Original Article

Osteoblast-derived extracellular vesicles are biological tools for the delivery of active molecules to bone†

Running title: Osteoblast-derived extracellular vesicles and bone

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Abstract

Extracellular vesicles (EVs) are newly appreciated regulators of tissue homeostasis and a means of intercellular communication. Reports investigated the role of EVs and their cargoes in cellular regulation and have tried to fine-tune their biotechnological use, but to date very little is known on their function in bone biology. To investigate the relevance of EV-mediated communication between bone cells, we isolated EVs from primary mouse osteoblasts and assessed membrane integrity, size and structure by transmission electron microscopy and fluorescence-activated cell sorting. EVs actively shuttled loaded fluorochromes to osteoblasts, monocytes and endothelial cells. Moreover, osteoblast EVs contained mRNAs shared with donor cells. Osteoblasts are known to regulate osteoclastogenesis, osteoclast survival and function by the pro-osteoclastic cytokine, Rankl. Osteoblast EVs were enriched in Rankl, which increased after PTH treatment. These EVs were biologically active, supporting osteoclast survival. EVs isolated from rankl\(^{-/-}\) osteoblasts lost this pro-osteoclastic function, indicating its Rankl-dependence. They integrated \(ex-vivo\) into murine calvariae, and EV-shuttled fluorochromes were quickly uptaken by the bone upon \(in vivo\) EV systemic administration. \(Rankl^{-/-}\) mice lack the osteoclast lineage and are negative for its specific marker TRAcP. Treatment of \(rankl^{-/-}\) mice with wildtype osteoblast EVs induced the appearance of TRAcP-positive cells in an EV density-dependent manner. Finally, osteoblast EVs internalized and shuttled anti-osteoclast drugs (zoledronate and dasatinib), inhibiting osteoclast activity in vitro and in vivo. We conclude that osteoblast EVs are involved in intercellular communication between bone cells, contribute to the Rankl pro-osteoclastic effect and shuttle anti-osteoclast drugs, representing a potential means of targeted therapeutic delivery. This article is protected by copyright. All rights reserved

Keywords

Extracellular vesicle, Osteoblast, Osteoclast, Rankl, Osteopetrosis, Targeted shuttling.
Introduction

Extracellular vesicles (EVs) represent a large group of complex membrane structures ranging from 40nm to 1000nm in diameter. They are phospholipidic bilayers encompassing cellular constituents, classified mainly according to their size and mechanisms of biogenesis. EVs generally comprise two different classes: small exosomes, of endocytic origin, and microvesicles, arising by budding from the plasma membrane.

When EVs were firstly observed, they were considered a product of cellular wasting and non-specific debris. Nowadays, the biologic meaning of EVs has dramatically changed, switching from a concept of a sophisticated detoxifying system of engulfed/dying cells, now attributed only to apoptotic bodies, to a programmed and regulated means of exchange of biologic information, modulated according to cellular needs and status. In this view, the mode of intercellular communication acquires one more paracrine mechanism in addition to cell-to-cell, autocrine, juxtacrine and systemic communications.

To date, there is evidence revealing that EVs are able to shuttle biologically active molecules, such as proteins, lipids, mRNAs and miRNAs, by many mechanisms of cellular interaction, including juxtacrine signaling, ligand/receptor interaction, protein shedding and cargo internalization. EVs can shuttle both ligands and receptors, thus activating new signaling pathways. Additionally, cell-derived EVs appear capable of transferring essential genetic information from one cell to another. Furthermore, they are under intense investigation for biotechnological and clinical applications, since EV cargoes can be manipulated to shuttle exogenous information, delivering molecules and drugs directly to the target cells.
Bone is a dynamic tissue hosting finely tuned metabolic processes aimed at guaranteeing the health of the bone tissue itself and that of other organs.\textsuperscript{(23)} To this aim, bone harbors an intricate and strictly synchronized remodeling process in which the bone-forming cells (osteoblasts), the bone-resorbing cells (osteoclasts) and the bone-sensing cells (osteocytes) have an intimate crosstalk, with coordinated and controlled coupling. This relationship induces a tightly regulated influence on the activities and functions of these cells. Since when the first pivotal molecule in osteoblast-osteoclast coupling was identified,\textsuperscript{(24,25)} more and more pathways and communication mechanisms between bone cells have been brought to light.

One of the most paradigmatic molecules implicated in the osteoblast-osteoclast crosstalk is the Receptor Activator of Nuclear Factor κ-B Ligand (Rankl).\textsuperscript{(25)} Rankl is predominantly a transmembrane protein, also occasionally cleaved into a soluble form, which in bone is expressed by osteoblasts and osteocytes and which binds its receptor, Rank, on the surface of osteoclasts and monocytes. The Rankl/Rank axis is a pivotal mediator of osteoclastogenesis, osteoclast function and osteoclast survival, and both human and murine osteopetrosis forms characterized by the absence of osteoclasts have been demonstrated to carry loss-of-function mutations of these genes.\textsuperscript{(26)}

Based on the complexity of the cellular cycle governing bone remodeling and the exchange of a plethora of molecules between cells in the bone microenvironment, the involvement of EVs in bone pathophysiology is a potential piece of the complex puzzle of bone cellular crosstalk. Despite the dramatic interest in EVs in many fields, their roles in bone biology are still poorly understood. Very few articles have reported the characterization of their molecular profile and activity, and it is possible that the real physiologic role of EVs in the bone microenvironment has been underestimated since only immortalized or tumor cells have been investigated so far, which exhibit intrinsic genetic abnormalities (i.e viral and pro-oncogenic genes).\textsuperscript{(27-29)}
In this study, we focused on mouse primary osteoblast-released EVs and characterized some of their biologic aspects, revealing that they have a previously unrecognized role in the bone cell crosstalk, and demonstrating their relevance as a useful tool to cleverly target bone cells.

**Materials and Methods**

*Animals*

Mice were employed for cell cultures and *in vivo* experiments in conformity with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, Dec. 12, 1987; Italian Legislative Decree, Gu n.61, 14/03/2014) and the NIH guide for the care and use of laboratory animals (8th edition. Washington (DC): National Academies Press (US); 2011). Their use was approved by the Institutional Review Board of the University of L’Aquila, Italy. At the end of the in vivo experiments and for primary cultures, mice were sacrificed by CO₂ inhalation. Mice treated with EVs starting at 5 days of life and subjected to blood collection were sacrificed by decapitation. For *in vivo, ex vivo* and *in vitro* experiments, wildtype mice were of the CD1 and the C57/BL6 backgrounds, whereas *rankl⁻/⁻* (*tnfsf11⁻/⁻*) mice were of the C57/BL6 background.

Mice were purchased by Charles River-Laboratories Italia S.r.l (Milan, Italy), housed in the animal facility of the University of L’Aquila, Italy at the following conditions: temperature, 20-24°C; humidity, 60±5%; dark/light cycle, 12/12 hours. They had access to food and water *ad libitum*, and have been fed with a normal diet (Mucedola code: 3KE25).

For the *in vivo* EV kinetics and biological effects, the sample size was calculated using dedicated software (SigmaPlot) based on the expected differences.
Cell cultures

Primary osteoblasts were obtained from skull bones of 7-10 days old CD1 mice (both genders). Calvariae were excised and sequentially digested three times with 1mg/ml *Clostridium histolyticum* type IV collagenase (Sigma, #C5138-1G) and 0.25% trypsin (Becton Dickinson) dissolved in Hank’s buffered salt solution (HBSS, Gibco). Osteoblasts derived from the second and third digestions were cultured in DMEM containing 10% Fetal Bovine Serum (FBS, Gibco), glutamine (5mM, Gibco) and penicillin/streptomycin (5U/ml, Gibco).

For transwell experiments, primary murine osteoblasts (2x10^5 cells/transwell-6 well) were centrifuged at 400g for 5 minutes and washed twice in PBS. Then, cell suspension was labeled with 1µM PKH26 (Sigma, #MINI26-1KT) for 5 min and washed twice (5min x 2) in complete DMEM (10% FBS). A transwell system with a 1µm pore size membrane (BD Falcon cell culture insert) was used for culturing PKH26 labeled osteoblasts in the upper chamber and unstained cells in the lower chamber. In both chambers, cells were plated at a density of 1x10^6/cm².

Mature osteoclasts were obtained from bone marrow buffy coat mononuclear cells (BMMs) of 10 days old CD1 mice (both genders). BMMs were seeded at a density of 1.2x10^6 cells/cm² and cultured in DMEM (containing penicillin, streptomycin and L-glutamine) supplemented with 10%FBS and recombinant human (rh)Macrophage Colony-Stimulating Factor (rhM-CSF, 50ng/ml, Peprotech). After 3 days, osteoclast differentiation was induced by adding rhRankl (120ng/ml, Peprotech) for additional 3 days. Mature osteoclasts were then harvested, seeded (1.5x10^5 cells/cm²), starved in DMEM supplemented with 0.2mg/ml in Bovine Serum Albumin (BSA, Sigma-Aldrich) for 12h and treated with EVs or drugs for 48h.
Primary mouse monocytes were obtained as described above, without the treatment with the rhM-CSF and rhRankl.

EA.hy926 human endothelial cell lines were obtained from the American Tissue Culture Collection.

***EV isolation and staining***

EV were isolated from untreated osteoblasts or from osteoblasts treated every 12h with $10^{-7}$M rhParaThyroid Hormone (rhPTH, Sigma-Aldrich). Upon reaching 80% confluence, cells were washed in PBS and starved in serum-free DMEM to prevent FBS EV contamination. After 24h, culture medium was harvested and sequentially centrifuged at 4°C at 5000g for 20 min to remove cell debris, 35000g for 20 min to remove apoptotic bodies, and 100000g for 70min to spin down EVs. The pellets containing EVs were then incubated for 30min at 37°C with either the membrane-permeant green dye 5-chloromethylfluorescein diacetate (CMFDA) (Thermo Fisher, #C7025) or with the SYTORNASElect Green Fluorescent Cell Stain (Thermofisher, #S32703), and for 5 min at 37°C with the cell membrane labeling linker PKH26. Then, the EVs were washed in PBS by ultracentrifugation at 100000g, at 4°C for 70 min. Finally, the pelleted EVs were re-suspended in a volume of PBS according to the application, and immediately used for the experiments or stored at -80°C. All cell treatments were performed using EV suspensions derived from 2.5x10^6 cells/cm^2.

***Western blot analysis***

Osteoblasts and their EVs were lysed in RIPA buffer and protein content was quantified by the BiCinchoninic Acid (BCA) assay (ThermoFisher, #23225). Proteins were separated by SDS-PAGE in non-reducing conditions, and blotted onto nitrocellulose membrane, which was incubated overnight at 4°C with primary CD63 antibody (1:200, Biolegend, #143902). After 1 hour incubation at room temperature with HRP-goat anti-rat IgG (1:2000) (Santa Cruz Biotechnology, #7074) or with the SYTORNASElect Green Fluorescent Cell Stain (Thermofisher, #S32703), and for 5 min at 37°C with the cell membrane labeling linker PKH26. Then, the EVs were washed in PBS by ultracentrifugation at 100000g, at 4°C for 70 min. Finally, the pelleted EVs were re-suspended in a volume of PBS according to the application, and immediately used for the experiments or stored at -80°C. All cell treatments were performed using EV suspensions derived from 2.5x10^6 cells/cm^2.
Cat. #sc-2006) bands were visualized by Super Signal West Pico chemiluminescent substrate (Thermo Fisher, #RA227125).

Transmission Electron Microscopy (TEM)

For TEM, 5µl of EV suspension in PBS were deposited onto Formvar-coated grids and left to air-dry for 20min. Grids were then washed with PBS and fixed by transfer onto 1% glutaraldehyde for 5min. After washing with distilled water, samples were contrasted with 4% uranyl-oxalate solution (4% uranyl acetate, 0.15M oxalic acid, pH 7) for 5min. Finally, grids were air-dried for 10 min and observed under a Philips CM 30 TEM at 80kV.

Fluorescence-Activated Cell Sorting (FACS) of EVs

EV pellets were loaded with CMFDA and incubated with a phycoerythrin (PE)-conjugated anti-Rankl antibody (1:100, BD Biosciences, Cat. #560295) for 30 min at 4°C, then CMFDA-loaded, Rankl-positive and -negative EVs were sorted by FACS. Nano Fluorescent standard particles (Spherotech, cat#NFPPS-52-4K) were used to set dimensional gate up to 1µM. Routine immunophenotype evaluation was performed on BD FACSCANTO II. Sorting of pure EV population was performed on BD FACSARIA II.

Gene expression

Conditioned media from osteoblasts were processed for EV isolation by ultracentrifugation as described above. Donor osteoblasts were massively washed in PBS (to eliminate any contaminants from the EVs present in the conditioned medium) and trypsinized. Osteoblast suspension was washed twice in PBS by centrifugation at 300g for 5 min at 4°C, then 1 million of these cells and the EV pellet were processed for RNA extraction by RNeasy mini kit (Qiagen, cat #74104), according to the manufacturer’s instructions, and quantified by Nanodrop 2000.
For large scale analysis, 32ng of total RNA were retrotranscribed by PreAMP cDNA Synthesis Primer Mix for mouse Osteoporosis, then the cDNA was ran on a mouse Osteoporosis PCR array (Qiagen, cat#PAMM-170Z).

For semi-quantitative RT-PCR of the RNA extracted from EVs, 100ng of RNA were reverse transcribed using the Moloney murine leukemia virus reverse transcriptase, subjected to PCR reaction that was performed by the DreamTaq Green master mix (Thermo Fisher) and verified by electrophoresis of PCR products in 2% agarose gel plus ethidium bromide. Primer pairs were: Gapdh, fwd 5’-TGGCAAAGTGAGATTGTTGC-3’; rev 5’-AAGATGGTGATGGGCTTCCCCG-3’; Col-1α1, fwd 5’-GTCCCTCTGGAAATGCTGAC-3’; rev 5’-GACCGGGAAGACCGACCA-3’.

For semi-quantitative RT-PCR of the RNA extracted from primary osteoblasts treated with PBS (control) or with osteoblast-derived EVs, 1µg of RNA was retrotranscribed and subjected to PCR as described above using the following primer pairs: Gapdh, fwd 5’-TGGCAAAGTGAGATTGTTGC-3’; rev 5’-AAGATGGTGATGGGCTTCCCCG-3’; Alp, fwd 5’-CTGGGAGTCTCATTGAGC-3’; rev 5’-CCAGCAGGTTTCTGTTTG-3’; Runx2, fwd 5’-GCCGGGAATGAGAACTA-3’; rev 5’-GGACCCTCCACTGTCACTTT-3’; Atf4, fwd 5’-TCGATGCTCATTTCGAATG-3’; rev 5’-AGAATGTAAAGGGGCAACC-3’; Osterix fwd 5’-AGAATCGTCTCCTCTCCA-3’; rev 5’-TGCTCCTGACTGAC-3’; Col-1α1, fwd 5’-GTCCCTCTGGAAATGCTGAC-3’; rev 5’-GACCGGGAAGACCGACCA-3’; Bglap, fwd 5’-CTTGGTTCTGACTGGGT-3’; rev 5’-GCCCTCTGCAGTGATAG-3’.

PCR conditions were 94°C for 30s, 60°C for 30s, and 72°C for 30s, replicated for 35 cycles.
**EV loading with anti-osteoclast drugs**

Sodium zoledronate (Sigma-Aldrich) was dissolved in PBS at a concentration of 14mM, and dasatinib (Selleckchem) was dissolved in DMSO at a concentration of 10mM. EVs from 2.5x10^6 osteoblasts/cm^2 were incubated with either drug for 1h at 37°C, then they were extensively washed in PBS and ultracentrifuged twice at 100000g for 70 min.

**In vitro EV biologic effects**

Mature osteoclasts were starved in serum-free DMEM as described above and treated for 48h with EVs (intact or drug-loaded) derived from 2.5x10^6 osteoblasts/cm^2. Osteoclasts were then fixed in paraformaldehyde/PBS (4% v/v), cytochemically stained for the osteoclast marker Tartrate-Resistant Acid Phosphatase (TRAcP) (Sigma, #387A-1KT) and counterstained with 1µM Hoechst 33342 (Sigma-Aldrich) to identify the nuclei.

**Ex vivo EV integration into bone**

Seven days old CD1 mice were sacrificed, their calvariae excised, plated in 35mm dish in DMEM with 10% FBS and incubated with PKH26-labeled EVs. After 48h, EV integration in the calvarial bones was evaluated by an Olympus Fv1000 confocal microscope.

**In vivo EV kinetics and tissue targeting**

Cryopreserved FACS-sorted Rankl-positive and PKH26-labeled EVs were injected intraperitoneally into 5 days old CD1 male mice. Groups of animals (n=6 each) were sacrificed after 1.5, 3, 6, 24 and 48 h and blood, liver, spleen, kidney and bones were collected and processed for PKH26 extraction in chloroform-methanol (2:1 vol/vol) and 0.125% sodium dodecyl sulfate. The PKH26 fluorescence intensity was measured by spectrofluorometry (excitation wavelength 550 nm; emission wavelength 567 nm) in the extracted lipid fraction.

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In vivo EV biologic effects

Five days old C57BL/6 rankl⁻/⁻ male mice were injected intraperitoneally daily for 5 days with 30000, 60000 or 120000 Rankl-positive EVs or with vehicle (PBS) as control. Then, mice were sacrificed and tibiae excised, fixed for 24h in 4% paraformaldehyde, dehydrated with ethanol and embedded in methoxyethyl methacrylate without decalcification. TRAcP histochemical staining was performed to identify and quantify TRAcP-positive cells belonging to the osteoclast lineage.

Eight weeks old C57BL/6J mice were treated for 4 days with 120 mg/kg body weight (bw) of retinoic acid (Sigma cat.#R2625, Vitamin A Acid) administered by oral gavage (31,32). Mice (n=5/group) were treated once, at day 1 of the experiment, by intraperitoneal injection of vehicles (PBS for sodium zoledronate and 0.1% DMSO for dasatinib), 70 µg/kg bw of sodium zoledronate, 12.5 mg/kg bw of dasatinib, osteoblast EVs loaded with a solution of 14 mM sodium zoledronate, osteoblast EVs loaded with a solution of 10 mM dasatinib, or naïve osteoblast EVs (2.5x10⁶ osteoblasts/cm²). One group of mice was also sham-treated by oral gavage with the retinoid acid vehicle (corn oil). At the end of the experiment, mice were sacrificed. The hind limb long bones were removed, fixed in 4% paraformaldehyde for methoxyethyl methacrylate embedding without decalcification, histochemically stained for TRAcP activity, and histomorphometrically analyzed for the osteoclast variables according to Dempster et al. (33). Counterstaining with hematoxylin was performed to evaluate osteoclast apoptosis.

For the in vivo experiments, 3-6 mice were employed per each group of treatment, allocated after randomization in number of 3 mice/cage. Treatments and measurements were performed in blind. No adverse effects were observed following treatment with EVs.
Statistics

Data were reported as mean±Standard Deviation (SD). Student’s $t$ test between two groups or One Way ANOVA using non parametric Kruskal-Wallis test between multiple groups were used as comparative statistical methods, with a significant p value <0.05 (GraphPad Prism 7.00). All in vitro experiments were performed with three technical replicates for each point and repeated at least three times or using three to six mice/group.
Results

Osteoblasts release membrane-derived structures into their surroundings

To investigate whether physiologic cell-to-cell communication in bone cells could involve EVs, we focused on osteoblasts, the active matrix-forming cell type in the bone, examining clues of intercellular communication based on EV exchange. Primary murine osteoblasts, labeled with the red fluorescent cell membrane dye PKH26, were plated in the upper chamber of 1µm pore transwell dishes, allowing the passage of EV-sized particles, while unstained osteoblasts were plated in the lower chamber (Figure 1A). The cultures were monitored and after 48h we observed the appearance of red fluorescence in the previously unstained osteoblasts in the bottom chamber (Figure 1B), indicating the fusion of donor cell labeled plasma membrane derivatives as well as their integration in the target cells.

EVs are harvestable from osteoblast conditioned media

To confirm the involvement of EVs in the transfer of membrane-bound dyes, we analyzed the release of EVs from cultured osteoblasts. We collected 24h serum-free conditioned media from primary mouse calvarial osteoblasts. After ultracentrifugation, the pellets were assessed for protein content (Figure 1C, left bar). To further evaluate at the molecular level whether or not our ultracentrifugation pellet contained EVs, we performed a Western blot for the commonly-reported exosome marker CD63. The enrichment of CD63 in our ultracentrifugation pellets compared to the total cell lysates indicated the isolation of a concentrated population of EVs (Figure 1D). The same evaluation performed in cryopreserved EVs demonstrated that this marker was not lost upon freezing (Figure 1D).

To confirm the presence of intact EVs obtained by ultracentrifugation, we loaded the EVs of the isolated pellets with the membrane-permeant fluorescent probe CMFDA. This dye is cleaved intracellularly by cytosolic esterases into a non-permeant molecule that does not leak out from...
intact membranes. By fluorescence microscopy, we observed in the pellets a population of green-fluorescent apparently integral vesicular structures (Figure 1E). Finally, a deeper morphologic evaluation by TEM analysis showed the expected heterogeneous population of intact and spheroid membrane bodies (Figure 1F), which matched the expected size distributions of EV populations.\(^{(1)}\)

Next, we questioned whether EV release was a physiologically controlled process. Since it has been reported that cells can respond to stimuli by increasing vesiculation,\(^{(34)}\) we treated osteoblast cultures with 10\(^{-7}\)M rhPTH, a potent regulator of osteoblast activity.\(^{(30)}\) While the donor osteoblast cell protein content was not affected by PTH (untreated osteoblasts, 1.1± 0.13 mg; PTH-treated osteoblasts, 1.05±0.02 mg; \(p=0.6\)), the protein content of the EV pellet obtained from the conditioned media of PTH-treated osteoblasts was higher than from control conditioned media (Figure 1C, right bar), supporting the hypothesis that this release was a hormonally regulated process.

Finally, we investigated whether EVs were able to target cells and transfer fluorophores. We incubated the CMFDA-loaded EVs with primary murine osteoblasts and monocytes, two of the most abundant cell types in bone and bone marrow, respectively, confirming the integration of this fluorescent probe in both recipient cells (Figure 1G,H).

**Qualitative and quantitative analysis of osteoblast-derived EVs by FACS**

To confirm the cellular origin and integrity of the membrane structures, we next performed a FACS analysis after incubation of EV pellets with CMFDA. We noted that 16.67±1.93% of events analyzed in the ultracentrifugation pellet represented CMFDA-positive structures up to 1\(\mu\)m in diameter, consistent with the size of EVs (Figure 1I,J). Given that CMFDA is a dye that does not leak out from integral membrane particles, we concluded that these structures were intact. The CMFDA-positive particles were sorted and their morphology was evaluated by TEM, confirming
the expected EV size distribution and apparent membrane integrity (Figure 1K).

We next assessed whether FACS-sorted EVs retained the ability to fuse with target cells. To this aim, we incubated primary osteoblasts with FACS-sorted CMFDA-loaded PKH26-labeled EVs. Microscopic analysis showed the integration of both fluorophores into target cells (Figure 2A, left panels). Trypsin/EDTA treatment of target cells did not compromise the presence of the dyes in their cytoplasm (Figure 2A right panels), confirming the complete integration of the fluorophores into their intracellular compartment.

**Osteoblast-derived EVs target and integrate into recipient cells**

We next investigated the kinetics of EV integration into target cells. To this aim, we performed a time-course experiment using osteoblasts incubated with FACS-sorted EVs loaded with CMFDA and labelled with PKH26. By confocal microscopy, we observed a time-dependent integration of EV fluorescent components in target cells starting after 24h from EV administration and persisting until at least 48h (Figure 2B). The analysis of the integrated dyes showed a typical vesicular distribution. Furthermore, Z-axis evaluation enabled us to distinguish also domains of the target cells enriched only in one of the two fluorophores with no co-localization in the same areas (Figure 2B). This observation suggests that target cells could process the individual constituents of EVs separately transferring them to different cellular compartments.

Finally, we investigated the integration of FACS-sorted EVs with other cell types, such as endothelial cells and monocytes, with which the osteoblasts exchange important molecular information. Fluorescence microscopy confirmed that both human umbilical vein cell line EA.hy926 and primary mouse monocytes internalized the fluorochromes shuttled by EVs (Figure 2C), suggesting also a mouse-human cross-species integration.
Osteoblast-derived EVs contain Rankl

Since EVs are reported to trigger ligand/receptor interactions in target cells,(16,17) we asked whether EVs from osteoblasts could shuttle Rankl, one of the most important intercellular crosstalk molecules in bone. We therefore investigated the presence of membrane-bound Rankl by FACS in osteoblast EVs and observed that 53.95±3.48% of them were Rankl-positive (Figure 3A). To further characterize the quality of FACS-sorted EV preparations, we performed TEM analysis of Rankl/CMFDA-double positive EVs and observed a heterogeneous population of intact spheroid bodies, with the expected EV morphology and size distribution (Figure 3B).

Since we noted that PTH increased EV production by osteoblasts (Figure 1C), and given that PTH is a well-known inducer of Rankl expression,(30) we evaluated the proportion of osteoblast-derived EVs which were Rankl-positive following treatment with 10^{-7}M rhPTH. FACS analysis showed that PTH increased the percent of Rankl-expressing osteoblasts as well as the mean PE-fluorescent units per osteoblast (Figure 3C,D). Furthermore, the percentage of RANK-L-positive events increased up to 63.6±4.20% of EVs in PTH-treated ultracentrifugation pellets vs. vehicle-treated osteoblasts (Figure 3E,F). Consistently, PTH increased also the total number of EVs (Figure 3G), the number of Rankl positive EVs (Figure 3F), the mean PE-fluorescent units per event (Figure 3H) and the percentage of Rankl-positive events (Figure 3I).

Biologic effects of osteoblast EVs

To assess the biologic effect of EVs on bone cells we investigated the phenotype of osteoblasts and osteoclasts exposed to osteoblast EVs. Semi-quantitative RT-PCR performed on control osteoblasts and osteoblasts treated for 48 hours with osteoblast EVs showed no modulation of Alp, Runx2 and Atf4, while osteoblast EVs significantly reduced osterix mRNA (Figure 4A,B). Likewise, the transcriptional expression of the matrix protein, Coll1a1 was not affected by osteoblast EVs, while Osteocalcin was significantly increased compared to control osteoblasts (Figure 4A,B). These data
suggest selective regulation of osteoblast genes by means of their own EVs.

Next, we evaluated the biologic effect of EVs on osteoclasts and noted that treatment with osteoblast-derived EVs improved osteoclastic variables, increasing osteoclast size and number of nuclei/cell (Figure 5A,B). Moreover, the EV-treated osteoclasts stained more strongly for TRAcp than vehicle-treated osteoclasts (Figure 5A). Taken together, these data confirmed a direct biologic effect of osteoblast-derived EVs in cells of the bone microenvironment and supported the concept that EVs are a means of Rankl mediated osteoblast/osteoclast crosstalk.

To establish the involvement of Rankl in the pro-osteoclastic effect of osteoblast-derived EVs, we took advantage of the rankl−/− mouse to isolate primary osteoblasts and collect rankl−/− EVs. We treated 12h-starved osteoclasts for 48h with wildtype or rankl−/− EVs, noting that rankl−/− EVs were less efficient than wildtype EVs in supporting osteoclast survival (Figure 5C,D,E). In fact, osteoclasts treated with rankl−/− EVs generally contained fewer nuclei and exhibited signs of nuclear degeneration (Figure 5C-E). Moreover we noted a higher percentage of retracted or disaggregated osteoclasts (Figure 5C-E), reminiscent of apoptotic cells. EV retrieval from wild type and rankl−/− osteoblasts was similar, as shown by the protein quantification of the EV pellets from the two genotypes (Figure 5F), ruling out that the observed differences were due to altered EV release by rankl−/− osteoblasts. These results demonstrated that EVs can exert a direct effect on osteoclast survival through their Rankl cargo.

**EVs are biotechnological tools to shuttle drugs to osteoclasts**

EVs have raised great interest as drug carriers. Since we found a clear effect of naïve osteoblast EVs on osteoclasts, we investigated whether they could be manipulated to shuttle anti-osteoclastic agents to target cells. To test this concept, we loaded osteoblast EVs with the clinically-approved anti-osteoclast drugs, sodium zoledronate (N-bisphosphonate) and the tyrosine kinase inhibitor

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dasatinib. We treated mature osteoclasts for 48h with EVs loaded with zoledronate (Figure 6A) (EV-Zol) or dasatinib (EV-Das) (Figure 6B), which induced cell death with high efficiency (Figures 6C,D).

Osteoblast-derived EVs shuttle RNAs to recipient cells.

EVs are reported to be involved in cell reprogramming via the shuttling of RNAs,\(^{(5,10,36)}\) so we examined whether osteoblast-derived EVs contained RNAs. To address this issue, we stained EV pellets with SYTO RNASelect, a cell-permeant dye that exhibits bright green fluorescence only when bound to single-stranded nucleic acids. We then treated both primary osteoblasts and monocytes with SYTO-stained EVs, noting the integration of green fluorescence into target cells, thus indicating the shuttling of RNAs (Figure 7A,B). FACS analysis revealed that most EVs (97.1±0.26\%) were SYTO-positive and contained RNAs (Figure 7C,D). Treatment of primary osteoblasts with SYTO-positive FACS-sorted EVs confirmed that green-stained RNAs were transferred into target cells (Figure 7E).

With this data in hand, we characterized the mRNA content of EVs. To achieve this goal we focused on a panel of 84 genes, known to be involved in osteoblast/bone physiology, using a Real Time Profiler PCR array. We found that EVs from osteoblasts were enriched especially in \(\text{Col1a1}, \text{Col1a2}, \text{Sparc}, \text{and Spp1}\) genes, typically involved in osteoblast function and bone formation (Table 1, Figure 7F). We confirmed the expression of the most abundant gene, \(\text{Col1a1}\), normalized vs. \(\text{Gapdh}\), by RT-PCR (Figure 7G). A further bioinformatics comparison of transcriptome profile between donor osteoblasts and released EVs showed no significant differences between them (Figure 7H), suggesting that in our conditions the EV transcriptome mirrors the osteoblastic transcriptomic profile.
We next investigated whether osteoblast-derived EVs were able to integrate into the bone tissue. To this aim, we incubated mouse calvariae *ex vivo* with PKH26-labeled osteoblast EVs for 48h. Confocal microscopy showed integration of PKH26 into the inner part of the bone (Figure 8A,B), supporting the hypothesis of an intrinsic osteotropism and integration of osteoblast EVs. It is known that peritoneal vessel allow EVs to pass into the circulation and reach target organs. Therefore, in order to demonstrate that EVs could target the bone tissue *in vivo*, we performed intraperitoneal injections of 30000 FACS-sorted Rankl positive EVs, labeled with PKH26, into 5 days old CD1 pups. Kinetic evaluation of PKH26 distribution in tissues revealed a fast uptake of PKH26 in bones, which peaked at 1.5h from EV injection, declining thereafter to a plateau within 24h (Figure 8C). PKH26 fluorescence was also observed in blood cells, liver, spleen and kidney, but with lesser intensity or slower kinetics (Figure 8C). In liver, a peak of PKH26 fluorescence was observed after 24h from EV injection (Figure 8C), suggesting clearance of the dye from the other tissues. Confocal microscopy on Optimal Cutting Temperature (OCT)-embedded liver sections showed the presence of PKH26 spots (Figure 8D,E), confirming the accumulation of PKH26 in the liver parenchyma.

To further investigate the effect of EVs on bone physiology, we focused on the osteoclast lineage and performed *in vivo* experiments in *rankl*<sup>−/−</sup> mice, a murine model of severe osteopetrosis characterized by the lack of osteoclasts and the consequent absence of the osteoclast biomarker TRAcP in the bone. We intraperitoneally injected increasing amounts of FACS-sorted Rankl-positive osteoblast EVs into *rankl*<sup>−/−</sup> mice. Histochemical analysis of tibial sections revealed the presence of TRAcP-positive cells in all EV treatments. In contrast, TRAcP positivity was totally absent in vehicle-injected mice (Figure 8F-K). The total number of TRAcP-positive cells did not vary between the EV treatment regimens (Figure 8J), but the total TRAcP-positive area increased with the number of injected EVs (Figure 8K). These results suggest that Rankl-positive EVs exhibit
Osteoclast activity is directly inhibited by in vivo osteoclastogenic potential.

**Osteoblast EV-encapsulated drugs inhibit osteoclast activity in vivo.**

To investigate whether osteoblast EV-encapsulated drugs could be used in vivo to inhibit osteoclast activity, we induced an acute osteoclast over-activation by administration of retinoid acid\(^{31}\) and treated the mice once, at day 1 of the experiment, with the free drugs or with EVs isolated from the conditioned media of osteoblasts, collected from one 125 cm\(^2\) flask/mouse (2.5x10\(^6\) osteoblasts/cm\(^2\)) and loaded with vehicle, zoledronic acid or dasatinib. After 4 days, mice were sacrificed, tibias were histochemically stained for TRAcP and evaluated histomorphometrically for the osteoclast variables. As expected, mice receiving retinoic acid underwent a significant reduction of body weight (Figure 9A), indicating the efficacy of the treatment\(^{31,32}\). They also showed an increase of the bone resorption marker, Collagen Type 1 C-Telopeptide (CTX) (Figure 9B), and of the osteoclast number (Figure 9C,D) and surface (Figure 9D) over bone surface. The increase of these variables was efficiently counteracted by treatment with free zoledronic acid or dasatinib, while EVs alone had no effect. Remarkably, osteoblast EV-encapsulated drugs reduced osteoclast over-activation (Figure 9A-E) and, in the case of EV-Zol, induced apoptosis (Figure 9F,G).

**Discussion**

The involvement of EVs in the communication between bone cells is very poorly understood. Authors have reported vesicle-like structures arising from osteocytes\(^{39}\) but with no data on their composition and function. Some EV protein characterization has been reported for osteoblast cell lines\(^{28,40}\) and “malignant” osteosarcoma cell EVs have been described to transport osteoclastogenic instruction\(^{41}\). Despite these reports, no functional data are available to understand the function of EVs in bone biology.
In our study, we used primary murine osteoblasts and assessed their release of EVs into the environment. We noted *in vitro* that osteoblasts shuttled lipophilic dyes to distant cells through transwell membranes with porosity up to 1µm. These results are consistent with the shuttle of EVs from donor cells to distant recipient cells. We confirmed the involvement of EVs in this process, isolating EVs from the conditioned media of osteoblasts and showing their ability to directly uptake, activate and retain cytoplasmic dyes, such as CMFDA. These results highlighted that our vesicular structures were cell-derived and that they showed integrity, as demonstrated by the presence of CD63 and by the TEM analysis. These EVs were able to integrate into target cells, including osteoblasts, monocytes, osteoclasts and endothelial cells, presumably transferring their cargoes. Confocal microscopy analysis showed different patterns of dye accumulation in target cells, suggesting that the integration of EV components is a dynamic and specific process. Different localization of dyes, with or without co-localization in different cell compartments, suggested that EV constituents are recycled in their target cells, likely in a programmed and regulated way, according to their needs.

It has been reported that EVs can exert their effect by ligand/receptor interaction.(16,17,27) Here we found evidence that in bone this type of intercellular communication includes the Rankl/Rank axis. In fact, FACS analysis showed that osteoblast-derived EVs contain Rankl on their surface. Previous reports have shown Rankl presence on EVs from the stromal/osteoblast cell lines UAMS-32P and SV-HFO.(27,28) Although these cells are established osteoblast-like lines, we believe that they cannot totally mirror the physiology of primary osteoblasts due to acquired genetic aberrations, being similar to tumor cells or viral-gene manipulated cells.(27)

We found Rankl on osteoblast-derived EVs in basal conditions, and this expression was upregulated by PTH. Our results showed that Rankl positive EVs affected osteoclast function *in vitro*, increasing their size and number of nuclei. Moreover, the TRAcP staining appeared more...
intense in EV-treated osteoclasts, suggesting more metabolically active cells. The effect seems to be Rankl-dependent, since $rankl^{-/-}$ EVs did not preserve osteoclast vitality, although the rate of EV retrieval was similar in wildtype and $rankl^{-/-}$ osteoblasts. Many osteoclasts showed signs of sufferance under $rankl^{-/-}$ EV treatment, with cellular retraction and loss of nuclei. These data are consistent with the deprivation of the well-known osteoclast pro-survival function of Rankl.\(^{(42)}\)

Interestingly, osteoblast EVs appear also to affect the expression of osteoblast-specific genes, suggesting selective autocrine regulation of osteoblast gene expression by means of their own EVs.

A key evaluation of the effect on the osteoclast lineage was represented by the \textit{in vivo} administration of wildtype osteoblast EVs in $rankl^{-/-}$ mice. The $rankl^{-/-}$ mouse is a model of human osteoclast-poor autosomal recessive osteopetrosis.$^{(26)}$ Like its human counterpart, this mouse model is characterized by the absence of the osteoclast lineage, and their bones are totally TRAcP-negative. We found an EV density-dependent appearance of TRAcP-positive cells in tibiae of $rankl^{-/-}$ mice treated with escalating numbers of our EVs, suggesting a biologic effect of the EV-shuttled Rankl. \textit{Ex vivo} tissue-specific integration experiments in calvariae confirmed the ability of EVs to integrate into the bone cells and \textit{in vivo} distribution experiments established that osteoblast-derived EVs are preferentially taken up very rapidly by the bone, further suggesting a tissue targeting specificity of the EVs released by osteoblasts.

EVs are known to contain and shuttle RNAs. Many reports over recent years have described the molecular content of EVs isolated from different cells, tissues and biologic samples.$^{(35)}$ Web compendia about these data, such as ExoCarta (http://exocarta.org/) and Vesiclepedia (http://microvesicles.org), display growing reporting lists of lipids, RNAs and proteins recognized in various classes of EVs.
To the best of our knowledge, no studies to date have investigated the mRNAs profile of EVs from 
bona fide primary osteoblasts. Here we found that our EVs contain a set of transcripts related to 
osteoblast activity. Although we are aware that our analysis is limited only to a very partial gene 
panel and does not measure the absolute quantity of transcripts, we believe that these results could 
have important biologic relevance. The 15 most abundant mRNAs in osteoblast EVs comprise a set 
of genes clearly associated with bone metabolism and osteoblast function. \textit{Col1a1} (ID: 12842) 
encodes the alpha-1 subunit of type I collagen, the most abundant extracellular matrix protein, 
mainly contained in bone, skin and tendon.\(^{(43)}\) \textit{Col1a2} (ID: 12843) encodes the alpha-2 subunit of 
the same type I collagen and, in association with the alpha-1 subunit, forms heterotrimeric type I 
procollagen for fibril formation.\(^{(44)}\) \textit{Bglap} (ID: 12096) encodes osteocalcin, one of the most 
abundant non-collagenous proteins in the mineralized matrix of bone.\(^{(44)}\) Osteocalcin is known to 
exert endocrine activities, regulating energy metabolism, neural development, male fertility and 
muscle function.\(^{(45-47)}\) Our observation that osteocalcin transcriptional expression is modulated in 
osteoblasts by their own EVs suggest that EVs could also contribute to the control of the osteoblast 
endocrine function. \textit{Sparc} (ID: 20692) encodes for the secreted acidic cysteine rich glycoprotein, or 
osteonectin, that in bone is involved mainly in the interaction between mineral crystals and 
collagen, probably triggering the nucleation of hydroxyapatite.\(^{(48)}\) Secreted phosphoprotein 1, or 
osteopontin, is the product of the \textit{spp1} gene (ID: 20750). It plays a role as an integral part of the 
mineralized matrix and is involved in important cell-matrix interactions.\(^{(49)}\) In fact, it has been 
reported that osteopontin works as a “bridge” between extracellular matrix and cells, acting as a 
ligand of integrin heterodimers, such as \(\alpha \nu \beta 3\) or \(\alpha \nu \beta 5,\(^{(50-53)}\)\) and driving osteoblast 
differentiation.\(^{(54)}\) A set of four transcripts are from structural and housekeeping genes, such as 
\textit{Gapdh} (ID: 14433, glyceraldehyde-3-phosphate dehydrogenase), \textit{Actb} (ID: 11461, actin beta), \textit{B2m} 
(ID: 12010, \(\beta -2\) microglobulin) and \textit{Hsp90ab1} [ID: 15516, heat shock protein 90 alpha (cytosolic) 
class B member 1]. A further comparison between EV and cellular transcriptomes did not reveal 
any preferential sorting or enrichment of mRNAs in EVs, therefore the osteoblast EVs seem to
mirror the transcript profile of their donor cells. This is at variance with what has been described in other cells, in which selective sorting of molecules into EVs was observed.\(^{(36)}\)

Due to their ability to shuttle specific cargoes and because of their targeted uptake in tissues, EVs are under intense investigation for various clinical applications. They could be used as “biomarkers” for diagnostic purposes, since they can reflect the molecular content of the donor tissues and are released into the circulation. Therefore, they could be useful in “liquid biopsies” to monitor the progression of diseases or scrutinize the success of therapies.\(^{(1,5)}\) Furthermore, they can be loaded with tracking molecules and drugs, widening the therapeutic index, narrowing the ED\(_{50}\) of drugs and reducing the risk of side effects because of their targeted delivery.\(^{(8)}\) Here, we showed that osteoblast-derived EVs can be loaded with anti-osteoclastic agents. In particular, we loaded osteoblast-derived EVs with dasatinib, a clinically approved inhibitor of tyrosine kinases for malignancies,\(^{(55-59)}\) or with zoledronate, a bisphosphonate clinically approved for osteoporosis.\(^{(60-62)}\)

Both agents are known to be very effective against osteoclasts. When encapsulated in EVs, both dasatinib and zoledronate still exerted their biologic effects. Das-EVs induced massive retraction and collapse of cells due to the inhibition of crucial tyrosine kinases, such as c-Src, involved in osteoclast cytoskeletal remodeling and actin ring formation.\(^{(63,31)}\) Zol-EVs induced vacuolization and apoptosis of osteoclasts, consistent with inhibition of the mevalonate and lipid pathways known to be disrupted by N-bisphosphonates.\(^{(64,65)}\) The EV-encapsulated drugs maintained their efficacy, as shown by the degeneration of osteoclasts similar to that induced by the free drugs. Most importantly, this anti-osteoclastic activity was observed also \textit{in vivo} in an acute mouse model of osteoclast over-activation, thus supporting their \textit{in vivo} use to shuttle pharmacological agents to bone cells.
The proof of principle that EVs can have a potential therapeutic effect in bone pathology was further uncovered by our *in vivo* results in Rankl deficient mice. Mice harboring mutations in the *tnsf11a* gene are the geno/phenocopy of human autosomal recessive osteopetrosis 2 (OMIM: #259710, OPTB2).(26) Presently, the patients are untreatable, have a very poor quality of life and a short life expectancy. They could take advantage of substitutive therapy with soluble Rankl,(66) but a pharmacological formulation of Rankl is not under development. On this basis, the hypothesis of the use of clinical-grade EVs from healthy donors or engineered from patient cells to carry the wildtype protein, could help setting a new strategy for treating, or at least alleviating the symptoms, of this lethal disease. More widely, we can image EVs as a shuttle for delivery of membrane bound molecules, cytokines and growth factors alike the Rankl reported in this study.

In conclusion, this report demonstrates that osteoblast EVs play a physiologic role in the interaction with other cells in the bone microenvironment, especially monocytes and osteoclasts. Moreover, we reported a proof of concept for the use of osteoblast-derived EVs for the treatment of bone diseases, since they are able to efficiently target the bone and induce clear biologic events, both by endogenous (Rankl) and exogenous (bisphosphonates and tyrosine kinase inhibitors) molecules, opening an avenue for their biotechnological use to treat skeletal disorders.

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Authors’ roles

Study design: AC, MM, AT. Study conduct: AC, AL, AM. Data collection: AC, AM, NR, AT. Data analysis: AC, AL, AM, NR, AT. Data interpretation: AC, AL, NR, MM, AT. Drafting manuscript: AC, AL, AT. Revising manuscript content: AC, AL, MM, AM, NR, AT. Approving final version of manuscript: AC, AL, MM, AM, NR, AT.
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Figure legends

Figure 1 – Characterization of osteoblast EVs. (A) Cartoon illustrating the experiment performed in transwells. (B) PKH26-labelled donor osteoblasts (left panel), unlabeled target osteoblasts at time 0 (inset phase contrast) (middle panel), and previously unlabeled osteoblasts after 48h of incubation in transwell with the PKH26-labelled osteoblasts (right panel). Scale bar: 5 µm. (C) Protein titration of EVs isolated by ultracentrifugation from serum-free media of vehicle- or PTH (10^{-7} M)-treated osteoblasts. (D) Western blot for CD63 of osteoblast cell lysate, freshly isolated EVs or frozen-thawed EV lysate from osteoblasts. (E) Fluorescence microscopy evaluation of a drop of control free CMFDA (left panel), and EV loaded with CMFDA (right panel). Scale bar: 1µm. Dotted line, drop edge. (F) Morphology of isolated osteoblast EVs (arrows) fixed, contrasted with uranyl-oxalate solution and observed by TEM. Scale bar: 1 µm. Phase contrast (left panels) and fluorescence microscopy (right panels) of (G) primary murine osteoblasts and (H) monocytes incubated for 48h with CMFDA-loaded osteoblast EVs. Scale bar: 5µm. (I) FACS setting for dimensional gate with commercial standard microbeads (purple: 0.23 µm; blue: 0.58 µm; green: 0.79 µm; red: 1.3 µm, Spherotech). (J) Dot plot of FACS-analyzed EV pellet isolated by ultracentrifugation from osteoblast conditioned media and loaded with CMFDA. (K) Ultrastructure of FACS-sorted CMFDA-loaded osteoblast EVs (arrows) observed by TEM. Scale bar: 1µm. Results are representative (B,D-K) or the mean+SD (C) of at least three independent experiments (Student’s t test).

Figure 2 – Osteoblast EV integration in target cells. (A) Evaluation by conventional epifluorescence microscopy of osteoblasts incubated for 2 days with osteoblast EVs previously labelled with PKH26 (red) and CMFDA (green) fluorescent dyes. Integration of the dyes in target cells before (left panels) and after (right panels) trypsin-EDTA treatment. Scale bar: 20 µm. (B) Confocal microscopy analyzing a time course experiment of osteoblasts incubated for 2 days with FACS-sorted osteoblast EVs labeled with PKH26. Scale bar: 5µm. (C) Evaluation by conventional
epifluorescence microscopy of endothelial cell line and primary murine monocytes incubated up to 48h with osteoblast EVs previously labelled with PKH26 (red) and CMFDA (green) fluorescent dyes, which were integrated in target cells. Scale bar: 20µm. Results are representative of three independent experiments.

Figure 3 – Analysis of RANKL expression in osteoblast EVs. (A) FACS analysis of CMFDA-loaded, PE-Rankl-negative (left gate) and -positive (right gate) EV populations isolated from primary osteoblast cultures. (B) Analysis by TEM of FACS-sorted Rankl-positive osteoblast EVs. Scale bar: 2 µm. (C) FACS quantification of the percentage of Rankl-positive osteoblasts and (D) Mean (PE)-Fluorescent Intensity (MFI) per event in primary osteoblast cultures treated with vehicle or PTH. (E) FACS analysis of CMFDA-loaded, PE-Rankl-negative (left gate) and -positive (right gate) EV populations isolated from primary osteoblast cultures treated with vehicle or PTH. (F-I) FACS quantification of (F) the percentage of Rankl positive EVs, (G) the total number of EVs, (H) the number of Rankl positive EVs and (I) the Rankl (PE)-positive MFI per event in EVs isolated from vehicle- and PTH-treated osteoblasts. Results are representative (A,B,E) or the mean±SD (C,D,F-I) of at least three independent experiments (Student’s t test).

Figure 4 – Autocrine effect of osteoblast EVs on osteoblast gene expression. (A) Transcriptional profile of mouse primary osteoblasts treated with PBS (Control) or with osteoblast-derived EVs (EVs) by semi-quantitative RT-PCR. (B) Densitometric analyses of the amplification bands of the indicated genes normalized by the housekeeping gene Gapdh. Data are representative (A) or the mean±SD (B) of 3 independent experiments. (Student’s t test).

Figure 5 – Effect of osteoblast EVs on osteoclast survival. (A) Cytochemical evaluation of TRAcP activity in primary murine mature osteoclasts treated with vehicle or osteoblast-EVs. Scale bar: 5µm. (B) Number of TRAcP-positive multinucleated osteoclasts (left panel), osteoclast area

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(middle panel) and number of nuclei/osteoclast in vehicle- (PBS) or osteoblast EV-treated osteoclast cultures. Microscopic evaluation of mature osteoclasts treated for 48h with EVs from (C) wildtype (WT) or (D) rankl−/− osteoblasts. Left panels, TRAcP cytochemical staining of osteoclasts; right panels, Hoechst staining of nuclei. Arrows, healthy osteoclasts; large arrowheads, osteoclasts with no nuclei (ghost cells); small arrowheads, retracted osteoclasts. (E) Quantification of the morphological features shown in (C) and (D). (F) Protein titration of EVs isolated by ultracentrifugation from serum-free conditioned media of wildtype (WT) or rankl−/− osteoblasts. Results are representative (A,C,D) or the mean+SD (B,E,F) of at least three independent experiments (Student’s t test).

**Figure 6 – EVs as drug delivery system.** (A) Cytochemical evaluation of TRAcP activity in mature primary murine osteoclasts incubated with osteoblast EVs, free zoledronate (Zol) (1 to 100μM) or Zol-loaded osteoblast EVs (EV-Zol). (B) Cytochemical evaluation of TRAcP activity in mature primary murine osteoclasts incubated with osteoblast EVs, free dasatinib (Das) (1 to 100μM) or Das-loaded osteoblast EVs (EV-Das). Scale bar: 5µm. (C) Quantification of the features shown in (A). (D) Quantification of the features shown in (B). Results are representative (A,B) or the mean+SD (C,D) of at least three independent experiments (One Way ANOVA).

**Figure 7 – Characterization of osteoblast EV RNA cargo.** Microscopic evaluation of (A) osteoblasts and (B) monocytes treated for 36h with EVs loaded with the RNA-specific green fluorescent dye, SytoRNA Select (unbound dye appears red). Scale bar: 5µm. (C) FACS dot plot of sorted SytoRNA select-stained EVs. (D) FACS dot plot of the SytoRNA select-positive and -negative osteoblast EV population shown in (C). (E) Primary osteoblasts incubated for 36h with EVs sorted in (D). Scale bar: 5µm. (F) Heat map (green=low expressed gene, red=high expressed gene) of genes evaluated on mRNA extracted from osteoblasts and osteoblast EVs by Real Time Profiler PCR array. (G) RT-PCR of mRNA from osteoblasts and osteoblast EVs for the most highly
expressed genes identified in (F). (H) Volcano plot assessing the statistical significance (p value) depending on fold change (FC) expression between the osteoblast and osteoblast EV genes listed in (F). Results are representative (A-G) or the p value (H) of three independent experiments (Student’s t test).

**Figure 8 – Osteoblast EV integration, targeting and biologic activity in bone.** (A) Confocal microscopy of murine calvaria incubated for 48h with PKH26-labeled osteoblast EVs (red). Planar acquisition. (B) Z-axis scan inside 32μm. Blue, nuclei stained with Hoechst. Scale bar: 5μm. (C) Time-dependent fluorimetric quantification of PKH26 in the indicated organs from 5 days old CD1 mice injected intraperitoneally with 30000 FACS sorted, PKH26-labeled, Rankl-positive osteoblast EVs/mouse. N=6 mice/group. (D,E) Histological evaluation by confocal microscopy of PKH26 red fluorescence (arrows) in the liver of mice injected with (D) vehicle (PBS) or (E) PKH26-labeled osteoblast EVs after 24h from injection. Scale bar: 5μm. (F-K) Histochemical evaluation of proximal tibia sections from 5 days old Rankl−/− mice treated 5 times, every other day, with (F) vehicle (PBS) or (G) 30000, (H) 60000 or (I) 120000 RANKL-positive osteoblast EVs/mouse. Sections were histochemically stained for the osteoclast-specific marker, TRAcP. Arrows, TRAcP-positive cells. Quantification of TRAcP-positive cell (J) number and (K) area. Results are representative (A,B,D-I) or the mean+SD (C,J,K) of 3-6 mice/group (One Way ANOVA).

**Figure 9 – In vivo effect of osteoblast EV-encapsulated sodium zoledronate and dasatinib on osteoclasts.** Eight weeks old C57BL/6J female mice were treated by oral gavage with corn oil (Sham) or with 120 mg/kg bw retinoic acid in corn oil to induce osteoclast over-activation. Retinoic acid-treated mice where then injected i.p. with vehicles (PBS or DMSO), 70 μg/kg bw of sodium zoledronate (Zol), 12.5 mg/kg bw of dasatinib, osteoblast EVs (EVs) or osteoblast EVs loaded with 14mM sodium zoledronate (EV-Zol) or with 10mM dasatinib (EV-DAS). After 4 days mice were (A) weighted, then they were sacrificed and blood samples were collected for (B) serum CTX.
quantification. (C) Proximal tibia sections were histochemically stained for the activity of the osteoclast-specific enzyme, TRAcP (purple stain). Scale bar: 100μm. (D) Histomorphometric evaluation of osteoclast number/bone surface (OcN/BS), (E) osteoclast surface/bone surface (OcS/BS) and (F) apoptotic osteoclasts (%). (G) High magnification of tibia sections stained for TRAcP (purple) and counterstained with hematoxylin. Arrows: apoptotic osteoclasts. Scale bar: 40μm. Results are representative (C,G) or the mean±SD (A,B,D,E,F) of 5 mice per group (unpaired Student's t-test).
TABLE 1 - List of the 15 most abundant genes (cut off, Ct < 27) evaluated by Real Time Profiler PCR array on mRNA extract from osteoblast EVs (n=3).

| Rank order | Gene Symbol | Ct Average | ST DEV |
|------------|-------------|------------|--------|
| 1          | Col1a1      | 20.62      | 1.32   |
| 2          | Hsp90ab1    | 21.88      | 0.40   |
| 3          | Gapdh       | 22.08      | 0.41   |
| 4          | Actb        | 22.29      | 0.50   |
| 5          | Col1a2      | 22.72      | 0.89   |
| 6          | Sparc       | 22.89      | 0.44   |
| 7          | B2m         | 24.77      | 1.04   |
| 8          | Igfbp2      | 25.55      | 0.55   |
| 9          | Timp2       | 25.66      | 0.57   |
| 10         | Spp1        | 25.72      | 0.50   |
| 11         | Mmp2        | 25.77      | 0.93   |
| 12         | Fgfr1       | 26.54      | 0.28   |
| 13         | Tgfb1       | 26.78      | 0.05   |
| 14         | Bglap       | 26.8       | 0.45   |
| 15         | Lrp1        | 26.84      | 0.21   |
Figure 2
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9