Original Article

DOI: 10.5582/irdr.2013.v2.2.59

A comparative proteomics study on matrix vesicles of osteoblast-like Saos-2 and U2-OS cells

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Summary **Matrix vesicles (MVs) play an important role in the initial stage of the process of bone mineralization, and are involved in multiple rare skeletal diseases with pathological mineralization or calcification. The aim of the study was to compare the proteomic profiling of osteoblast-like cells with and without mineralization ability (Saos-2 and U2-OS), and to identify novel mineralization-associated MV proteins. MVs were extracted using ExoQuick solution from mineralization-induced Saos-2 and U2-OS cells, and then were validated by transmission electron microscopy. A label-free quantitative proteomic method was used to compare the protein profiling of MVs from Saos-2 and U2-OS cells. Western-blots were used to confirm the expression of MVs proteins identified in proteomic studies. In our proteomic studies, we identified that 89 mineralization-related proteins were significantly up-regulated in Saos-2 MVs compared with U2-OS MVs. We further validated that two MVs proteins, protein kinase C α and ras-related protein Ral-A, were up-regulated in MVs of Saos-2 cells compared to those of U2-OS cells under mineralization-induction. Our findings suggest that protein kinase C α and ras-related protein Ral-A might be involved in bone mineralization as MVs components.**

Keywords: Matrix vesicle, osteoblasts, mineralization, proteomics

1. Introduction

Matrix vesicles (MVs) are small vesicles with a diameter of 50-200 nm, which contain abundant phospholipid and protein components and play an important role in the process of bone mineralization (*1*). It has been reported that a MV protein deficiency might participate in the pathological mineralization process of many rare skeletal diseases (*2*). Recently, the protein profiling of MVs from different origins were analyzed in several proteomic studies, and more than 2,000 proteins have been identified in MVs including several validated mineralization-related proteins such as alkaline phosphatase (TNAP), and annexins (*3-6*). However, the roles of most of the MV proteins in mineralization regulation remain unclear.

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Saos-2 is a human osteoblast-like cell line having significant mineralization ability under osteogenic induction. Meanwhile, another human osteoblast-like cell U2-OS has low phosphate enzyme activity and could not be mineralized after induction (*7*). Therefore, we speculated that the difference among MV proteins between Saos-2 and U2-OS cells could provide novel clues to clarify the exact roles of MVs during the mineralization process.

In this study, we performed a label-free LC-MSMS proteomic approach to compare the MV protein profiles between Saos-2 and U2-OS cells in order to further screen functional MV proteins for mineralization regulation.

2. Materials and Methods

2.1. *Cell culture and osteogenic induction*

Human Saos-2 and U2-OS cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). U2-OS and

Saos-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Thermo Scientific, Logan, UT, USA) and McCoy's 5A Medium (Gibco, Life Technologies, Carlsbad, CA, USA) respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Beyotime, Haimen, Jiangsu, China) at 37°C under 5% (v/v) CO_2 in a humidified atmosphere. Mineralization was induced on confluent cells in induction medium supplemented with 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich).

2.2. *Mineralization detection by Alizarin Red staining*

Saos-2 and U2-OS cells were plated in 24-well plates for induction, and mineralization levels were assessed by alizarin red staining during induction (0 days, 3 days, and 7 days). The cells were washed with PBS and fixed with 4% paraformaldehyde. Then the cells were washed with PBS and stained with 0.5% (w/v) alizarin red S solution for 1 h. After washing with PBS, the stained cultures were photographed.

2.3. *Matrix vesicles extraction by ExoQuick solution*

Cells were washed with PBS without calcium-magnesium ions 3 times, and then collagenase (2 mL, 1 mg/mL) was added. Supernatant was collected and centrifuged at 3,000 rpm for 30 min at 4°C, transferred into a 100 kD ultrafiltration concentrator tube (Millipore, Billerica, MA, USA) and centrifuged at 3,000 rpm for 30 min at 4°C to concentrate to about 1 mL. The concentrated liquid was mixed with the ExoQuick Exosome Isolation Reagent (System Biosciences, Mountain View, CA, USA) at 4°C overnight. The mixture was centrifuged at 3000 rpm for 30 min at 4°C to sediment MVs.

2.4. *Transmission electron microscopy validation for matrix vesicles*

Freshly isolated MVs pellets were first fixed with 3% glutaraldehyde at 4°C for 2 h, and then post-fixed in 1% osmium tetroxide for 1 h. After dehydration in a graded ethanol series with acetone, samples were embedded in epoxy resin. 75 nm thick semithin sections were mounted on copper grids, and stained with uranyl acetate while lead citrate solutions were used to enhance the contrast. Electron micrographs were observed on a H800 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Tokyo, Japan).

2.5. *LC-MS/MS analysis and label-free quantification*

MVs protein samples were extracted by adding 200 μL SDT buffer containing 4% SDS, 150 mM Tris (pH 8.0). After heating and sonication, the lysates were centrifuged (10,000 g, 1 h, 4°C) and the supernatants were collected. The protein concentrations were assayed with a standard Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 150 μg of MVs protein samples from U2-OS and Saos-2 cells (with and without mineralization induction) were digested by a filter-aided sample preparation (FASP) method as described previously (*8*). Mass analyses were performed on a Q Exactive mass spectrometer. MS/MS spectra were searched against the nonredundant Inter-national Protein Index (IPI) human protein database or Swiss-prot database. Label-free quantitative analyses were performed as described previously (*9*). MaxQuant output files were uploaded into a Perseus package for calculation of significant scores. Proteins with scores that varied more than 2 fold were defined as significantly differentially expressed.

2.6. *Western-blots*

Equal amounts (25 μg) of MVs protein samples were separated on a 12% SDS-PAGE gel, and transferred to PVDF membranes (Millipore), and then probed with primary antibodies against protein kinase C α (Ptglab, 1:1000 dilution) and ras-related protein (Ptglab, 1:1000 dilution) overnight at 4°C, followed by peroxidase-conjugated secondary antibodies. Finally, the bands were visualized using ECL reagents (Millipore) according to the manufacturer's instructions.

3. Results

After osteogenic induction, Saos-2 cells demonstrated a time-dependent increase in mineralized nodule formation assessed by Alizarin Red staining, while mineralization was absent in the matrix of U2-OS cells (Figure 1). Using ExoQuick reagents, MVs were successfully precipitated, and were validated by

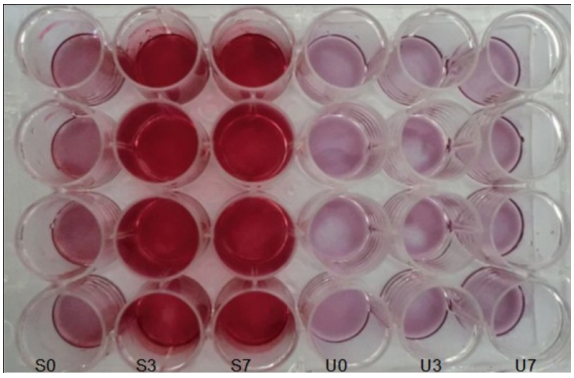

Figure 1. Alizarin Red test for the mineralization of U2-OS and Saos-2 cells. Saos-2 cells for 0 day, 3 days and 7 days after mineralization induction, respectively (S0,3,7); U2-OS cells for 0 day, 3 days and 7 days after mineralization induction, respectively (U0,3,7). Saos-2 cells appear significantly mineralized after induction for 3 days, while in U2-OS cells mineralization was still not clear after induction for 7 days.

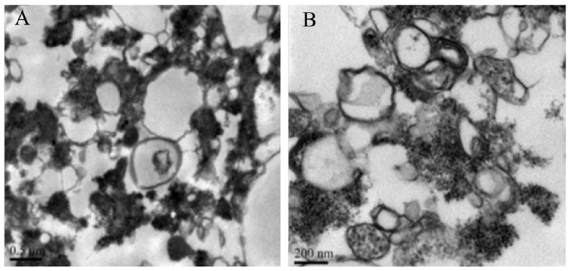

Figure 2. Representative images of Transmission electron microscopy on MVs. (A) Saos-2 MVs with mineralization nodules after induction for 7 days; **(B)** U2-OS MVs without mineralization nodules after induction for 7 days.

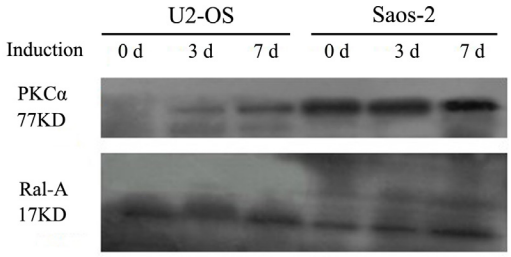

Figure 3. Western-blot analysis on protein kinase C α (PKCα) and ras-related protein Ral-A on MVs from Saos-2 and U2-OS cells at different induction times. Saos-2 MVs had higher PKCα expression levels than U2-OS MVs; Saos-2 MVs showed additional bands of Ral-A.

Table 1. Top list of GO molecular function on up-regulated MVs proteins in Saos-2 cells compared with U2-OS cells

GO Term	Count	*p*-Value	*q*-Value
GO:0005515 protein binding	51	7.16E-51	1.15E-48
GO:0000166 nucleotide binding	34	3.84E-46	3.09E-44
GO:0005524 ATP binding	20	2.44E-26	1.07E-24
GO:0005525 GTP binding	13	1.98E-22	6.84E-21
GO:0005509 calcium ion binding	12	1.68E-15	4.51E-14
GO:0003924 GTPase activity	10	3.88E-19	1.25E-17
GO:0016491 oxidoreductase activity	10	9.55E-14	2.00E-12
GO:0051082 unfolded protein binding	8	1.08E-16	3.08E-15
GO:0003779 actin binding	7	3.66E-11	6.09E-10

Table 2. Top list of GO molecular function on up-regulated MVs proteins in Saos-2 cells compared with U2-OS cells

GO Term	Count	*p*-Value	*q*-Value
GO:0044419 interspecies interaction between organisms	26	1.04E-53	2.50E-51
GO:0002474 antigen processing and presentation of peptide antigen *via* MHC class I	19	4.73E-48	5.71E-46
GO:0006955 immune response	19	5.59E-28	3.00E-26
GO:0019882 antigen processing and presentation	18	3.59E-40	2.48E-38
GO:0015031 protein transport	11	9.69E-15	2.46E-13
GO:0055114 oxidation reduction	10	4.31E-14	9.91E-13
GO:0007264 small GTPase mediated signal transduction	8	1.76E-11	3.14E-10
GO:0007165 signal transduction	8	6.27E-05	3.74E-04
GO:0008152 metabolism	8	0.009911	0.015593

transmission electron microscopy. As seen in Figure 2, the precipitation by ExoQuick reagents recognized spherical membrane-bound vesicle structures with diameters ranging from 50 to 200 nm, and part of the vesicles contained electron dense material.

In the proteomic study, we identified a total of 175 differentially expressed proteins (fold > 2) in Saos-2 MVs compared with U2-OS MVs, including 89 up-regulated proteins and 86 down-regulated proteins (Supplemental data, Table 1S (*http://www.irdrjournal.com/docindex.php?year=2013&kanno=2*)). Among the up-regulated MVs proteins, alkaline phosphatase (ALP) ranked as the most significantly increased MVs protein in Saos-2 cells, which has been proved in many studies. 89 up-regulated proteins were further classified on Gene Ontology in terms of biological processes (Table 1) and molecular function (Table 2) using the MAS 3.0 software (CapitalBio, Beijing, China). In particular, we observed that 12 up-regulated MVs proteins of Saos-2 cells belong to calcium ion binding proteins (GO: 0005509).

To confirm the results of our proteomic study, two up-regulated MV proteins in Saos-2 cells (protein kinase C α and ras-related protein Ral-A) were selected for validation by Western blotting. We detected that both MVs proteins were overexpressed in Saos-2 cells compared to U2-OS cells, which is consistent with our proteomic findings (Figure 3).

4. Discussion

Matrix vesicles have been implicated in pathological mineralization events, which are characteristic of multiple rare diseases. In this study, we compared the MVs protein profiles between mineralization-competent cells Saos-2 and mineralization incompetent cells U2-OS. We identified a panel of differentially expressed proteins at MVs levels associated with mineralization, which could provide novel clues for mining the regulatory details of mineralization.

We performed a GO analysis on 89 up-regulated MV proteins in Saos-2 cells. In molecular function analysis, differentially expressed proteins were mainly

categorized into protein binding, nucleotide binding, ATP-binding, GTP-binding and calcium ion binding groups. In particular, a subgroup of calcium ion binding proteins were identified including mannose receptor [C type 2], protein kinase C [alpha] (PKCα), profilin 1, heat shock protein 90 kDa beta, macrophage migration inhibitory factor, S100 calcium binding protein A6, annexin A6, NAD-dependent malic enzyme [mitochondrial], S100 calcium binding protein A13, and transketolase. Most of these proteins have been proved to be involved in mineralization process regulation, such as, annexin A6 (*10,11*). However, several calcium ion binding proteins identified in this study have not been reported. We selected one of them, protein kinase C α (PKCα), for further validation. Our Western-blot study confirmed that Saos-2 MVs had an increased expression level of PKCα compared to U2-OS during mineralization induction. Previous studies indicated that PKCα plays an important role in osteoblast differentiation and mineralization. Bawden *et al*. (*12*) found that PKCα was localized in differentiating odontoblasts and the PKC signal transduction pathway may be involved in key inductions in the early stages of dentin and enamel formation. Miraoui *et al*. (*13*) demonstrated that PKCα acts as an important regulator in FGFR2-induced osteogenic differentiation of mesenchymal cells. Our data for the first time found that PKCα could also be located in MVs and up-regulated in mineralization-competent cells which suggests PKCα might be engaged in the mineralization process at the MVs level, however, its exact mechanism needs further investigation.

Phospholipase D is an important enzyme for the mineralization process (*14*). Phospholipase D is engaged in a high rate of hydrolysis of neutral phospholipids and a lower rate of degradation of anionic phospholipids, which allows mineral formation in MVs (*15*). It has been proposed that ras-related protein Ral-A is involved in the tyrosine kinase-mediated, Ras-dependent activation of phospholipase D (*16*). In our study, we confirmed that Ral-A also exists as a MVs component, and suggests that its interaction with phospholipase D in MVs might participate in the regulation of the mineralization process.

In conclusion, in this study we identified a series of MVs proteins specifically up-regulated in mineralization competent osteoblasts, which might provide new clues to study the mechanism for mineralization control in the extracelluar matrix. We also confirmed that protein kinase C α and ras-related protein Ral-A are novel MVs proteins and might be involved in bone mineralization as MVs components.

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(Received April 25, 2013; Revised May 16, 2013; Accepted May 17, 2013)