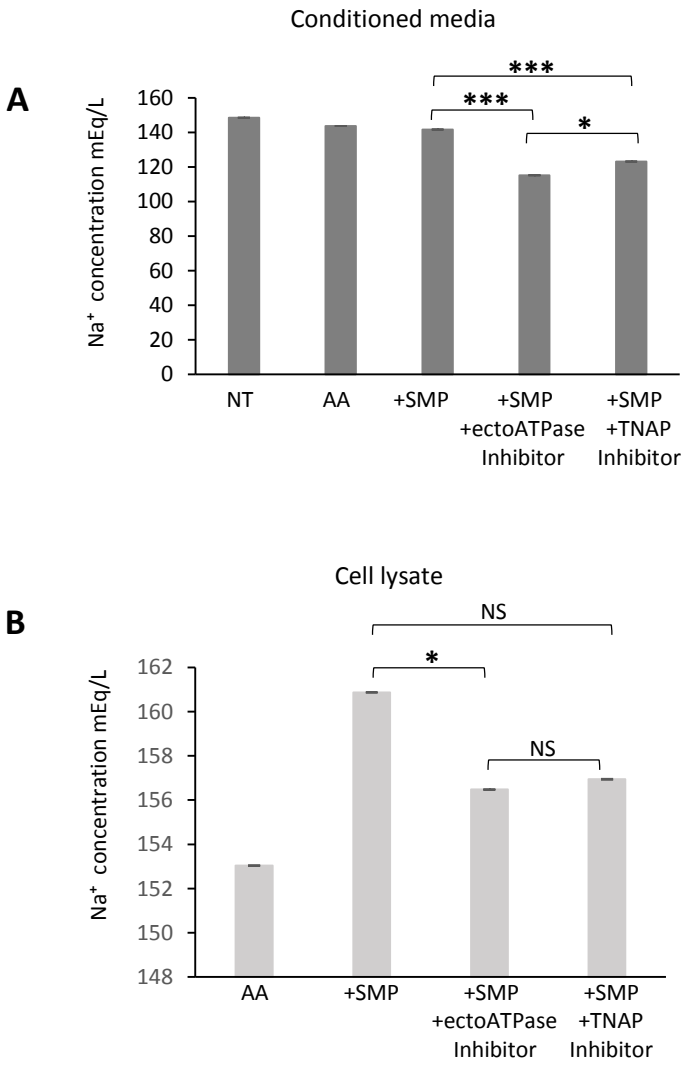

Figure 21. Sodium levels were altered in SMP-mediated mineralization. MC3T3-E1 cells were cultured in media supplemented with SMP and treated with ectoATPase or TNAP inhibitor. On day 12, Sodium levels (Na^+) were measured in media and cell lysates . **(A)** Both inhibitors significantly decrease sodium levels in the media. **(B)** Data shows deceased sodium levels in cell lysates with ectoATPase and TNAP inhibitors comparing to the control (+SMP). Data analysis indicates that Na^+ concentrations in cell lysates are more significantly declined with ectoATPase inhibitor. NS (no significance), * $P \leq 0.05$, ** $P \leq 0.001$. Error bars represent SEM.

concentrations of Na^+. However, data analysis demonstrated that Na^+ levels more significantly reduced with ectoATPase inhibitor (Fig. 21B).

4.6 Effect of ectoATPase ARL67156 on MC3T3-E1 osteopontin levels

OPN protein is considered as an important regulator of mineralization by many in vivo and in vitro studies [88-90]. Hence we examined if its expression was altered in the conditions that we have used. OPN mRNA expression was examines via RT-PCR and protein levels via Western blotting.

OPN mRNA expressions were evaluated at day 12. Total RNA were isolated from cell layers and analysed for OPN mRNA. As seen in Figure 22A, OPN mRNA was dramatically affected in non-mineralized cultures treated with ectoATPase inhibitor.

OPN protein levels were analyzed in MC3T3-E1 cultures treated with ectoATPase inhibitor and compared it to cultures treated with TNAP inhibitor MLS-0038949. At day 12 of culturing, total proteins were extracted with RIPA buffer and OPN levels were analyzed by Western blotting. MC3T3-E1 cultures treated with βGP and ectoATPase inhibitor showed a dramatic decrease of OPN protein levels. However, TNAP inhibitor MLS-0038949 has less effect on OPN expression (Fig. 22B). On the other hand, OPN protein strongly expressed in SMP-mediated cultures in presence or absence of both inhibitors. Importantly, SMP cultures supplemented with ectoATPase inhibitor lack dephosphorylated OPN band (Fig. 22C). Actin expression confirms equal loading of protein.

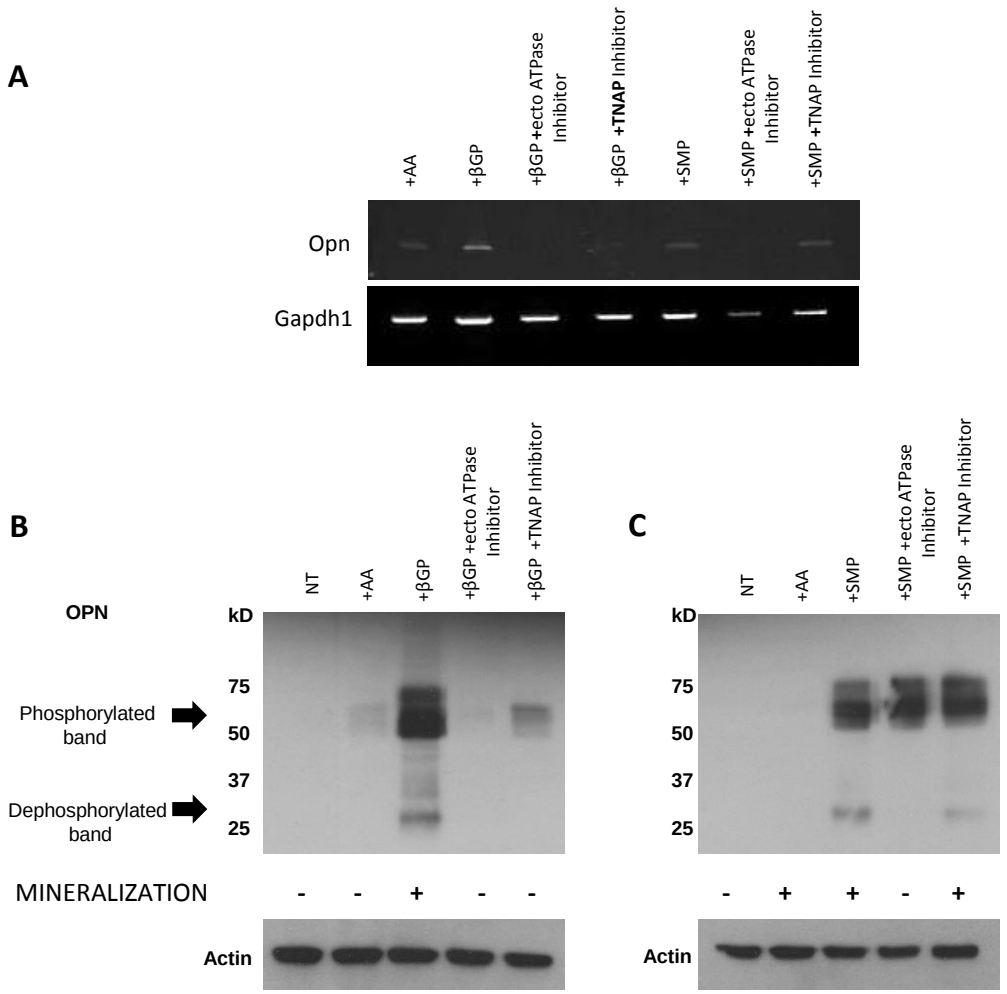

Figure 22. Osteopontin analysis at mRNA and protein levels in MC3T3-E1 cultures. On day, 12 RIPA buffer was used to extract cell layers from cultures treated with growth media only (NT), supplemented with ascorbic acid (AA) and with Pi sources (βGP and SMP) in presence and absence of mineralization inhibitors, ectoATPase and TNAP inhibitor. **(A) RT-PCR indicated that OPN expression is affected by ectoATPase inhibitor in both** βGP and SMP treated cultures. Also, cultures supplemented with βGP and TNAP inhibitor shows decreased levels of OPN mRNA. **(B)** In βGP-treated cultures**,** OPN protein expression is dramatically down-regulated with ectoATPase inhibitor. To less extent, OPN protein level is diminished with TNAP inhibitor. Both inhibitors show no mineralization. **(C)** SMP-mediated mineralization displays a comparable expression of OPN that seen in SMP and SMP with TNAP. Interestingly, non-mineralized SMP cultures supplemented with ectoATPase inhibitor strongly express phosphorylated band of OPN and devoid of dephosphorylated one. Actin expression confirm consistent loading of proteins.

5. Discussion

Biomineralization is a multifaceted process that is precisely regulated by many systemic and local factors. Generally, mineralization requires adequate collagen-rich ECM, boost of Pi and Ca^{2+} levels at site of mineralization and a tight regulation of ePi/ePPi balance. Bone ECM is enriched with phosphatases that are able to generate ePi and ePPi. Also, osteoblasts have a unique distribution of ion pumps that regulate ions transportation essential for HA formation. This thesis has explored the role of ATP in mineralization.

Previously, our laboratory demonstrated that ATP is an in vitro source of Pi ions [39]. Crystals structurally similar to HA were deposited along collagen fibrils when MC3T3-E1 cultures supplemented with ATP at concentration of 4-5 mM [39]. ATP can be hydrolyzed either by specific ATPase and/or TNAP. Earlier, we investigated role of TNAP in ATP-mediated mineralization using levamisole hydrochloride, a TNAP inhibitor [39] and those results showed normal mineralization suggesting that TNAP would not be involved in ATP-mediated mineralization. The results presented in this thesis, however, showed a new TNAP inhibitor MLS-0038949 is capable of inhibiting ATP-mediated mineralization. This specific potent inhibitor abolished mineralization in ATP-treated cultures without affecting either cell viability or collagen ECM scaffold. Also, our new data indicated that MLS-0038949 significantly reduced amount of Pi ions in media of ATP-treated cultures. This finding suggests that ATP is a substrate for TNAP and it is likely that TNAP activity is essential for ATP degradation. In absence of TNAP activity, extracellular ATP molecules are more likely to be a substrate for other phosphatases including NPP1 [22]. NPP1 mainly generates PPi from ATP. ePPi accumulate due to lack of TNAP activity and

interfere with HA growth. Therefore and as stated before, ATP is a pivotal local molecule for mineralization. However, whether ATP is acting as a mineralization promoter or inhibitor is likely based on different factors.

In the work presented in this thesis, we separated TNAP functions from any other ATPase activity using specific ectoATPase inhibitor ARL67156. This inhibitor cannot enter the cell and thus inhibits only extracellular ATPase activity. Surprisingly, ARL67156 abolished mineralization in all cultures including SMP-treated ones that do not require enzymatic activity to generate Pi for mineralization. This unexpected result indicates that ATPase activity may not only required for raising Pi levels, but also for other mechanism (s) that are essential to nucleate or initiate HA deposition. This was supported by the results that compared affects of TNAP inhibitor and ARL67156. In contrast to TNAP inhibitor, ectoATPase inhibitor ARL67156 did not lower Pi levels in the media. However, and regardless of the availability of free Pi in the media, MC3T3-E1 were unable to form mineral nodules. Interestingly, lysates from cells treated with ARL67156 show significantly lower Pi amounts compared to controls and this correlated with lack of mineralization. This result raised a question whether ARL67156 interferes with Pi transport and/or iPi generation. However, since that ARL67156 is negative charged ATP analogue, it would be unlikely to cross cell membrane and its function most probably be extracellulary or at cell membrane.

It is well established that Pi crossed osteoblasts/MVs membranes primarily via sodium-dependent cotransporters (PiT1 and PiT2) [91]. This Pi transportation is essential to elevate intracellular/intravesicular Pi to levels permissive for HA nucleation and initiation [91]. Interesting study demonstrated that Inhibition of PiT1 using

phosphonoformic acid (foscarnet) and arsenate abolished Pi uptake in aortic smooth muscles and prevented pathologic mineralization [10]. However, mineralization capability of cells lacking PiT1 indicates the existence of alternative Pi entry pathways, presumable a backup mechanism [91].

Na^+-K^+ ATPase, fueled by ATP, maintains proper sodium membranous gradients that is essential for Pi and Ca^{2+} homeostasis via PiT1 and NCX, respectively [66, 92]. The fact that about one-third of body's Na^+ is stored in bone ECM indicates the importance of Na^+ homeostasis in bone biology [93]. Chronic low extracellular Na^+ levels, hyponatremia, was linked to osteoporosis and increased osteoclasts activity [93, 94]. Further, a study showed that Na^+/H^+ is essential for osteoblasts differentiation and matrix formation [95]. However, still little is known regarding functions of Na^+ in osteoblasts [10, 94]. In this work we have investigated levels of Na^+ in mineralized and non-mineralized MC3T3-E1 cultures. Our preliminary results indicated that a relation between Na^+ amounts and mineralization exists. We showed that Na^+ levels clearly decreased in cell lysates of cultures treated with βGP and ectoATPase inhibitor. This effect was not seen when TNAP inhibitor was used. Low Na^+ level was associated with low Pi levels in cell lysates and lack of mineralization, presumably due to inactive PiT1 as a consequence of deactivated Na^+-K pump. On the other hand, Na^+ levels were decreased in SMP-treated cultures in presence of both inhibitors, ectoATPase and TNAP inhibitor. However, more significant reduction in the amount of Na^+ was recorded in presence of ectoATPase inhibitor. Thus, it is conceivable to speculate that ARL67156 likely targets Na^+-K^+ ATPase which results in disturbed cellular Na^+ levels. The improper Na^+ gradients affect ion transportation,

specifically deactivate PiT1 as well as reverse NCX direction. This disrupted ion homeostasis resulted in impaired mineralization (Fig. 23, proposed mechanism).

As described above, analysis of the mineralizing media supplemented with ectoATPase inhibitor demonstrated high levels of ePi whether the source was organic (βGP) or inorganic Pi (SMP). ePi has been shown to regulate gene expression in different cell types via specific signaling pathways [96]. In osteoblasts, there is a cross talk between ePi level and OPN gene and protein expression [83]. A study showed that OPN was upregulated in MC3T3-E1 cultures when supplemented with 10 mM Pi [97]. Further, OPN was shown, in vitro, to be a potent inhibitor for HA nucleation/growth [98]. Therefore, we investigated OPN levels in these cultures. OPN is multifunctional phosphorylated glycoprotein with an important regulatory roles in bone remodelling and mineralization [15, 99]. Yet, the exact role is not fully determined. Several studies suggested that OPN could play an inhibitory or promoter function depending on its concentration and phosphorylation state [15, 98]. It is believed that highly phosphorylated OPN exerts an inhibitory effect in normal and pathological mineralization [99]. Further, certain phosphorylated OPN peptides were also capable of inhibiting hydroxyapatite formation in vitro [100]. Consistently, dephosphorylation of OPN eliminates its inhibitory effect [101]. The fact that the level of phosphorylation can be regulated, together with the existence of many OPN isoforms differing in their level of phosphorylation, implies an important role for post-translational phosphorylation in modulating OPN function [100]. Our data suggested that ectoATPase inhibitor affects OPN synthesis and also likely its phosphorylation levels (Fig. 22). Firstly, βGP mineralized cultures inhibited by ARL67156 showed no OPN production. Secondly, presumably dephosphorylated OPN may be

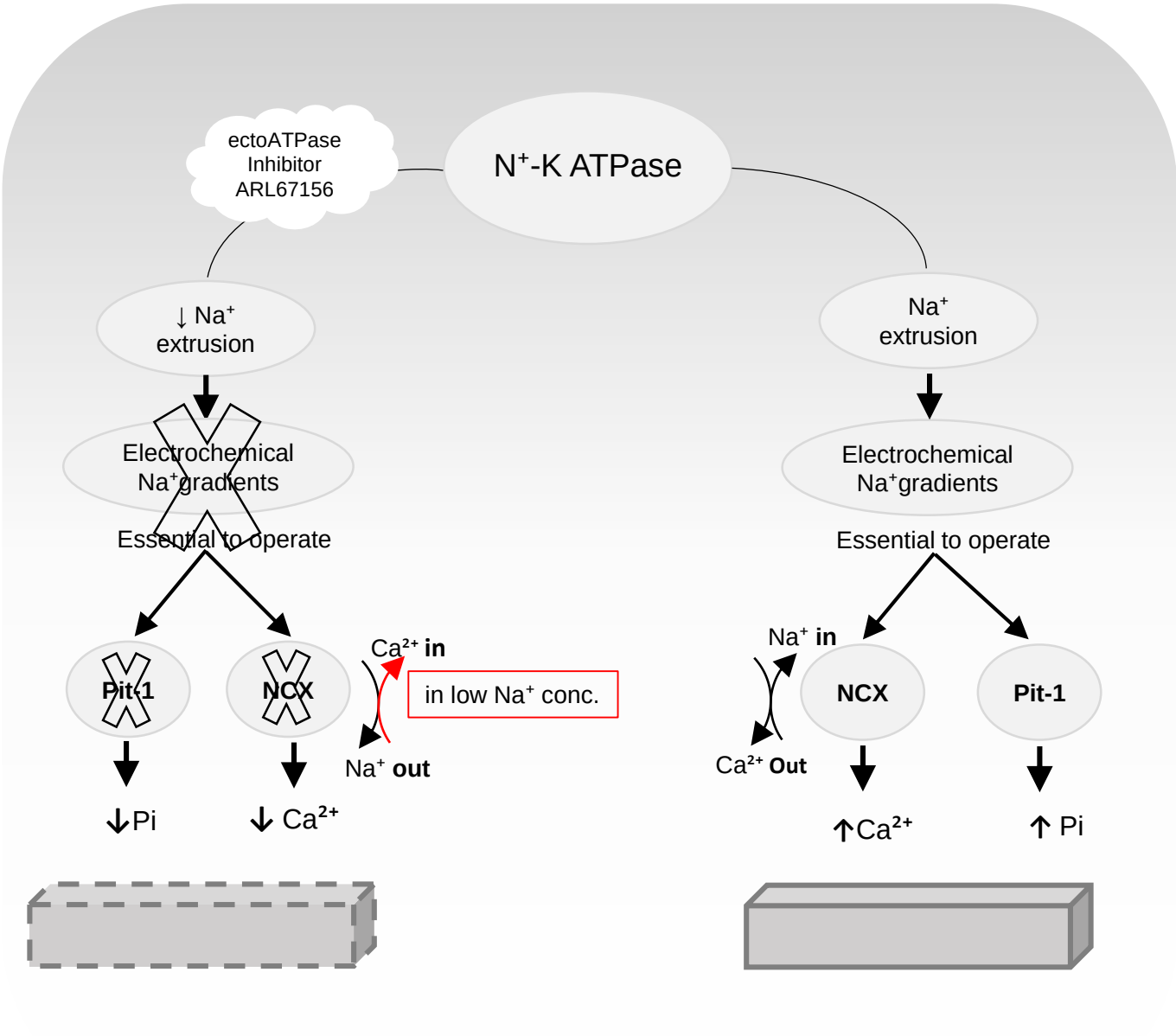

Figure 23. **Proposed mechanism.** Na^+-K^+ ATPase regulates membranous Na^+ gradient which is critical for osteoblasts Ca^{2+} and Pi homeostasis. Both Pit1 and NCX are driven by sodium concentrations. Physiologic Na^+ levels allow Pi transport and Ca^{2+} extrusion at site of bone mineralization and result in normal bone formation. Inhibition of ATPase activity with ARL67156 targets likely Na^+-K^+ ATPase which results in imbalance in cellular Na^+ levels, inactive Pit1 as well as reversed action of NCX that reduce amount of Pi and Ca^{2+} in the ECM of osteoblasts.

missing from these cultures treated with ARL67156. It has been shown that OPN protein migrates on SDS-PAGE/Western blots as a broad band ranging from 52 to 65 kDa. This broad band is a result of the high degree of posttranslational modifications. Dephosphorylated OPN is visible as a separate band smaller than the 52-kDa marker [102]. Our OPN Western blot analysis demonstrated that all mineralized cultures (βGP, SMP and SMP with TNAP inhibitor) displayed both bands, the strong broad bands and separate small bands. Importantly, OPN signals in all non-mineralized cultures (βGP with ectoATPase inhibitor, βGP with TNAP inhibitor and SMP with ectoATPase inhibitor) devoid of the smaller band (dephosphorylated OPN). Interestingly, non-mineralized SMP-treated cultures supplemented with ectoATPase inhibitor showed only large molecular size OPN and lacked the smaller band. This may indicate that the accumulation of the phosphorylated OPN in the cultures might contribute to the inhibition of mineralization. Interestingly, OPN signal slightly raised above background levels in non-mineralized βGP-treated cultures in presence of TNAP inhibitor. This may be because of high ePPi levels in the cultures - OPN is upregulated also by ePPi in presence of levamisole as well as upregulated in *Akp2*$^{-/-}$ mice [18, 103]. Thus, it has been suggested that OPN is upregulated by ePi and ePPi but via two different pathways [18]. Interesting study suggested that OPN could regulate the mineralization via its ability to sense and respond to changes to the ePi/ePPi ratio [103].

OPN mRNA levels were also altered. At mRNA levels, ePi-mediated OPN upregulation was extremely sensitive to ectoATPase inhibitor. Both βGP and SMP cultures supplemented with ectoATPase inhibitor showed dramatic decrease in OPN mRNA. Interestingly, OPN mRNA concentration does not reflect in protein expression

levels in SMP cultures treated with ectoATPase inhibitor. Since the final protein concentration is controlled at translational and/or post-translational levels [99], it would be possible that SMP promotes OPN protein synthesis in some manner that is not reflected at mRNA levels. Perhaps, SMP/ePi is able to stimulate specific pathways that affect protein translation. However, related literatures indicated that mRNA levels could be independent of final protein concentration [104].

Taken together, these findings lead us to speculate that inhibition of ATPase activity by using ectoATPase inhibitor ARL67156 interferes, indirectly, with Pi cellular entry. This can be seen by lack of mineralization as well as OPN mRNA levels. Osteopontin expression is strongly upregulated in response to elevated ePi levels. The ability of Pi transporter inhibitor, foscarnet, to block OPN expression [18] might be an indication that Pi must be elevated intracellularly in order to induce OPN upregulation. However, interesting study indicated that PiT1 could play a sensory role and regulates gene expression independently from the Pi transport function [77, 105, 106]. As blocking of PiT1 does not affect viability of cells, there must be another pathways for Pi entry. These backup Pi entry mechanism is likely not enough to initiate HA formation.

6. Future Work

For better understanding of previously discussed results, we are planning to focus on the following concepts and questions:

1. The role of iPi in mineralization. Do phosphonoformic acid or Foscarnet (Pit inhibitor) have an inhibitory effect on mineralization of SMP-treated cultures versus βGP-treated cultures?
2. The role of Na-K ATPase in mineralization. Inhibiting this with diphenylhydantoin (specific inhibitor of Na-K ATPase) would show if this pump is involved in mineralization and if so, this inhibitor would be expected to have similar effect on mineralization as ectoATPase inhibitor on MC3T3-E1 cultures treated with either βGP and SMP.
3. Confirmation that alterations in Na+ levels affect mineralization. Adding exogenous Na+ to cultures to counter the lower Na+ and examining of it rescues mineralization.
4. PHOSPHO1 is presumed to elevate iPi levels to initiate HA formation. Testing the effect of PHOSPHO 1 specific inhibitors (MLS-0390838 and MLS-0263839) on iPi levels in βGP- and SMP-treated cultures would demonstrate the role of PHOSPHO1 in regulation of iPi.
5. Does ATPase activity affect OPN production and phosphorylation status via affecting mineralization or via regulating iPi levels? Detailed analysis for OPN expression and phosphorylation status in MC3T3-E1 cultures.

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