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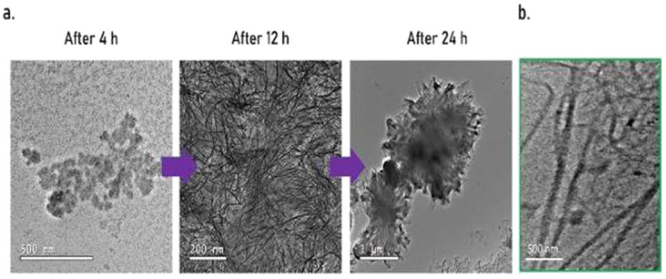
Unravelling the role of phosphatidylserine on the precipitation of calcium phosphate by mineralizing-extracellular vesicles

Marcos Antonio Cruz[a], Claudio Ferreira[a], Saida Mebarek[b], Rene Buchet[b], Luciano Caseli[c], Pietro Ciancaglini[a], Ana Paula Ramos[a]
[a]*Department of Chemistry, University of Sao Paulo, Ribeirao Preto, Brazil*
[b]*UFR Chimie Biochimie, Universite Lyon 1, Lyon, France*
[c]*Instituto de Ciências Ambientais Químicas e Farmacêuticas, Federal University of Sao Paulo, Sao Paulo, Brazil*

Bone biomineralization is an exquisite process by which cells control the deposition of a hierarchically mineralized matrix. There is growing evidence about the involvement of extracellular vesicles in the formation and delivery of the very first mineral nuclei to the bone growth front. Our aim is to unravel the molecular mechanisms behind phospholipid-mediated calcium phosphate nucleation and to get insight on how phospholipid-mineral complexes are involved in the mineralization of collagen. To this end, we used self-assembled lipid monolayers mimicking the lipidic composition of matrix vesicles (MVs). We evidenced that the enrichment of phosphatidylserine (PS) on MVs is responsible to control the nucleation of the mineral. Using self-assembled monolayers mimicking the lipid composition of MVs, we noticed by *in situ* characterization techniques (vibrational spectroscopy, transmission electron microscopy and potentiometric measurements of free Ca^{2+}) that mineralization was achieved within 4 h on supersaturated solutions only when PS was present (Fig. 1). Amorphous calcium phosphate nucleated by PS was able to further convert to apatite after 24 h. Moreover, we also evidenced that phospholipid-mineral complexes were able to deliver a precursor mineral phase to type I-collagen fibrils after 24 h, attesting that these vesicles can act as a confined medium to specifically nucleate calcium phosphate in a PS-mediated manner. PS is localized in the inner leaflet of MVs, where calcium phosphate complexes and apatites are formed. Our findings indicated that PS is enough to induce apatite precipitation. We believe that this mechanism is not exclusive to MVs but universal to other mineralizing-EVs.



(a) TEM of mineralized PS-rich monolayers (b) Collagen fibrils infiltrated by the mineral after 24h.

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Fra1 is dispensable for the function of Runx2-expressing osteoblasts

Julia Luther, Mona Neven, Olga Winter, Lana Rosenthal, Michael Amling, Jean-Pierre David
Institute for Osteology and Biomechanics (IOBM), University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Ubiquitous overexpression of the transcription factor activator protein-1 (AP-1) member Fra1 causes progressive osteosclerosis, a phenotype due to a cell-autonomous accelerated osteoblast differentiation resulting in an increased number of mature osteoblasts. In addition to the bone phenotype, Fra1 overexpression opposes adipocyte differentiation by an adipocyte-specific inhibition of Cebpa expression. Conversely, epiblast-specific deletion of Fra1 by *More-Cre* causes an osteopenic phenotype most likely due to a cell autonomous decreased bone formation by osteoblasts. Moreover, Fra1 has additionally been shown to enhance osteoclastogenesis in vitro.

To confirm the cell autonomous bone phenotype, we generated osteoblast-, as well as osteoclast-specific *Fra1*ko mice. Cell-specific deletion of Fra1 in osteoclasts by *LysM-Cre* did not affect trabecular bone mass in 12 week-old nor in 1 year old-mice (BV/TV: 14.23±3.49% (control) versus 12.85±2.733% (*LysM-Cre*;$Fra1^{fl/fl}$) at the age of 1 year). Deletion of Fra1 in osteoblasts by *Runx2-Cre* did not significantly change bone mass in young as well as in aged *Runx2-Cre*;$Fra1^{fl/fl}$ mice compared to control (trabecular BV/TV: 15.53±2.243% (control) versus 13.36± 0.7766% (*Runx2-Cre*;$Fra1^{fl/fl}$), cortical thickness: 0.2011±0.02263 mm (control) versus 0.1923±0.02356 mm (*Runx2-Cre*;$Fra1^{fl/fl}$) at the age of 1 year). In addition, we could not observe a significant change in the number of osteoblasts in these mice. However, interestingly, osteoblast specific deletion of Fra1 causes reduced adipose tissue mass accrual and reduced maturation of adipocytes in the epididymal fat tissue.

Therefore, expression of Fra1 in the later stage of osteoblast differentiation is not required for bone mass acquisition of adult mice but regulates adipose tissue metabolism. These data suggest that the bone anabolic function of Fra1 would be driven by its expression in early mesenchymal osteoblast progenitors rather than in commited Runx2-positive pre-osteoblasts.

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Epigenetic priming of BMP-mediated osteogenesis and bone repair

Amel Dudakovic[a], Rebekah Samsonraj[a], Christopher Paradise[a], Catalina Galeano-Garces[a], Merel Mol[b], Daniela Galeano-Garces[a], Pengfei Zan[a], M. Lizeth Galvan[a], Mario Hevesi[a], Oksana Pichurin[a], Roman Thaler[a], Dana Begun[a], Peter Kloen[b], Marcel Karperien[c], A. Noelle Larson[a], Jennifer Westendorf[a], Simon Cool[d], Andre van Wijnen[a]
[a]*Mayo Clinic, Rochester, United States*
[b]*Amsterdam University Medical Center, Amsterdam, Netherlands*
[c]*University of Twente, Enschede, Netherlands*
[d]*Institute of Medical Biology, Singapore, Singapore*

Bone formation can be enhanced by bone morphogenetic proteins (e.g., BMP2), parathyroid hormone (PTH), and targeting of WNT inhibitors. Previously, we established that the inhibition of the epigenetic enzyme Ezh2 (i.e., reduction in methylation of histone H3 at lysine 27) is bone anabolic and osteo-protective. These biological effects are linked to the ability of Ezh2 inhibition to enhance bone-stimulatory signaling pathways. For example, Ezh2 loss stimulates Wnt ligand (e.g., Wnt10b and Wnt10a) expression, enhances PTH receptor (Pthr1h) expression, and increases Smad1/5 phosphorylation (i.e., BMP2 signaling cascade activation). Because of high cost and side-effects associated with clinical use of BMP2, we investigated whether BMP2 dosing can be reduced by concurrent Ezh2 inhibition. Co-administration of BMP2 and GSK126, a selective Ezh2 inhibitor, enhances MC3T3 osteoblast differentiation. Interestingly, GSK126 and BMP2 co-administration results in synergistic activation of osteogenic genes, alkaline phosphatase activity, and alizarin red staining. Dual BMP2 (10ng/ml) and GSK126 (5μM) administration is synergistic and as effective as 50ng/ml BMP2 at inducing MC3T3 osteoblastogenesis. mRNA-Seq analysis reveals robust increases in osteoblast/osteocyte markers with BMP2 and GSK126 co-administration. In support of the findings with MC3T3 osteoblasts, dual GSK126 and BMP2 administration enhances osteogenic gene expression (e.g., SP7 and IBSP) in human BMSCs undergoing osteogenic differentiation. BMP2 (300ng local) and