Original Article

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Label-free quantification proteomics reveals novel calcium binding proteins in matrix vesicles isolated from mineralizing Saos-2 cells

Xiaoying Zhou[1,2], Yazhou Cui[1], Jing Luan[1], Xiaoyan Zhou[1], Genglin Zhang[1], Xiumei Zhang[1], Jinxiang Han[1,*]

[1] *Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **Matrix vesicles (MVs) involved in the initiation of mineralization by deposition of hydroxyapatite (HA) in their lumen are released by the budding of mineralization-competent cells during skeletogenesis and bone development. To identify additional mineralization-related proteins, MVs were isolated from non-stimulated and stimulated Saos-2 cells in culture *via* an Exoquick™ approach and the corresponding proteomes were identified and quantified with label-free quantitative proteome technology. The isolated MVs were confirmed by electron microscopy, alkaline phosphatase activity (ALP), biomarkers, and mineral formation analyses. Label-free quantitative proteome analysis revealed that 19 calcium binding proteins (CaBPs), including Grp94, calnexin, calreticulin, calmodulin, and S100A4/A10, were up-regulated in MVs of Saos-2 cells upon stimulation of mineralization. This result provides new clues to study the mechanism of the initiation of MV-mediated mineralization.**

Keywords: Mineralization, matrix vesicles, Exoquick™, label-free LC-MS/MS, calcium-binding proteins

1. Introduction

Skeletogenesis and bone development occur by a series of physicochemical and biochemical processes. Together, these processes facilitate the mineralization of the extracellular matrix (ECM), in part by promoting the deposition of hydroxyapatite (HA) crystals in the sheltered interior of matrix vesicles (MVs). The MVs are membrane-invested bodies with diameters ranging from 30 to 400 nm that perform specialized roles in the initiation of matrix mineralization (*1,2*). These roles include transporting amorphous calcium phosphate (ACP), managing mineral nucleation, regulating the Pi/PPi ratio in the intra- and extra-cellular fluid, controlling calcium ion and Pi homeostasis, and interacting with the surrounding ECM to direct HA localization and growth (*3-7*). MVs possess protein and lipid machinery, highly enriched in certain mineralization-related proteins, that is essential to carrying out these functions (*8*). Proteomic studies of MVs isolated from different mineralization-competent cells have recently been reported (*1,2,9*), but whole-proteome pattern analysis and quantification of changes in MVs during mineralization process have yet to be performed.

Human osteosarcoma Saos-2 cells undergo the entire osteoblastic differentiation sequence from proliferation to mineralization (*10*) and release mineralization-competent MVs (*11*). Therefore, Saos-2 cells serve as a model of pro-mineralizing cells and were selected to analyze the functions of MVs. MVs are traditionally isolated from conditioned media by serial ultracentrifugation (*1,2*). Recently, a rapid proprietary method of exosome isolation called Exoquick™,

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.
E-mail: samshjx@sina.com

became commercially available; this method is reported to be as effective as ultracentrifugation (*12,13*). The current study used Exoquick™ to isolate MVs from non-stimulated and stimulated Saos-2 cell cultures. Label-free quantitative liquid chromatography-tandem mass spectrometry (label-free LC-MS/MS) was used to analyze the protein expression of two MV preparations. Results revealed novel calcium-binding proteins (CaBPs), including Grp94 (endoplasmin/HSP90), calnexin, calreticulin (calregulin), calmodulin, S100A4/A10, and plastin-3. These proteins were found to be highly expressed in MVs isolated from stimulated Saos-2 cells. Their functions in relation to mineralization are discussed here in conjunction with existing knowledge of the mineralization process.

2. Materials and Methods

2.1. *Cell culture and treatment*

Human osteosarcoma Saos-2 cells, acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were cultured in McCoy's 5A (Gibco, Carlsbad, CA, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Invitrogen, Carlsbad, CA, USA). Mineralization was induced by treatment of confluent Saos-2 cells (6 days to reach confluence) for 6 days in growth medium supplemented with 7.5 mM β-glycerophosphate (β-GP) (Sigma-Aldrich, St. Louis, MO, USA) and 50 μg/mL ascorbic acid (AA) (Sigma-Aldrich) (*14*).

2.2. *Isolation of MVs*

MVs were harvested from non-stimulated and stimulated Saos-2 cell cultures after 6 days using Exoquick™ (System Biosciences Inc., Carlsbad, CA, USA) (*13*). Saos-2 cells were digested with 1 mg/mL collagenase Type IA (Sigma-Aldrich) in Hank's balanced salt mixture (Solabio, Shanghai, China) at 37°C for 3 h. Cells were then pelleted by centrifugation at 3,000 g for 30 min. The supernatant was filtered through a 100K Amicon Ultra filter (Millipore Corporation, Billerica, MA, USA) to the appropriate volume. The supernatant was then added to Exoquick™ and mixed well by inversion. After refrigeration overnight (at least 12 h), the Exoquick/biofluid mixture was centrifuged at 1,500 g for 30 min to collect MVs, and all traces of supernatant were removed by aspiration. The collected MVs appeared as a white pellet and were stored at –80°C.

2.3. *Transmission electron microscopy*

Freshly obtained MV pellets were fixed in situ with 2.5% glutaraldehyde in 0.1 M sodium cacodylate acidulated buffer at 4°C for 2 h; after fixation, pellets were incubated in a 1% osmium tetroxide phosphate buffer solution for 1 h. The samples were dehydrated in a graded ethanol series with acetone and then embedded in epoxy resin. Semithin sections of approximately 75 nm were prepared, mounted on copper grids, and stained with uranyl acetate and lead citrate solutions to enhance the contrast. Electron micrographs were taken with an H800 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Japan).

2.4. *Alkaline phosphatase activity analysis*

The alkaline phosphatase (ALP) activity of MVs was determined using *p*-nitrophenyl phosphate (*p*-NPP) (Sigma-Aldrich) as a substrate and absorbance was recorded at 405 nm. An aliquot of MV pellets was lysed by addition of 100 μL buffer containing 25 mM Tris-HCl (pH = 7.4) and 0.5% Triton X-100 (*15*). Then, 1 μL of MVs lysate was mixed with 100 μL *p*-NPP. The reaction was stopped by addition of 50 μL NaOH (3 M) and absorbance was detected at 405 nm using Bio-Tek Synergy HT. ALP activity was normalized to total protein content using the Bradford method.

2.5. *Mineralization analysis*

Freshly collected MVs were suspended in mineralizing buffer (*14*) (100 mM NaCl, 12.7 mM KCl, 0.57 mM $MgCl_2$, 1.83 mM $NaHCO_3$, 0.57 mM Na_2SO_4, 3.42 mM NaH_2PO_4, 2 mM $CaCl_2$, 5.55 mM D-glucose, 63.5 mM sucrose, and 16.5 mM TES, pH = 7.5) and incubated for 12 h at 37°C. The calcium phosphate mineral complex was divided into two parts, centrifuged at 8,800 g for 15 min, and washed with water. One part of the resulting pellet was dried and incorporated by pressing it into KBr pellets. Mineral composition were determined in the range 400 – 4,000 cm^{-1} using a Nicolet 6700 Fourier transform infrared (FTIR) spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector. The mineral deposit produced a spectrum identical to that of the HA standard (Aladdin Industrial Inc., Shanghai, China). Another portion of the resulting pellet was solubilized with 0.1 M HCl for 3 h. The calcium content of the HCl supernatant was measured using a Calcium Assay Kit (Bioassay Systems, Carlsbad, CA, USA) (*16*) and normalized to total protein content using the Bradford method.

2.6. *LC-MS/MS*

Nano-flow LC-MS/MS was performed on a Thermo Easy-nLC 1000 equipped with a C18 reverse phase (RP) column (0.15 × 100 mm, Thermo EASY column SC200). The mobile phases were 2% acetonitrile

(ACN) with 0.1% formic acid (phase A and the loading phase) and 84% ACN with 0.1% formic acid (phase B). Samples were loaded onto a Thermo EASY column SC001 trap column (RP-C18, 0.15 × 2 mm) and equilibrated with 100% phase A. Samples were then subsequently separated with the C18 RP column at a flow rate of 400 nL/min. To achieve proper separation, a 120 min linear gradient elution was used: 0-100 min with 0% to 45% phase B; 100-108 min with 45% to 100% phase B; and 108-120 min with 100% phase B. Analysis of tryptic peptides was performed using a Q-Exactive mass spectrometer (Thermo Scientific). The mass spectrometer was operated in data-dependent mode and all analyses were performed using the positive nano-electrospray ion mode. The normalized collision energy value was set at 30%. Previously fragmented peptides were excluded for 25 s.

2.7. *Protein database search and label-free quantification*

Continuum LC-MS data were processed and searched using MaxQuant software (version 1.3.0.5). Proteins were searched against a "decoy database" based on the Homo sapiens protein sequence database (downloaded September 2012) from UniProt (*http://www.uniprot.org/*) and combined with common contaminants using the Andromeda search engine (*17*). Quantitative analyses were performed using a label-free approach, as previously described (*18*). A minimum of one quantification was required to establish the intensity value for each peptide. MaxQuant output files were subsequently uploaded into Perseus (version 1.2.0.17) for calculation of significance. The level of significance was determined with a 2-fold change. Analysis of data was completed using MAS 3.0 and *http://string.embl.de/* workstation.

2.8. *Western blotting*

MV pellets were homogenized in radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors on ice for 60 min and centrifuged at 12,500 g for 5 min at 4°C. The protein concentration of the MV lysate was determined using the Bradford method (BioRad Laboratories, Carlsbad, CA, USA). Samples (20 µg) were suspended in Laemmli loading buffer and incubated at 95°C for 6 min. Proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a polyvinylidene fluoride (PVDF) membrane. After 1 h of blocking with 5% low fat milk in TBST (10 mM Tris, 100 mM NaCl, and 0.05% Tween-20), membranes were incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-human CD63, CD9, and Hsp70 (1:1,000) from System Biosciences, Inc. (Carlsbad, CA, USA); mouse monoclonal anti-human ALP (B-10) (1:200), goat polyclonal anti-human Grp94 (C-19) (1:400), mouse monoclonal anti-human Calnexin (E-10) (1:1,000), and mouse monoclonal anti-human Calregulin (A-9) (1:100) from Santa Cruz Biotechnology (Carlsbad, CA, USA); mouse monoclonal anti-human Actin (1:1,000) from Beyotime (Shanghai, China); and rabbit polyclonal anti-human S100A10 (1:1,000) from Proteintech Group, Inc. (Wuhan, Hubei, China). Primary antibodies were immunostained with the appropriate peroxidase-conjugated secondary antibodies. Finally, the blots were visualized using ECL reagents (Millipore Corporation, Billerica, MA, USA) in accordance with the manufacturer's instructions.

2.9. *Statistical analysis*

For quantitative data, results are expressed as the mean ± S.D. of *n* observations. Statistical significance between groups was determined using an unpaired Student's *t* test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. *AA and β-GP stimulated Saos-2 cell mineralization and the release of mineralization-competent MVs*

AA and β-GP are two osteogenic factors known to accelerate osteoblastic differentiation and mineralization (*14*). As expected, mineralization was greatly enhanced by the concomitant addition of 50 mg/mL AA and 7.5 mM β-GP in Saos-2 cell cultures over 6 days. MVs were isolated from non-stimulated and stimulated Saos-2 cells after 6 days using Exoquick™. The intact MVs were confirmed by electron microscopy and assayed for ALP activity, expression of biomarkers, and mineral formation. As seen in TEM, freshly isolated MVs were recognized as spherical membrane-bounded vesicle structures with diameters ranging from 30 to 400 nm (Figures 1A and 1B). MVs isolated from stimulated cells contained electron-dense material (Figure 1B), which was previously reported to have mineralizing capabilities (*1*). As expected, MVs isolated from stimulated Saos-2 cells exhibited greater ALP activity than those from non-stimulated cells (Figure 1C). Moreover, after incubation in mineralizing buffer for 12 h MVs that were isolated from stimulated cells were able to continue Ca^{2+} uptake (Figure 1E) and form mineral HA, a finding that was confirmed using FTIR (Figure 1D). Proteins including CD63, CD9, and Hsp70, which are known to be reliable biomarkers of MVs and exosomes (*6,19*), were present in both types of MV preparations (Figure 2).

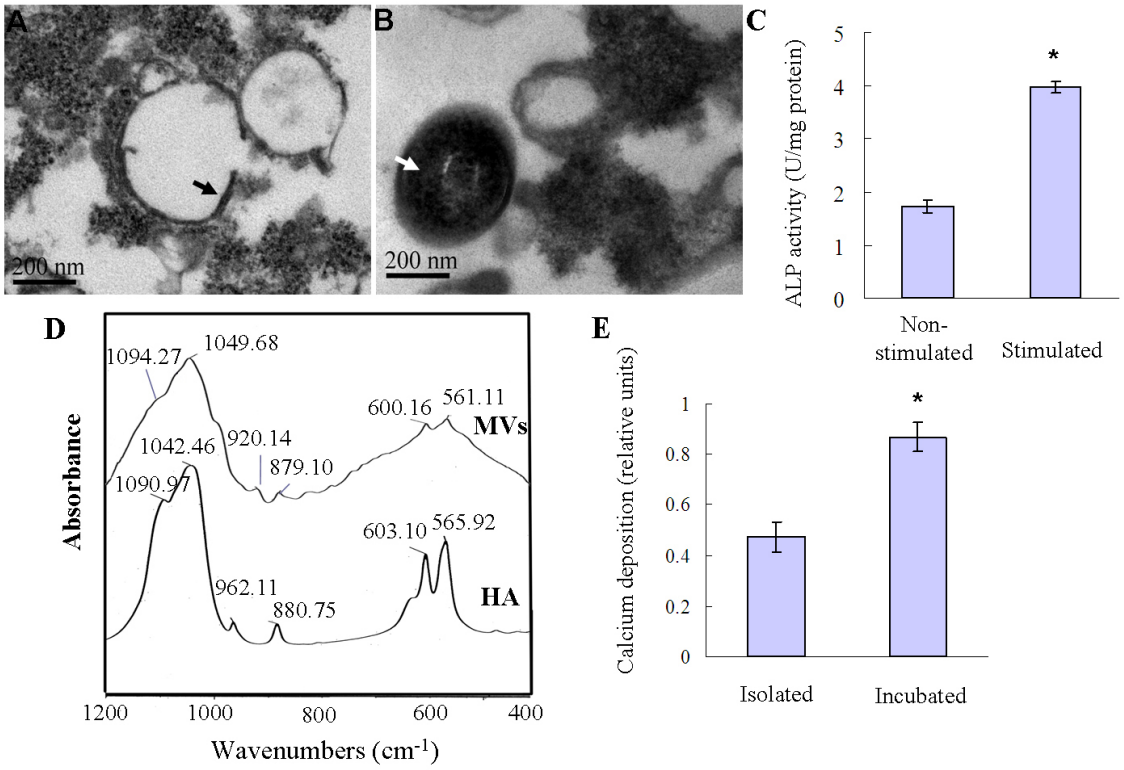

Figure 1. Ultrastructural studies, ALP activity, and functional studies of MVs. (A, B) TEM view of MVs isolated from non-stimulated and stimulated Saos-2 cells. The black arrow indicates MVs that were membrane-bounded bodies. The white arrow shows a vesicle containing electron-dense material, previously reported to be signs of calcification. (**C**) ALP activity of MVs isolated from non-stimulated and stimulated Saos-2 cells (Results are mean ± SD, $n = 3$). * $p < 0.05$, *vs*. non-stimulated cells. (**D, E**) Mineralization assay of MVs. MVs isolated from stimulated Saos-2 cells were incubated at 37°C in mineralizing buffer for 12 h. Infrared spectra (**D**) indicated that the mineral formed by MVs was hydroxyapatite. Isolated MVs were able to continue taking up Ca^{2+} (**E**) when incubated in mineralizing buffer (Results are mean ± SD, $n = 3$). * $p < 0.05$, *vs*. isolated group.

3.2. *Results and classification of MV proteins identified by LC-MS/MS*

In total, 255 different proteins were obtained from MVs from non-stimulated and stimulated Saos-2 cells according to software analysis (fold ≥ 2). Of these, 186 proteins increased and 69 proteins decreased. MV proteins that were previously reported to be related to mineralization, such as ALP and NPP1 (inorganic pyrophosphatase, ectonucleotide pyrophosphatase phosphodiesterase 1) (*8*), were highly expressed in the mineralized MVs, demonstrating the usefulness of this model for discovery of new proteins involved in the mineralization process. The different proteins were classified based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification using the MAS 3.0 workstation.

Analysis of molecular function based on the GO classification indicated that 19 CaBPs and 4 calmodulin-binding proteins (CaMBPs) were up-regulated in MVs of Saos-2 cells upon stimulation of mineralization (Table S1, *http://www.biosciencetrends.com/docindex.php?year=2013&kanno=3*). Analysis based on KEGG pathway classification revealed that 7 proteins associated with calcium signaling pathways (CaSP) were highly expressed in mineralized MVs

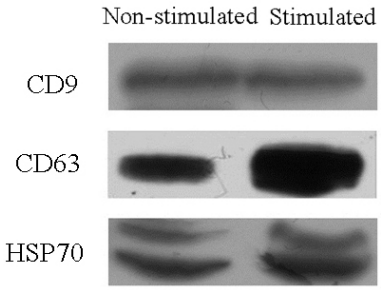

Figure 2. Presence of exosomal protein markers. CD9, CD63, and Hsp70 were present in both MV preparations.

(Table S1, *http://www.biosciencetrends.com/docindex.php?year=2013&kanno=3*).

3.3. *Validation of MV proteins identified by Western blotting*

To confirm both the accuracy of protein identification by mass spectrometry and label-free quantification, five proteins were selected for validation by Western blotting. Consistent with label-free quantification, four detected CaBPs (Grp 94, calnexin, calregulin, and S100A10) were more highly expressed in the MVs isolated from stimulated cells than those from non-

stimulated cells (Figure 3). Furthermore, ALP, used as a marker of mineralization and identifier of MVs (*20*), was also enriched in the mineralized MVs (Figure 3). Together, data from proteomics and Western blotting results suggest that the current study reliably identified MV proteomes of Saos-2 cells.

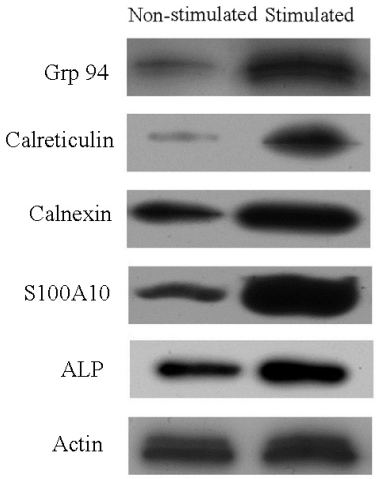

Figure 3. Enrichment of MVs with Grp 94, calnexin, calregulin, S100A10, and ALP upon stimulation.

4. Discussion

This study confirmed that the Exoquick™ approach, used to isolate biofluid and cell culture-derived exosomes (*12,13*), was effective at isolating quality MVs from Saos-2 cells. The MVs isolated from stimulated cells can still take up Ca ions and form HA when incubated in mineralizing buffer, thus indicating that MVs have their own proteins to carry out mineralization. Label-free LC-MS/MS helped to shed light on subtle differences between MVs from non-stimulated and stimulated Saos-2 cells.

The intravesicular initiation of mineralization requires Ca^{2+} influx into MVs and formation of phosphatidylserine (PS) nucleation complexes (*21*). The CaBPs, which bind Ca ions in specific domains, are known to actively participate in numerous cellular functions such as Ca^{2+} homeostasis and Ca^{2+} signal pathways (*22*). The current results showed that 19 CaBPs were up-regulated in different ratios; this was partly confirmed by Western blotting (Figure 3). In addition, Grp94, calnexin, calreticulin, calmodulin, and plastin-3 were previously identified as MV proteins (*1,2,9,23*), but they have not been cited as important components of MV-mediated mineralization. Both Grp94 and calreticulin are reported to bind HA (*24,25*). A recent study determined that purified native Grp94 can directly bind both ACP and HA in the presence of excess calcium and phosphate but that its binding to ACP is inhibited by ATP, bisphosphonates, and pyrophosphate (*24*). That study also noted a novel Grp94-calreticulin-annexin A5-mineral complex generated after massive calcium influx in SiHa cells (*24*). Alternatively, abundant CaBPs including Grp94 and calreticulin are responsible for the storage of calcium to protect cells from massive calcium influx (*26*). Therefore, these CaBPs are presumed to be involved in the initiation of mineral nucleation. When mitochondrial Ca^{2+} is released *via* mitochondrial transition pores into the Pi-rich intracellular vesicles (*7*), the released Ca^{2+} interacts simultaneously with both Pi and PS, forming PS-Ca^{2+}-Pi complexes, and it interacts with PS and CaBPs to form PS-Ca^{2+}-CaBPs-Pi complexes. ACP is a major component of both PS-Ca^{2+}-Pi and PS-Ca^{2+}-CaBPs-Pi complexes (27). After that interaction, vesicles containing ACP are transported to the extracellular space and deposited on collagen fibrils. These vesicles continue to accumulate Ca and Pi, which stimulate the formation of HA crystals from the immature mineral present in the lumen, initiating mineralization (*7*). During HA crystal propagation, CaBPs are also needed to bind the influx of free Ca^{2+} to the mineral. In addition, the increased regulation of calumenin is associated with mouse cartilage development (*28*) and healing of closed mid-diaphyseal fractures (*29*). Plastin-3 has a potential Ca^{2+}-binding site near the N terminus and may also contribute to the assembly and maintenance of plasma membrane protrusions. These protrusions, from which MVs appear to originate, are characteristic of growth plate chondrocytes *in vivo* (*27*) and MC3TC cells *in vitro* (*1*). This group of CaBPs may take part in the biogenesis of MVs, managing mineral nucleation, transporting Ca^{2+}, and controlling calcium homeostasis in the MVs. Moreover, further studies are needed to ascertain the possible function of each CaBP.

Calmodulin, one of the major calcium sensors and Ca^{2+} signal mediators, is not confined to its Ca^{2+}-bound form because the Ca^{2+}-free form can also recognize different target proteins including calmodulin kinase II, calcineurin, and many ion channel proteins (*30*). The calmodulin pathway is associated with regulation of osteoblast growth and differentiation (*30,31*). The inhibition of both calmodulin and calmodulin kinase II can decrease ALP activity and cell mineralization (*31*). Calmodulin can interact selectively and non-covalently with CaMBPs, both in its calcium-bound and calcium-free states. The current study identified 4 CaMBPs including MYO1C/1B, MARCKS, and IQGAP1, that were up-regulated in the mineralization process. Fortunately, all four have previously been identified as MV proteins (*2,9,32*), but their roles in mineralization are unknown. These CaMBPs may interact with calmodulin or regulate the calmodulin pathway to affect biomineralization.

In addition, the current study found that S100A4, A6, A10, A11, and A13 were expressed in two MV samples. Only S100A4 and A10 were significantly up-

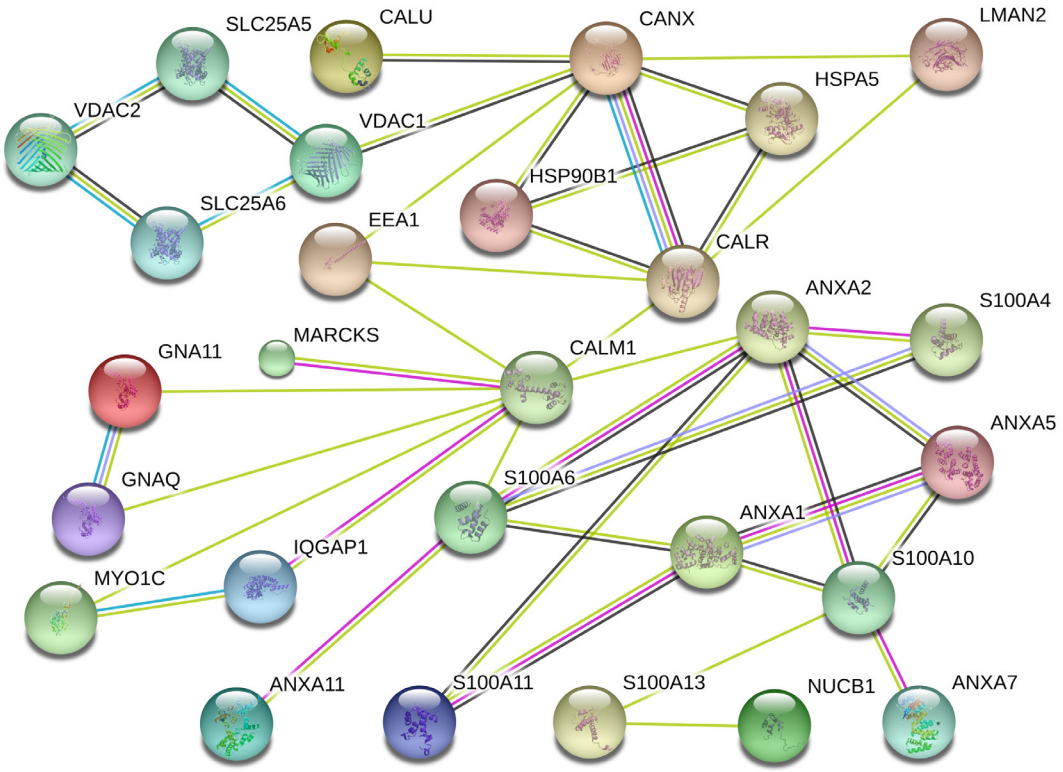

Figure 4. STRING analysis of direct (physical) or indirect (functional) associations among the calcium-associated proteins analyzed. The network nodes are proteins in the illustration shown. The lines in different colors represent the types of evidence for the associations.

regulated in MVs from stimulated cells. To date, there has been little evidence suggesting the enrichment of S100 families in mineralized MVs compared to non-mineralized MVs (*11*). In agreement with a previous study (*11*), the current results suggest that S100A10 may be associated with MV-mediated mineralization. However, determination of the specific function of S100A10 will require additional study.

The annexins have previously been described as important to MV function through channel-directed Ca ion uptake into MVs (*11,33*). Nevertheless, a recent study found that Annexin A5 and Annexin A6-deficient mice developed no abnormal mineralization (*34*). The same group also found that the depletion of Annexin A5 and Annexin A6 in mice caused no gross changes in MV-mediated mineralization (*35*). The current results revealed no significant differences in Annexin A1, 2, 4, 5, 6, 7, or 11 expression for MVs from non-stimulated and stimulated cells. Therefore, annexins may not be essential or directly linked to the MV-mediated mineralization process.

STING workstation (*36*) was used to analyze the interaction among the calcium-associated proteins indicated earlier (Table S1, *http://www.biosciencetrends.com/docindex.php?year=2013&kanno=3*). Figure 4 shows that calnexin, calreticulin, and calmodulin act as connection centers. Thus, further studies are clearly needed to determine whether these CaBPs play an important role in bone mineralization as MV components.

In conclusion, MVs were isolated from non-stimulated and stimulated Saos-2 cell cultures *via* an Exoquick™ approach and their proteomes were identified and quantified using label-free quantitative proteome technology. The proteomes analysis indicated that 19 CaBPs were up-regulated in MVs of Saos-2 cells upon stimulation of mineralization. Understanding the role of CaBPs and their potential partners in MV functions may provide novel insights into the mechanism of physiological bone mineralization.

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