Extracellular Membrane Vesicles Derived from 143B Osteosarcoma Cells Contain Pro-Osteoclastogenic Cargo: A Novel Communication Mechanism in Osteosarcoma Bone Microenvironment1,2,3,4

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Abstract

The bone microenvironment (BME) is the main hub of all skeletal related pathological events in osteosarcoma leading to tumor induced bone destruction, and decreasing overall bone quality and bone strength. The role of extra-cellular membrane vesicles (EMVs) as mediators of intercellular communication in modulating osteosarcoma-BME is unknown, and needs to be investigated. It is our hypothesis that osteosarcoma-EMVs contain pro-osteoclastogenic cargo which increases osteoclastic activity, and dysregulated bone remodeling in the osteosarcoma-BME. In this study, EMVs were isolated from the conditioned media of 143B and HOS human osteosarcoma cell cultures using differential ultracentrifugation. Nano-particle tracking analysis determined EMVs in the size range of 50-200 nm in diameter. The EMV yield from 143B cells was relatively higher compared to HOS cells. Transmission electron microscopy confirmed the ultrastructure of 143B-EMVs and detected multivesicular bodies. Biochemical characterization of 143B-EMVs detected the expression of bioactive pro-osteoclastic cargo including matrix metalloproteinases-1 and -13 (MMP-1, -13), transforming growth factor-β (TGF-β), CD-9, and receptor activator of nuclear factor kappa-β ligand (RANKL). Detection of a protein signature that is uniquely pro-osteoclastic in 143B-EMVs is a novel finding, and is significant as EMVs represent an interesting mechanism for potentially mediating bone
Introduction
Osteosarcoma is an aggressive malignancy of bone, mainly affecting adolescents and young adults. Interactions between osteosarcoma and bone microenvironment (BME) promote tumor growth and osteoclastic bone destruction. The main goal of this study is to understand the role of extracellular membrane vesicles (EMVs) as potential modulators of osteosarcoma BME and to identify the key biochemical components of EMVs mediating cellular dynamics and dysregulated pathologic remodeling of the matrix and bone. EMVs are membrane-invested structures that are derived from a number of cells including osteosarcoma cells [1,2]. In recent years, EMVs have received much attention for their role in various diseases and as biomarkers of therapy and disease burden [3]. Recent studies report that tumor cell–derived EMVs support cancer cell growth, survival, metastasis, and angiogenesis, evade host immune surveillance, modulate tumor microenvironment (TMN), and initiate the formation of premetastatic sites [4–12]. Tumor-derived EMVs, in general, originate through the fusion of multivesicular bodies (MVBs) with the plasma membrane (exosomes) or by budding (shed vesicles or microvesicles), followed by exocytotic release [13–16]. Detection of EMVs and osteoblastic and osteoclastic lesions in the bioluminescent osteosarcoma orthotopic mouse (BOOM) model provides a strong rationale to investigate the role of EMVs in modulating osteosarcoma BME [2]. Biochemical analyses of EMV cargo will be informative as it will identify the key EMV mediators underlying osteosarcoma pathobiology.

Biomechanical stress in the bone TMN leads to increased intracellular calcium levels that, in turn, may promote EMV biogenesis, increase the expression of extracellular remodeling enzymes such as matrix metalloproteinases (MMPs), and stimulate exocytotic delivery of bioactive cargo. These biochemical events may result through the activation of G protein–coupled receptors (GPCRs) or calcium-dependent signaling pathways. A study by Ancha et al. showed the role of H2 receptor, a GPCR, in the regulation of MMP-1 expression and secretion in cultured gastric cells [17]. Savina et al. demonstrated that increased intracellular calcium concentrations in K562 leukemia cells trigger Rab11-mediated fusion of MVBs with the plasma membrane and release exosomes [18]. Another study suggested the role of cAMP/protein kinase A pathway in the release of tumor necrosis factor receptor I–associated exosomes [19]. In the osteosarcoma BME, neither the role of cAMP/protein kinase A pathway nor of calcium-dependent pathway and their downstream effects on cytoskeleton rearrangements leading to vesicle biogenesis are known and are subjects of the current study.

Functional implications of EMVs depend on the cargo composition that, in turn, is governed by the metabolic status of the donor cell from which they originate. For instance, EMVs containing MMPs and proteases such as plasminogen activator promote tumor invasion and metastases, whereas those enriched in cytokines such as transforming growth factor β (TGF-β) evade host immune response. Little is known about the mechanisms underlying EMV-mediated intercellular dynamics in the TMN. Peinado et al. reported a role for melanoma exosomes in establishing premetastatic niches by reprogramming bone marrow–derived cells [20]. Exosomes derived from prostate, breast, and lung cancer cells activate fibroblasts or mesenchymal stem cells by increasing their motility and rendering them resistant to apoptosis [21,22] or by stimulating myofibroblastic differentiation [23,24].

Extracellular matrix remodeling is an important process mainly mediated by metalloproteinases, such as MMPs in the tumor BME, which enable the tumor cells to grow, invade, and metastasize. Another important role of MMPs besides extra cellular matrix (ECM) degradation is in the activation of membrane-associated proteins and regulation of cell signaling pathways. Increased expression of MMP-1, MMP-2, and MMP-9 and down-regulation of micro RNA (miRNA) 143, which targets MMP-13, correlates to poor prognostic outcomes in patients with osteosarcoma [25–28]. A recent study by Husmann et al. clearly outlines the importance of MMP-1 in osteosarcoma pathobiology where in short hairpin RNA (shRNA)-mediated down regulation of MMP-1 expression in 143B cells generated smaller primary tumors and fewer micrometastases and macrometastases in the lungs, and overexpression of MMP-1 in nonmetastatic HOS cells resulted in osteolytic primary tumors and lung metastasis [29].

It is our hypothesis that osteosarcoma EMVs contain pro-osteoclastogenic cargo that increases osteoclastic activity and dysregulated bone remodeling in the osteosarcoma BME. In this study, we demonstrate that 143B osteosarcoma cells generate EMVs by mechanisms involving cAMP/calcium-dependent signaling pathways and contain pro-osteoclastic cargo.

Materials and Methods
Detection of Tumor-Induced Histopathologic Changes in the Osteosarcoma BME
For evaluating tumor-induced histopathologic changes in the osteosarcoma BME, excised tibias (tumor bearing) from the BOOM model [2] were fixed in 4% paraformaldehyde (pH 7.4). The in vivo studies using the BOOM model were done with the assistance of the Proof of Concept Laboratory at The University of Kansas Cancer Center, with the approval of the University of Kansas Institutional Animal Care and Use Committee. For histopathologic evaluation, tibias were decalcified in 10% EDTA (pH 7.5) for 2 weeks before sectioning and paraffin embedding. The sections were processed for hematoxylin and eosin staining and immunohistochemistry (IHC). To detect osteoblastic-mediated mineralization in the tumor tissue, von Kossa staining was done using non-decalcified tumor tissue.
sections. To detect the immunoexpression of MMPs in the tumor tissue of the BOOM model, MMP-1 and MMP-13 IHC was done using primary antibodies (MMP-1, RB-1536; MMP-13, MS-825) purchased from Lab Vision Thermo Scientific (Kalamazoo, MI), followed by detection. The detection reagents were purchased from Biocare Medical (Concord, CA) and Dako (Carpinteria, CA). For negative control, primary antibody was excluded, and human placenta tissue sections were used as positive control in MMP IHC.

Preparation of Conditioned Media from 143B Cell Cultures

Human osteosarcoma cell lines 143B (highly aggressive and metastatic; k-ras activated) and HOS (nonaggressive and nonmetastatic; k-ras wild type) were purchased from American Type Culture Collection (Manassas, VA). The 143B cells were genetically engineered to express luciferase gene (FUW-Luc-mCherry-puro), and cultured in Dulbecco’s modified Eagle’s medium according to the manufacturer’s recommendations. EMVs were engineered to express luciferase gene (FUW-Luc-mCherry-puro) and cultured in serum-free media for 24 hours and subjected to differential ultracentrifugation for isolation of EMVs.

Isolation, Quantitation, and Characterization of EMVs Derived from the CM of 143B and HOS Cell Cultures

Differential ultracentrifugation. We used differential ultracentrifugation (low speed followed by ultracentrifugation at 110,000g for 2 hours) to isolate EMVs from the CM prepared from osteosarcoma cells according to the scheme shown in Figure 1.

Nanoparticle tracking analysis. To determine the EMV concentration and size distribution profile of EMVs isolated from CM of osteosarcoma cell cultures, vesicles were analyzed using the NanoSight (Amesbury, UK) NTA 2.3: Nanoparticle Tracking and Analysis instrument and software (release version build 11 RC1, 2012, hardware: LM14). The samples were injected in the sample chamber according to the manufacturer’s recommendations. EMVs were analyzed in phosphate-buffered saline solution under Brownian motion at 22°C to 24°C with laser wavelength at 638 nm. Multiple video frames were captured for 60 seconds per reading. Screen gain remained at 1.0, and detection threshold ranged from 13 to 14. The number of readings for EMVs, at dilutions 1:5000, 1:2000, 1:1000, and 1:100, ranged from 5 to 20 measurements. Analysis displayed plots describing particle diameter (size) distribution cumulative of each reading, 2-dimensional (2-D) and 3-dimensional (3-D) particle light intensity against particle concentration per milliliter, as well as a video snapshot.

Alkaline phosphatase assay. The alkaline phosphatase (ALP) activity of EMVs was assayed using ALP colorimetric kit (AnaSpec, Fremont, CA). Briefly, 50-μg vesicles were incubated with a colorimetric substrate, para-nitrophenyl phosphate, and the conversion of para-nitrophenyl phosphate to p-nitrophenol on release of phosphatase ions was monitored at 405 nm. The protein concentration of the EMV samples was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Flow cytometry and fluorescence microscopy. For detection of mCherry fluorescence on EMVs derived from mCherry-labeled, 143B luciferase–expressing, puromycin-resistant cells, EMV suspensions were examined microscopically using the Olympus (Center Valley, PA) IX71 inverted fluorescent microscope equipped with a x100 arc lamp and monochromatic complementary metal oxide semiconductor camera. In addition, flow cytometric data were acquired on EMV suspensions using the BD LSR II flow cytometer integrated with FACSDiva software (BD Biosciences, San Jose, CA, USA).

Transmission electron microscopy (TEM) analyses. For TEM, 143B EMV pellets were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide (OsO4), dehydrated, embedded in epon resin, and cut into ultrathin sections. The sections were stained with uranyl acetate and lead acetate before mounting on EM grids. The sections were examined and photographed using a JEM 1400 electron microscope (JEOL USA, Inc., Peabody, MA, USA) (80 kV).

Western blot analyses. To determine the biochemical composition of the 143B EMV cargo, Western blot analyses were performed according to the previously described method [30]. 143B EMVs were homogenized in Tris lysis buffer (20 mM Tris, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and 1 mM DTT). Crude lysates of 143B cells (12.5-25 μg) and EMVs (25-40 μg) were denatured in sodium dodecyl sulfate sample buffer, electrophoresed on 12% denaturing polyacrylamide gels, and visualized by Ponceau stain. For immunoblot analysis, the proteins from the gel were transferred on to a polyvinylidene fluoride (PVDF) membrane and incubated with the following primary antibodies: anti-MMP-1 and anti–MMP-13 (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); 200 μg/ml each) at 1:200, anti–CD-9 (SBP: System Biosciences (Mountain View, CA, USA); 0.25 mg/ml) at 1:1000, and anti-RANKL and anti–TGF-β (GeneTex (Irvine, CA, USA); 1 mg/ml) at 1:1000 dilution. Detection of the immunostained bands was done by ECL chemiluminescence detection system (Thermo Scientific, Rockford, IL). Image acquisition was done using LabWorks Image Acquisition and Analysis Software 4.6.00.0 (UVP Bioimaging Systems, Upland, CA) and Image Lab software for the ChemiDoc MP system (Bio-Rad Laboratories) at incremental exposure time frame of 15, 30, 60, 180, and 300 seconds.

Calcium sensitization and 143B EMV biogenesis. To measure the effect of intracellular rise in calcium concentrations on EMV biogenesis, mCherry-labeled 143B cells were cultured on 35-mm dishes to subconfluence and prepared for calcium imaging. Briefly, 143B cells were loaded with 1 μM Fura-2 AM, a fluorescent dye which binds to free intracellular calcium by incubating at 37°C for 30
minutes according to previously described methods [31]. The ratio of Fura-2 excitations at 340 to 350 nm and 375 to 380 nm of light corresponds to the intracellular calcium concentration \([Ca^{++}]\). Specifically, we evaluated the effects of two agents that modulate intracellular \([Ca^{++}]\): ionomycin, an ionophore, which increases intracellular calcium levels through store-dependent mechanisms and forskolin, an activator of cAMP generating adenylate cyclase on EMV biogenesis. Osteosarcoma cells were either stimulated with ionomycin (alone) at three different concentrations, i.e., 1, 3, and 10 \(\mu M\), or pretreated with forskolin at 10 \(\mu M\) before the addition of ionomycin. Measurements of increase in calcium concentrations in 143B osteosarcoma cells were recorded using a Photon Technology International (PTI Technologies Inc, Birmingham, NJ) automated spectrofluorometer connected to an inverted microscope (Leica DMI-4000B; Leica Microsystems, Wetzlar, Germany) equipped with a 14-bit CoolSNAP charge-coupled device camera (Photometrics, Tucson, AZ). Data acquisition, calibration, and analysis were done using the EasyPro (PTI) software. Changes in the cellular morphology and induced EMV biogenesis on forskolin and/or ionomycin stimulation were observed in high power (×40) by fluorescence microscopy. Forskolin pretreatment was done using 10 \(\mu M\) concentrations at 37\(^{\circ}\)C for 5 minutes. Ionomycin stimulation was done at 1, 3, and 10 \(\mu M\). Intracellular calcium concentration was estimated from the Fura ratio by using Grynkiewicz equation [32].

**Statistical analysis.** Data presented represent means (+ SD) from three or more independent experiments. Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA). All experimental data are presented as means ± SD. Student’s t test and one-way analysis of variance were used for determining statistical significance between resting cells (before stimulation) versus ionomycin or forskolin + ionomycin–treated cells. A P value of <0.05 was considered statistically significant.

**Results**

**Rapidly Growing Tumor Induces Histopathologic Changes in the Osteosarcoma BME**

Histopathologic studies on the tumor tissue obtained from the BOOM model detected remarkable tumor-induced morphologic changes as evidenced by varying cortical bone thickness and destruction of tibia of tumor-bearing mice (Figure 2, A and B). Detection of resorptive pits and multinucleate osteoclasts in the tibial sections of the BOOM model demonstrates high osteoclastic activity (Figure 2C). Intense von Kossa staining of tumor-bearing bones suggests tumor-induced prolific osteoblastic activity (Figure 2D). Light microscopy revealed the presence of numerous osteocytes in the tumor-bearing bone (Figure 2, A and B). Whether those osteocytes are transformed by 143B EMVs is unknown at present and is a subject for future investigation. Furthermore, detection of numerous vesicles in the vicinity of the resorption pit suggests an active catabolic role for osteoclasts in osteosarcoma pathobiology (Figure 2E). Ultrastructural examination of the extracellular matrix of the tumor tissue from the BOOM model revealed the presence of EMVs interspersed among collagen fibrils (Figure 2F). Immunohistochemical studies detected the expression of MMP-1 and MMP-13 in the tumor and nontumor cells such as osteocytes, osteoclasts, and osteoblasts of the osteosarcoma BME (Figure 3).

**Isolation and Characterization of 143B EMVs Includes a Multimodality Approach Using Differential Centrifugation, Nanoparticle Tracking Analysis, and TEM**

Osteosarcoma EMVs were isolated from the CM of mCherry + ve, 143B-luc, and HOS cells by differential ultracentrifugation (Figure 1). The size distribution profile of isolated EMVs as determined by
nanoparticle tracking analysis (NTA) was in the range of 50 to 200 nm (Figures 4, A and B, and W1). The EMV yield generated from 143B cells was higher as reflected by their mean EMV number per milliliter (711 × 10^8 bEMVs per milliliter) and protein concentration (1.2 mg/ml) compared to HOS cells (mean EMV number per milliliter = 7.3 × 10^8 hEMVs per milliliter) and protein concentration (0.33 mg/ml) (Figure W2). Because 143B EMV output was greater (100 ×) than HOS EMVs, and for the sake of focus of the current study, further characterization was done on 143B EMVs. Ultrastructural characterization of EMVs derived from 143B cells revealed the presence of numerous vesicles in the size range of 50 to 200 nm (Figure 4, C and D). TEM revealed the presence of MVBs and perivesicular mineral clusters in the osteosarcoma BME (Figure 4, C and D). Presence of ALP enzyme activity in 143B-derived EMVs confirmed their mineralization competence as observed by TEM (Figure 5A). Flow cytometry and fluorescence microscopy detected the retention of mCherry fluorescence in EMVs derived from mCherry + ve, 143B luciferase-expressing cells (Figure 5, B and C).

**143B EMVs Contain Pro-Osteoclastogenic Cargo**

Biochemical characterization of cargo proteins of 143B-derived EMVs by Western blot analysis demonstrates the expression of a pro-osteoclastogenic cargo, which includes MMPs (MMP-1 and MMP-13), CD-9, RANKL, and TGF-β (Figure 6). Detection of a clear band at 52 kDa in 143B EMV lysates corresponds to the predicted band size for MMP-1 as previously reported by Husmann et al., in the 143B cell lysates [29] (Figure 6A). This band is likely to be a proenzyme as reported previously [33]. Immunodetection for MMP-13 expression revealed the presence of a major band at 68 to 70 kDa that was selectively enriched in 143B EMVs (Figure 6A). This band is very likely to be the proenzyme form of MMP-13 as previous studies report the detection of the proenzyme or the latent form at 60 to 65 kDa, whereas the active form is detected at 30 to 48 kDa [34,35].

Further characterization revealed that 143B EMVs contain pro-osteoclastogenic cargo, i.e., CD-9, RANKL, and TGF-β (Figure 6C). All the three proteins are potent stimulators of osteoclastogenesis [36–40], but their presence in EMVs derived from osteosarcoma has never been previously reported. Immunoblot analysis of 143B EMVs with CD-9 antibody detected a band at 48 to 50 kDa, which is very likely the trimeric form. Recent studies have reported the presence of multimeric forms of CD-9 detected at 24 kDa (monomeric), 38 kDa (homodimer), 52 to 54 kDa (trimer), and 70 to 72 kDa (tetramer), which most likely form due to spontaneous intermolecular disulfide bonding of membrane-proximal cysteine residues [41,42]. Immunoblot analysis of 143B EMVs with anti-RANKL antibody revealed the presence of multimeric form of RANKL at 48 kDa. Previous studies report the existence of the following three different RANKL isoforms: RANKL1, which is similar to the original RANKL, contains both the intracellular and transmembrane spanning domain; RANKL2, which has a shorter intracellular domain than RANKL; and RANKL3, which lacks the...
transmembrane domain, constitutes the soluble form of RANKL and inhibits osteoclastogenesis [43]. Immunoblot analysis of 143B EMVs with anti-TGF-β antibody revealed the presence of latent form of TGF-β at 52 kDa, which was also detected in exosomes derived from brain tumors [44].

**Figure 5.** Determination of ALP activity and detection of mCherry fluorescence in 143B EMVs. A shows bar graphs comparing ALP activity (U/ml) in 143B EMVs versus 143B cell lysates (n ≥ 3). B shows detection of mCherry fluorescence in EMVs derived from 143B-luc-mCherry OS cells (× 20). C shows mCherry-positive 143B EMV forward scatter (FSC) and side scatter (SSC) by flow cytometry.

**Ionomycin and Forskolin Stimulate Calcium Mobilization within 143B Cells Leading to Vesicle Biogenesis**

Calcium imaging studies revealed that 143B cells actively mobilize calcium in the presence of ionomycin, a calcium ionophore, and cause cytoskeleton rearrangements leading to vesiculation. Confocal
microscopy showed that ionomycin induced morphologic changes within 143B cells such as loss of cell-cell contact, distortion of cellular margins, changes in the cytoskeleton architecture, formation of membrane blebs, and accumulation of intracellular, perinuclear vesicles (Figure 7, A1 and B1). Addition of 1, 3, and 10 μM ionomycin to 143B cells induced a significant increase ($P < 0.0001$) in intracellular [Ca++] within 300,000 milliseconds (Figures 7 C1, and W3). Pretreatment with 10 μM forskolin, an adenylate cyclase activator, increased calcium mobilization in both naïve and ionomycin-sensitized 143B OS cells and resulted in increased intracellular [Ca++] within 100,000 milliseconds (Figures 7 D2, and W3). The above events stimulated cytoskeleton rearrangements within 143B cells leading to vesicular biogenesis (Figure 7, A2, B2, and C2).

**Discussion**

Emerging evidence suggests the role of EMVs in supporting tumor microenvironment niches and as potential mediators of intercellular communication mainly through horizontal transfer of oncogenic cargo [45,46]. Although EMVs were previously detected in the

![Image of Western blot analysis](image)

**Figure 6.** Detection of pro-osteoclastogenic cargo in 143B EMVs by Western blot analysis. Crude lysates of 143B cells (12.5-25 μg) and EMVs (25-40 μg) were analyzed for MMP-1, MMP-13 (A and B), TGF-β, RANKL, and CD-9 (C) expression by Western blot analysis. All samples were analyzed in triplicate.

**Figure 7.** Calcium mobilization and induction of EMV biogenesis in 143B OS cells in the presence of forskolin and ionomycin. A1 and A2, show morphologic changes within ionomycin-sensitized 143B cells leading to the accumulation of intracellular, vesicles, whereas B1 and B2, show intracellular vesiculation in forskolin-pretreated and ionomycin-sensitized 143B cells as observed by confocal microscopy. C1 and D1, show kinetic changes in the Fura-2 ratio in ionomycin (C1) and in forskolin-pretreated, ionomycin-sensitized (D1) 143B cells. C2 and D2, compare Fura 350/375 between resting versus ionomycin (alone)–treated 143B osteosarcoma cells (C2) and resting versus forskolin-pretreated and ionomycin-sensitized (forskolin + ionomycin combination) 143B osteosarcoma cells (D2).
BOOM model [2], their role as potential drivers of cancer-induced bone destruction and as key mediators of osteolytic activity in the osteosarcoma BME needs further investigation. This study for the first time reports isolation and characterization of EMVs derived from 143B human osteosarcoma cells and its potential implications on the TMN. It clearly demonstrates that majority of the EMVs derived from 143B cells are in the size range of 50 to 200 nm in diameter. The use of NTA allows quantitative and rapid determination of EMV sample size, size range, and concentration. It is highly reliable for accurately determining the size distribution of cell-derived EMVs as it is based on Brownian motion, does not consider the refractive index of the nanoparticle, and is free from sample shrinkage artifacts commonly encountered during fixation for microscopy [47]. Vesicles obtained from 143B CM were devoid of contaminating vesicles from FBS [48]. Detection of MVBs by TEM in 143B EMV samples suggests that the mode of biogenesis and release of EMVs is most likely through endocytic invagination followed by the formation of early endosomes that mature to form MVBs. Size range of 143B EMVs as determined by NTA (50-200 nm), evidence of MVBs by TEM, and the presence of CD-9, an exosome-specific biomarker as listed in ExoCarta database (Bundoora, Victoria, Australia), suggest that 143B EMVs contain exosomes.

To our best knowledge, this is the first study to report the presence of a pro-osteoclastogenic cargo in EMVs isolated from 143B cells. Detection of MMPs (MMP-1 and MMP-13) in 143B EMVs is an important and novel finding because MMP-1 and MMP-13 (MMP)–expressing EMVs could be used as disease biomarkers for evaluating osteosarcoma prognosis. Detection of RANKL in osteosarcoma EMVs is novel and significant as it plays an important role in the activation of MMPs and for stimulating osteoclastogenesis. Targeting MMP-1 expression and activity through RANKL inhibition is promising as recent studies by Casimiro et al. demonstrates a role of RANKL in the activation of MMP-1 expression and activity in breast cancer metastasis [49]. Whether selective inhibition of EMV-derived RANKL and/or MMP-1 and MMP-13 inhibits osteosarcoma pathology remains to be investigated. Targeting RANK/RANKL osteoprotegrin (OPG) signaling in osteosarcoma is currently under intense investigation, and studies with OPG and RANK-Fc demonstrate inhibition of osteolytic lesions in mouse models and improved survival rates [50,51].

Detection of TGF-β in 143B EMVs is an important finding especially in the context of regulating the bone TMN. In the BME, TGF-β is generated mainly from the mineralized bone matrix by osteoclastic resorption and further stimulates the production of osteolytic and proneoplastic factors [52,53]. It can stimulate migration of osteoblast progenitors and osteosarcoma cells either directly [54] or indirectly through osteoclast-mediated chemokine (C-X-C motif) ligand 16 (CXCL16) chemokine secretion [55]. It plays an important role in the osteoclastogenic differentiation of uncommitted monocytes by stimulating RANKL and/or tumor necrosis factor α (TNF-α)-induced nuclear factor of activated T-cells cytoplastic, calcineurin dependent 1 (NFATc1) expression [38]. Tumor exosomes and microvesicles secrete TGF-β that blocks the differentiation of monocytes and increase the accumulation of immature myeloid cells including myeloid-derived suppressor cells (MDSCs) [56]. Recently, the role of MDSCs in the osteolytic bone tumor microenvironment in promoting osteoclastic bone resorption was demonstrated [57,58]. Whether EMV-derived TGF-β increases MDSC-mediated osteoclastic resorption in the OS BME is currently unknown and is the subject of our future studies. Blocking exosome-derived TGF-β is an attractive therapeutic strategy to reduce osteoclastic activity from MDSCs in the tumor microenvironment and increase the efficacy of antitumor immune therapies.

Detection of CD-9, a tetraspanin protein in the EMVs derived from 143B cells, is a novel finding. To our best knowledge, the role of this protein in osteosarcoma pathobiology has never been investigated. Besides being a designated exosome-specific marker, CD-9 is also a pro-osteoclastogenic fusogenic protein as it regulates osteoclast differentiation and the formation of mature polynuclears [36,59]. It is overexpressed in osteotropic cancers and not only promotes the homing of cancer cells in the bone marrow but also induces osteoclastic bone resorption [37]. Studies report that inhibition of CD-9 by KMC8, a widely used antibody against CD-9, suppresses osteoclastogenesis [60], whereas RANKL-stimulated expression of CD-9 and other fusogenic genes such as CD-47 in osteoclast precursors promotes mature polykaryotic, tartrate-resistant acid phosphatase and osteoclast-specific transmembrane protein expressing osteoclast phenotype [61]. A recent study demonstrated the role of CD-9 in mediating MMP-9–induced migration and invasion in fibrosarcoma cells [62].

Elevation of intracellular calcium concentration on forskolin pretreatment and ionomycin sensitization of 143B cells leads to changes in the cytoskeleton architecture and vesicle biogenesis. This finding is important especially in the context of osteosarcoma BME where actively metabolizing cancer cells maintain energy homeostasis by regulating cytosolic calcium through induction of oscillatory events that eventually trigger cytoskeleton rearrangements and vesicle biogenesis. Previous studies have reported that elevated intracellular calcium concentration (Ca2+), cAMP levels, and P2X7 receptor (purinergic receptor ion channels mediating calcium and influx across the plasma membrane) activation modulate the pool of EMV output and sorting of cargo by regulating docking, priming, and exocytosis of vesicles [19,63,64]. Identification of targets associated with EMV biogenesis in response to elevated calcium or adenylyl cyclase remains to be elucidated. Therapies targeting the osteosarcoma BME could be designed to either inhibit EMV biogenesis directly or inactivate their bone-destructive, proneoplastic cargo.

In conclusion, this study suggests a novel role of EMVs in driving osteoclastic bone resorption by virtue of their pro-osteoclastogenic cargo and disrupting bone remodeling homeostasis in the osteosarcoma BME.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2014.04.011.

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