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The role of PHOSPHO1 in matrix vesicle-driven biomineralization

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Mineralization-competent cells release a special class of extracellular vesicles, named matrix vesicles (MVs), involved in the deposition of apatite minerals at the sites of bone and dental calcification. Current knowledge describes MVs as harbouring the complete biochemical machinery necessary to drive biomineralization. Two phosphatases, tissue-nonspecific alkaline phosphatase (TNAP) and orphan phosphatase 1 (PHOSPHO1) have been identified as non-redundant master drivers of MV-driven biomineralization. Recent studies from our group have shown that MVs isolated from *Phospho1*$^{-/-}$ mice are reduced in number, less filled high-mechanical strength compounds and less able to form apatite minerals compared with WT vesicles. These data indicate a possible role of PHOSPHO1 in the biogenesis and function of MVs. The purpose of this study is to shed light on the role of PHOSPHO1 in MV-driven biomineralization and the functional interplay between PHOSPHO1 and TNAP. Primary chondrocytes were isolated from the growth plate of WT and *Phospho1*$^{-/-}$ mice and cultured in an osteogenic media (OM) in the presence or not (control) of PHOSPHO1 inhibitor (MLS0263839) and/or TNAP inhibitor (SBI-425). The ability of chondrocytes to mineralize was assessed by Alizarin Red assay, while gene expression was assessed by RT-qPCR. The intracellular localization of PHOSPHO1 protein was assessed by confocal microscopy. Results showed that the treatment of WT cells with MLS0263839 and SBI-425 (separately and combined) led to a significant decrease in the mineralization ability of chondrocytes in comparison with control cells. *Phospho1*$^{-/-}$ chondrocytes treated in OM with or without SBI-425 also showed a reduced mineralizing ability in comparison with WT cells. The inhibitors (separately and mixed) affected TNAP activity in both WT and *Phospho1*$^{-/-}$ cells, the differentiation of the cells to an osteoblast-like phenotype, and the expression of genes encoding the main enzymes of the MVs' biochemical machinery. Confocal microscopy showed that PHOSPHO1 appeared to accumulate in the perinuclear region before the addition of the OM and partly translocate to the nucleus after the addition of the OM. Thus, our study shows that PHOSPHO1 regulates both the differentiation of chondrocytes to an osteoblast-like phenotype and the function of MVs by controlling the assembly of the MVs' biochemical machinery. Importantly, our study suggests a crosstalk between the biochemical pathways mediated by PHOSPHO1 and TNAP.

Role of PHOSPHO1 on the biogenesis and function of matrix vesicles: a preliminary study

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Bone ossification is a result of the work of a special class of extracellular vesicles (EVs), called matrix vesicles (MVs). MVs are released by outward budding from the apical microvilli of chondrocytes and osteoblasts. Studies have shown that released MVs are pre-filled with amorphous aggregates of Ca^{2+} and inorganic phosphate (P_i) complexed with phospholipids and proteins, also referred to as the nucleational core (NC). Upon further accumulation of Ca^{2+} and P_i ions in the MVs' lumen, the NC form crystalline minerals that propagate onto collagen fibrils after the release in the ECM. Current knowledge describes MVs as harbouring the complete biochemical machinery necessary to drive the maturation of the NC and the propagation of apatite minerals. Orphan phosphatase 1 (PHOSPHO1, *Phospho1*) has been identified as crucial for the accumulation of P_i ions in the MVs' lumen. Recent studies from our research group have shown that PHOSPHO1 also has a crucial role in the biogenesis of MVs as well as in the assembly of the NC in the MVs' lumen. In this talk, I will describe our current knowledge on the role of PHOSPHO1 in the biogenesis and biological functions of MVs.

Proteoliposomes as a model of matrix vesicles and bone mineralization

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Chondrocytes and osteoblasts mineralize the extracellular matrix (ECM) by promoting the synthesis of hydroxyapatite (HA) seed crystals in the inner part of matrix vesicles (MVs) during endochondral bone formation. Several lipids and proteins present in the membrane of the MVs mediate the interactions of these vesicles with the ECM and regulate the initial mineral deposition and further propagation. Among the proteins of MV membranes, ion transporters control the availability of phosphate and calcium needed for initial HA deposition. Phosphatases (orphan phosphatase 1, ectonucleotide pyrophosphatase/ phosphodiesterase 1 and tissue-nonspecific alkaline phosphatase) play a crucial role in controlling the inorganic pyrophosphate/inorganic phosphate ratio that allows MVs mediated initiation of mineralization. The lipidic microenvironment can help in the nucleation process of first crystals and also plays a crucial physiological role in the function of MVs associated enzymes and transporters (type III sodiumdependent phosphate transporters, annexins and Na^+,K^+-ATPase). The whole process is mediated and regulated by the action of several molecules and steps, which make the process complex and highly regulated. Liposomes and proteoliposomes, as models of biological membranes, facilitate the understanding of lipid–protein interactions with emphasis on physicochemical and biochemical processes.

In this presentation, we discuss the use of proteoliposomes as multiple protein carrier systems intended to mimic the various functions of MVs during the initiation and propagation of mineral growth in biomineralization. We focus on studies applying biophysical tools to characterize the biomimetic models in order to improve the understanding of the importance of lipid-protein and lipid-lipid interfaces throughout the process. (Financial Support: FAPESP 2019/08568-2; 2019/25054-2; CNPq 304021/2017-2).

Role of extracellular vesicles in ectopic calcification

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Cardiovascular calcification has emerged as a predictor of and contributor to cardiovascular morbidity and mortality. Evidence suggests that arterial calcification, particularly when present as microcalcification, reduces plaque integrity and triggers rupture. Calcifying extracellular vesicles released by vascular smooth muscle cells are the smallest nidus to form microcalcification. On a molecular level, post-translational modification of the lysosomal sorting receptor sortilin, regulates the calcification propensity of these extracellular vesicles. By better understanding the biogenesis and downstream functional consequences of calcifying extracellular vesicles we will gain novel insights into the pathogenesis of vascular calcification.

TNAP activation by inflammation in vascular smooth muscle cells and release into extracellular vesicles: possible functions in atherosclerotic plaques

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Atherosclerosis is characterized by a multi-step process of chronic vascular inflammation and subsequent plaque formation. Inflammation is involved in all stages of the atherosclerotic process. During the formation of a lesion nidus, vascular smooth muscle cells (VSMCs) present in the media layer of the artery migrate toward the intima and are exposed to oxidized lipids and cytokines. Then, VSMCs change their phenotype to become osteochondrocytes or foam cells. VSMCs, trans-differentiated or not, may release several types of extracellular vesicles (EVs) including exosomes, microvesicles (MVs)/microparticles and apoptotic bodies. Emerging evidence in the field suggests that these smooth muscle cell-derived EVs can contribute to intercellular communication during the development of atherosclerosis via the transfer of cellular contents such as protein and microRNA, which may prevent or promote disease progression depending on the context.

Our objectives are to analyze the phenotype of VSMCs cells under inflammatory condition and to isolate and characterize the different type of EVs secreted by these cells. To this purpose, we used Human Coronary Artery Smooth Muscle Cells (HCASMCs).

We observed that the proinflammatory cytokine interleukin-1β (IL1-β) induced a phenotypic switching of HCASMCs. Quantitative PCR and western blot experiments revealed an increase in the expression of *RUNX2*, *SOX9, OPG and TNAP* in HCASMC cells cultured under inflammatory conditions, after seven, fourteen and twenty-one days. We isolated and characterized each class of microparticles produced by HCASMCs stimulated after fourteen days by IL-1β. We observed an enrichment of EVs in TNAP, suggesting that the increase in TNAP in EVs upon inflammation is a trigger mechanism to initiate calcification which could be sustained by calcification-induced inflammation and by enriched-TNAP EVs.

The function of matrix vesicles in physiological and pathological mineralization

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Mineralization-competent cells, such as chondrocytes and osteoblasts, release a special class of extracellular vesicles, named matrix vesicles (MVs), that contribute to the initiation of skeletal/dental mineralization. MVs are also released by calcifying vascular smooth muscle cells (VSMCs) at the sites of medial vascular calcification. Our current understanding of the biochemical pathways involved in matrix vesicles (MVs)-mediated initiation of skeletal and dental calcification is compatible with the following sequence of events: MV biogenesis appears to be controlled by the function of cytosolic PHOSPHO1. In the ECM, MVs initiate mineral deposition by accumulation of P_i generated by a dual mechanism: a) intravesicularly by the action of PHOSPHO1 on phosphocholine generated from sphingomyelin by the action of SMPD3, as well as b) via P_i transporter P_iT-1-mediated incorporation of extracellular P_i generated extravesicularly by TNAP and/or NPP1 on ATP. Then, the extravesicular propagation of mineral onto the extracellular matrix (ECM) is primarily controlled by the pyrophosphatase activity of TNAP that restricts the concentration of this potent mineralization inhibitor to establish a PP_i/P_i ratio conducive to controlled calcification. Additionally, osteopontin, particularly in its phosphorylated form (p-OPN), is another potent mineralization inhibitor and also a natural substrate of TNAP, that binds to hydroxyapatite as soon as it is exposed to the extracellular fluid. The p-OPN/OPN ratio further restricts the degree of ECM mineralization. During both physiological and ectopic calcification, MVs are thought to be released by outward budding of the apical microvilli, bind to collagen fibrils, and function as nanoreactors to initiate and propagate mineralization onto the collagenous scaffold. However, how MVs associate with the collagenous matrix and help propagate mineralization is still poorly understood. The significance of TNAP for biomineralization has been exploited therapeutically. Our laboratory has contributed much of the pre-clinical data validating mineral-targeted TNAP (asfotase alfa) for the treatment of hypophosphatasia (HPP, approved in 2015 for pediatric-onset HPP) and we have now developed a TNAP inhibitor (SBI-425) that reduces soft-tissue calcification in mouse models of pseudoxanthoma elasticum, chronic kidney disease-mineral bone disorder and atherosclerosis. This inhibitor is expected to enter Phase II clinical trials in 2020-2021. We have also developed pharmacological inhibitors of PHOSPHO1 and shown i*n vitro* that the simultaneous inhibition of TNAP and PHOSPHO1 leads to a more profound suppression of calcification than using either inhibitor alone. The underlying hypothesis of our current work is that MV biogenesis and function is required not only for normal skeletal and dental mineralization but also for ectopic calcification. We surmise that the dual phosphatase inhibition strategy is more effective due to the combined effect of suppressing MV biogenesis, initiation and propagation of mineralization.

Extracellular vesicles as a part of secretory phenotype of senescent vascular smooth muscle cells and its influence on T cells.

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Aging is a complicated biological process leading to progressive deterioration of physiological function and increased vulnerability to death and diseases. Recently it was demonstrated that aging at the cellular level – cellular senescence, contribute to aging of the organism but also promote development of age-related diseases. One of the most important feature of senescent cells, named *Senescence Associated Secretory Phenotype* (SASP), is increased secretion of many bioactive factors like cytokines, growth factors, chemokines and matrix modifying enzymes. Apart from myriad of soluble proteins also extracellular vesicles (EVs) were shown to participate in SASP. Factors secreted by senescent cells influence microenvironment and promote low grade inflammation associated with aging. Atherosclerosis is commonly recognized inflammatory disease that affect elderly people. Importantly it was demonstrated that senescent cells accumulates in atherosclerotic plaque. However little is known about the influence of SASP on immune cells that contribute to plaque development. Thus our study aimed in revealing the role of extracellular vesicles secreted by senescent human vascular smooth muscle cells (hVSMCs) in the modulation of T cell functioning. We performed unbiased proteomic analysis of EVs, which revealed mark differences in the EVs composition derived from senescent cells (senEVs) comparing to young hVSMCs. Moreover we found that senEVs carry proteins involved in the regulation of immune response. Accordingly, we demonstrated that EVs secreted by senescent cells influence production of cytokine by T cells and monocytes. Altogether our results suggest that EVs secreted by senescent VSMCs may facilitate inflammation having deleterious impact on atherosclerosis development.

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Regulation of human bone cells mineralization competence by apigenin

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Osteoblasts as the bone mineralization-competent cells participate in the initial steps of mineral formation. They secret matrix vesicles (MVs), where mineralization is initiated. Mineralization carried out by MVs is a complex process requiring the involvement of low molecular weight (LMW) compounds and various proteins, among them annexins (Anx) and tissue-nonspecific alkaline phosphatases (TNAPs). TNAP hydrolyzes inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i) and AnxA6 can form ion channels across the membrane, being responsible for and calcium ions (Ca^{2+}) uptake into MVs. MVs, containing relatively high concentrations of Ca^{2+} and P_i, create an optimal environment to induce the formation of hydroxyapatite (HA). An important role in mineralization is also played by LMW compounds. Recently published observations suggested the effects of flavonoids on processes regulating bone metabolism. They can modulate enzymes activity, interact with extracellular matrix (ECM) proteins and cell-surface receptors, as well as with transcription factors resulting in changes in the expression of genes important in the mineralization process. One of the promising and frequently studied flavonoid is apigenin.

The aim of this study was to analyze the potency of apigenin to affect mineral formation by two mineralization-competent human cell lines: osteoblastic hFOB 1.19 and osteosarcoma Saos-2. For this purpose, cell lines were cultured for 7 days under resting conditions or after stimulation with ascorbic acid (AA) and β-glycerophosphate (β-GP) in the presence of apigenin. Ability of cells to mineralize was confirmed by staining with AR-S observed under light microscope and determination of the TNAP activity by ELISA assay. Results showed that apigenin may affect the mineral formation, making minerals more compact. What is more, low concentrations of apigenin (1 - 2 μM) can cause increase of TNAP activity, while higher concentrations (5 - 10 μM) its decrease. Based on analysis of quality of minerals in MVs produced by both cell lines using TEM imaging with EDX microanalysis and ion mapping, it has been found that apigenin can affect the structure and composition of minerals.This flavonoid could also disturb the intracellular distribution of AnxA6 and TNAP, especially blocking TNAP attachment to the membrane, as examined by fluorescent microscopy analysis of their co-localization.

Obtained results may help to understand the mechanisms of apigenin action, and to develop novel therapies using this flavonoid for bone cancer treatment. (Financial Supports: This work was implemented as a part of Operational Programme Knowledge Education Development 2014-2020 co-financed by the European Social Fund No. POWR.03.02.00-00-I007/16-00. Support from the POLONIUM2020 project co-financed by NAWA is also acknowledged.)

Molecular Regulation of Matrix Vesicles Biogenesis

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Virtually all cell types secrete extracellular vesicles (EVs) that carry biologically active molecules. EVs are highly heterogeneous and dynamic in nature. Their molecular composition reflects their cell-type of origin, pathophysiological cell state, biogenesis pathway, and biological function. A specific population of EVs, called matrix vesicles (MVs), has a unique function of supporting the initiation of the mineralization of cartilage, mantle dentin and woven bone, and has been implicated in pathologic mineralization of blood vessels in chronic hyperphosphatemic conditions. The mineralization-supporting function of MVs depends on the presence of tissue-nonspecific alkaline phosphatase (TNAP) on their surface. We have previously demonstrated that elevated extracellular phosphate (P_i) is a potent stimulator MVs release from mineralization-competent cells. In this study, we used 17IIA11 cell line (a model of committed osteogenic cells) to identify molecular players in P_i-stimulated MVs release and the effect of P_i on MVs composition. MVs were isolated from extracellular matrix (ECM) by enzymatic digestion followed by differential ultracentrifugation and characterization using nanoparticle tracking analyses and Western blot. MVs protein composition was analyzed by mass-spectrometry followed by gene ontology assignments using PANTHER 11.0 software. These analyses identified 421 proteins significantly more abundant in MVs released upon stimulation with 10mM P_i (hyperphosphatemic conditions), 117 proteins present only in MVs released in hyperphosphatemic conditions, and 116 proteins present only in MVs released in normophosphatemic conditions. Interestingly, while TNAP was highly abundant in MVs released under normophosphatemic and hyperphosphatemic conditions, elevated P_i reduced the TNAP protein levels in 17IIA11 cells but not in MVs. To investigate the biological significance of this downregulation of TNAP, we used previously generated 17IIA11 cells deficient in mineralization-regulating transcription factor Trps1 (Trps1-KD cells). In comparison with parental 17IIA11 cells, Trps1-KD cells have dramatically decreased expression of TNAP, do not respond to P_i, and have impaired MVs biogenesis. Interestingly, restoring TNAP expression in Trps1-KD cells was sufficient to restore their ability to respond to P_i. In summary, our data suggest that the extracellular P_i not only stimulates the MVs release but also significantly affects their protein composition. Furthermore, our studies identified a novel role of TNAP in MVs biology that extends beyond its enzymatic activity and suggests TNAP is required for the MVs biogenesis as a component of P_i receptor/sensor complex.

The role of phosphatidylserine in biomineralization assessed by means of Langmuir monolayers

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The role played by phosphatidylserine (PS) in the formation of mineral nuclei in matrix vesicles (MVs) extracted from growing-femurs of chicken embryos was investigated by a combination of *in situ* investigations and *ex vivo* analysis. By using self-assembled Langmuir monolayers, we reconstructed the nucleation core - a PS-enriched motif thought to trigger mineral formation in the lumen of MVs. *In situ* infrared spectroscopy of Langmuir monolayers and ex situ analysis by transmission electron microscopy evidenced that mineralization was achieved in supersaturated solutions only when PS was present. PS nucleated amorphous calcium phosphate that converted into biomimetic apatite. By using monolayers containing lipids extracted from native MVs, mineral formation was also evidenced in a manner that resembles the artificial PS-enriched monolayers. PS-enrichment in lipid monolayers creates nanodomains for local increase of supersaturation, leading to the nucleation of ACP at the interface through a multistep process. We posited that PS-mediated nucleation could be a predominant mechanism to produce the very first mineral nuclei during MV-driven bone/cartilage biomineralization. (Financial Support:FAPESP 2019/25054-2 ; 2017/20846-2 ; 2019/08568-2)

Role of annexins and fetuin-A in TNAP activation and tissue calcification

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The mineralization process is initiated by osteoblasts and chondrocytes during intramembranous and endochondral ossifications, respectively. Both types of cells release matrix vesicles (MVs), which accumulate P_i and Ca^{2+} and form apatites in their lumen. Tissue non-specific alkaline phosphatase (TNAP), a mineralization marker, is highly enriched in MVs, in which it removes inorganic pyrophosphate (PP_i), an inhibitor of apatite formation. MVs then bud from the microvilli of mature osteoblasts or hypertrophic chondrocytes and, thanks to the action of the acto-myosin cortex, become released to the extracellular matrix (ECM), where they bind to collagen fibers and propagate mineral growth. In addition, sarcoma proto-oncogene tyrosine-protein (Src) kinase and Rho-associated coiled-coil (ROCK) kinases, which are involved in vesicular transport, may also regulate the mineralization process.

To further study the role of TNAP we compared the mineralization ability of human fetal osteoblastic cell line (hFOB 1.19 cells) with that of osteosarcoma cell line (Saos-2 cells). The composition of P_i and Ca^{2+} compounds in cytoplasmic vesicles was distinct from that in MVs released after collagenase-digestion. Apatites were identified only in MVs derived from Saos-2 cells, while MVs from hFOB 1.19 cells contained amorphous calcium phosphate complexes. In addition, annexins (Anxs), nucleators of mineralization in MVs, increased mineralization in the sub-membrane region in strongly mineralizing Saos-2 osteosarcoma, where they co-localized with TNAP, whereas in less mineralizing hFOB 1.19 osteoblasts, Anxs co-localizations with TNAP were less visible in the membrane. We also observed a reduction in the level of fetuin-A (FetuA), an inhibitor of mineralization in ECM, following treatment with levamisole and K201, TNAP and Ca channels inhibitors, respectivelly. Moreover, a fraction of FetuA was translocated from the cytoplasm towards the plasma membrane during the stimulation of Saos-2 cells, while this displacement was less pronounced in stimulated hFOB 19 cells. The addition of PP2, which is an inhibitor of Src kinase, significantly inhibited the mineralization process. In contrast, the addition of Y-27632, which is an inhibitor of ROCK kinase, did not affect significantly the mineralization process as denoted by AR-S staining and TNAP activity assay.

In summary, osteosarcoma Saos-2 cells had a better ability to mineralize than osteoblastic hFOB 1.19 cells. The formation of apatites was observed in Saos-2 cells, while only complexes of P_i and Ca^{2+} were identified in hFOB 1.19 cells. This was also evidenced by a more pronounced accumulation of Anxs and FetuA in the plasma membrane, where they were partly co-localized with TNAP in Saos-2 cells, in comparison to hFOB 1.19 cells. This suggests that both activators (Anxs) and inhibitors (FetuA) of mineralization were recruited to the membrane to activate TNAP and take part in the process of tissue calcification which seems to be differently regulated by Src and ROCK kinases. (Financial Supports: HC Partnership Programs POLONIUM 2018/2019 and 2021/2022 from NAWA to ASK; TRI-BIO-CHEM grant from NCRD co-financed by the European Social Fund POWR.03.02.00-00-I007/16-00 to JM; ERA-NET ERA-CVD/MICROEXPLORATION/4/2018 grant from NCRD to SP and the statutory funds of the Nencki Institute of Experimental Biology PAS).

Osteoblast extracellular vesicles: beyond matrix mineralization

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Bone cells communicate by many means, including extracellular vesicles (EVs). The bone tissue is complex and host a variety of cell types, among which osteoblasts and osteoclasts play critical roles in bone growth and remodelling. Bone can also host primary and metastatic cancer cells, which colonise the bone/bone marrow microenvironment disrupting the communication among the physiologic cells. Classical matrix vesicles released by mineralizing chondrocytes and osteoblasts are devoted to the induction of the mineralization process. Beyond this, EVs instead play complex roles in the orchestration of bone cell activities. We have demonstrated that osteoblast EVs carry the most potent osteoclastogenic cytokine RANKL, through which they induce osteoclast formation and promote osteoclast survival. These EVs integrate into osteoblasts and osteoclasts and transfer many molecular pathways including proteins and nucleic acids. Furthermore, they can be used as biotechnological means for molecular delivery, targeting osteoclasts physiologically through the RANKL pathway and pharmacologically when they are loaded with anti-resorptive or anti-tumoral drugs. Osteotropic cancer cells also employ EVs for pathological communication with osteoblasts, osteoclasts and vascular endothelium, disrupting the physiologic cycle orchestrating their function. As a result, cancer cell EVs target the osteoblasts to modify their EV molecular profile, reducing osteoblastogenesis and enhancing osteoclastogenesis and angiogenesis. New discoveries in the bone EV-mediated physiologic and pathologic communication are expected to recognise new targetable pathways potentially useful to treat bone diseases. In conclusion, investigations of EVs are likely to revolutionise the research in the bone field, allowing innovative observations in health and diseases (Financial Supports: Italian Association for Cancer Research; Italian Ministry of University).

The role of annexins in calcification of human coronary artery smooth muscle cells

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Vascular calcification (VC) is accompanied by the expression of tissue nonspecific alkaline phosphatase (TNAP), a crucial enzyme for calcification in vascular smooth muscle cells (VSMCs). Furthermore, it has been shown that membrane-binding annexin A6 (AnxA6) is abundant in sites of calcification and that its depletion reduces VSMC this process. In chondrocytes, where the physiological mineralization takes place, which is a similar process to the pathological VC, the non-specific calcium channel blocker K201 inhibits up-regulation of expression of terminal differentiation marker genes (*TNAP, AnxA2, AnxA4, AnxA6*) expression. Matrix vesicles released from K201-treated chondrocytes contain significantly lower amounts of AnxA2, AnxA5 and AnxA6 and lower TNAP activity, as described in Wang et al. (2003) J Biol Chem 278, 3762-3769.

Our aim was to identify the role of annexins in VC. We used human coronary artery smooth muscle cells (HCASMCs) cultured in an osteogenic medium for 7 and 21 days to promote their transition to osteochondrocyte-like cells. In order to inhibit the activity of annexins, K201 at various concentrations was added. The calcification in the cell cultures was examined via Alizarin Red-S (AR-S) staining. Subsequently, the minerals were extracted using cetylpyridinium chloride (CPC) in order to analyze their quantity. Also the TNAP activity after the addition of K201 was analyzed. Another experiment used to explore the influence of annexins on VC was the addition of proteoliposomes containing AnxA6-FITC to HCASMCs cultures for the period of 14 days. Transmission electron microscopy (TEM) was used to identify extracellular vesicles (EVs) and the presence of minerals in samples of HCASMC lysates. The obtained results show that K201 markedly decreases the calcification in HCASMC cultures. It also seems to reduce the activity of TNAP in HCASMCs. Addition of proteoliposomes containing AnxA6-FITC to atherosclerotic cells strongly stimulates their calcification, examined by the AR-S/CPC assay, and slightly induces TNAP, examined by ELISA assay, both prevented by K201. Calcium minerals can be observed under the TEM only in lysates of HCASMC cultures not treated with K201.

In conclusion, annexins seem to play a significant role in VC together with TNAP and the molecular mechanisms of their activity should be further investigated.
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Na,K-ATPase supports *in vitro* mineralization: Comparative study with MVs

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Na,K-ATPase (NKA) is of vital importance in the regulatory functions of many cells and tissues. It is also found in appreciable concentration in the matrix vesicles (MVs) membrane [1]. We hypothesized that the NKA-membrane orientation may be used to understand the process of MVs biogenesis. Therefore, rightside/inside-out orientations could reveal whether the vesicles were protruded from the apical cell membrane or if it`s first invaginated and then released. Here, collagenase released MVs (212 nm diameter) were isolated from chondrocytes extracted from the growth-plate of chicken embryos [2]. ^{31}P-NMR and colorimetric techniques estimated MVs activity in the presence of ouabain is ±95%, Levamisole and SBI-425 were more effective because they inhibit TNAP, which is the most important phosphatase enzyme in MVs. To determine whether NKA could propagate mineral formation proteoliposomes were designed. Turbidity changes of synthetic cartilage lymph (SCL) in the presence of MVs were used to evaluate the inhibitors influence in the ability to nucleate and propagate apatite mineral. FTIR was used for precipitates chemical analysis. Proteoliposomes mimetizing MVs were prepared using co-solubilization with $C_{12}E_8$ and the phospholipids DPPC and DPPC:DPPE (1:1 w/w lipid ratio). These lipid compositions are known to randomly distribute different positions of NKA into the membranes. AFM images revealed the formation of protrusions related to the NKA insertion into the liposomes´s membrane, associated with the height dimensions of the αβ unit and protein orientation [3]. The α subunit domain, containing the active site of NKA, has a 4 nm height, while the β subunit domain is 8 nm high. The mean height of the protrusions found in DPPC-NKA and DPPC:DPPE-NKA proteoliposomes were 2.1 nm and 0.5 nm, respectively, revealing the exposure of ATP binding site outwards in the presence of DPPE. As a consequence, the DPPC-NKA proteoliposomes preserved 61% of the solubilized-NKA activity, while DPPC:DPPE-NKA proteoliposomes preserved 91%. Thus, proteoliposomes with reconstituted NKA achieved mineral propagation by ATP hydrolysis and may be helpful to probe the mechanism of MVs biogenesis.

[1]. Thouverey et al. (2011) J. Proteomics, 74:1123; [2] Buchet et al. (2013) Methods Mol Biol. 1053:115; [3] Sebinelli et al. (2019) Soft matter, 15:2737.