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Supplemental Information

Extracellular Vesicles Derived from Osteogenically Induced Human Bone Marrow Mesenchymal Stem Cells Can Modulate Lineage Commitment

Margarida Martins, Diana Ribeiro, Albino Martins, Rui Luís Reis, and Nuno Meleiro Neves

SUPPLEMENTAL FIGURE 1

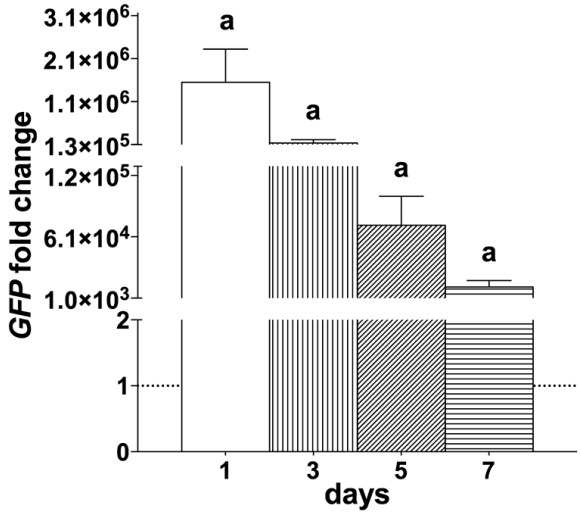
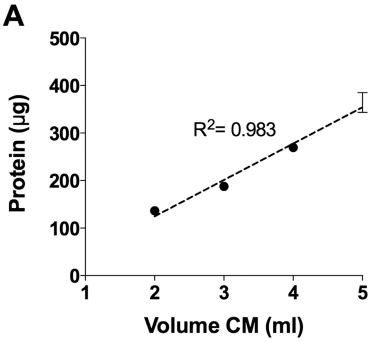


Figure S1. **hBMSCs RUNX2-transfection stability - related to Figure 2**
GFP expression, tagged to *RUNX2*, is shown as mean ± SEM (3 donors, with 3 technical replicates). BM was considered as calibrator at each time point and is indicated as a dashed line. **a**, denotes significant differences compared to BM.

SUPPLEMENTAL FIGURE 2

A



B

Experiment	EV-related protein amount (µg)	
	Spiked	Recovered
1	649.9 ± 33.9	558.8 ± 34.8
2	324.9± 17	324 ± 5.8

Figure S2. **Efficiency of the EVs enrichment method - related to Figure 1**
(**A**) Method linearity, showing a positive correlation between EVs protein and the conditioned medium (CM) volume. (**B**) Method recovery using high (experiment 1) and medium (experiment 2) amounts of EVs, showing a recovery efficiency ≥ 86%. The data is shown as mean ± SEM (n=3, with 3 technical replicates).

SUPPLEMENTAL TABLE

Table S1. Compilation of primers used for qPCR, related to Figure 3

Name	Primer sequence 5'-3' Forward/ reverse	Tm (°C)	Product size (bp)	Sequence Reference (NCBI)
BGLAP	GTGCAGAGTCCAGCAAAGG TCAGCCACTCGTCACAGC	59.4	549	DQ007079.1
BMP2	TGAATCAGAATGCAAGCAGG TCTTTTGTGGAGAGGATGCC	56.3	250	NM_001200.2
COL1A1	AAGAACCCCAAGGACAAGAG GTAGGTGATGTTCTGGGAGG	58.4	159	NM_000088.3
GFP	AACACCCGCATCGAGAAGTA CTTGAAGTGCATGTGGCTGT	57.3	279	pCMV6-eGFP Origene
GAPDH	ACAGTCAGCCGCATCTTCTT GACAAGCTTCCCGTTCTCAG	58.4	308	NM_002046.4
IBSP	ACTGAGCCTGTGTCTTGAAA CTTCCAACAGCCAATCACTG	56.3	102	AH002985.1
RUNX2	TTCCAGACCAGCAGCACTC CAGCGTCAACACCATCATTC	58.1	242	NM_001145920.1
SP7	CCCTTTACAAGCACTAATGG ACACTGGGCAGACAGTCAG	57.1	216	NM_001173467.1
SPP1	CCCACAGACCCTTCCAAGTA GGGGACAACTGGAGTGAAAA	58.4	244	AF052124.1

Tm, melting temperature; bp, base pairs

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

RUNX2 plasmid construct

Genetically- induced hBMSCs osteogenic commitment

The commercial available plasmid pCMV6-AN-GFP (PS100019, Origene, USA; 6.6 kb) was modified to express runt-related transcription factor 2 gene (*RUNX2*, NM_001024630). This vector carries the cytomegalovirus (CMV) promoter, the human growth hormone polyA signal downstream the insert, the ColE1 ori for replication in bacteria, the Simian vacuolating virus 40 ori for replication in mammalian cells, the f1 ori for the recovery of single-stranded plasmids and an ampicillin resistance gene for plasmid selection purposes in *Escherichia coli*. The open reading frame cloned in this vector is expressed with a tagged turbo Green Fluorescent Protein (GFP). Competent *E. coli* DH5α grown at 37°C on Luria-Bertani medium (Gibco) supplemented with ampicillin (Sigma, Spain) (150 μg/mL) was used as host for the *RUNX2* plasmid amplification after heat shock transformation (Sambrook and Russell, 2001). Plasmid DNA isolation was performed using the Plasmid Midi kit (Qiagen, IZASA, Portugal) according to the manufacturer's instructions. Determination of the plasmid DNA concentration and purity was performed by microspectrophotometry (NanoDrop 1000, Thermo Scientific, Portugal).

For the genetically induced commitment, cationic-lipid transfection (Lipofectamine LTX with Plus Reagents, Life Technologies, Alfagene) was used. The transfection mixture was composed by 500 ng of plasmid DNA and a lipid to DNA ratio of 2 ×. The lipid/DNA complexes formed in Opti-MEM were added to 1 h adhered cells. After 5 h, the cells were rinsed with phosphate buffer saline (PBS; Sigma) and fed with 1 ml of BM. The transfection efficiency was evaluated by the quantitative real-time polymerase chain reaction (qPCR) *GFP* reporter gene expression (Figure S1). hBMSCs cultured in BM were set as control.

Isolation of EVs

To exclude large cell debris, membranes and/or apoptotic bodies the conditioned medium (CM) was filtered (pore size 0.22 μm) (Thery et al., 2006). EVs were isolated from the CM using a polymeric precipitation solution (ExoQuick-TC; System Biosciences, BioCat GmbH, Germany). This method is based on the trapping of EVs, under salt conditions at 4°C. EVs are pelleted by low-speed centrifugation. Upon resuspension of the recovered sample the residual polymer is diluted and the EVs are liberated (Peterson et al., 2015). One ml of solution was mixed with 5 ml of CM and incubated overnight at 4°C. After centrifugation at room temperature, 1500 × *g*, 30 min (Eppendorf, Fisher Scientific, Portugal), the supernatant was discarded and the tubes were centrifuged at 1500 × *g*, 5 min. The pelleted EVs were resuspended in phosphate buffered saline (PBS) for all the EVs characterization assays but the CD63 protein ELISA assay, for which exosome binding buffer (EXOEL CD63; System Biosciences) was used; or in BM for the "Transfer of osteogenically committed hBMSCs-EVs into uncommitted homotypic cells" assays, and used fresh.

For control experiments, BM was processed as the CM and after centrifugation no pellet was detected, excluding potential contamination by fetal bovine serum FBS-EVs. The EVs isolation procedure was validated using a pool of CM obtained from hBMSCs cultured for 7 days. To evaluate the linearity, a variable amount of CM (2 to 5 ml) in a final volume of 5 ml was mixed with ExoQuick solution (Figure S2A). To evaluate the recovery, two known amounts of EVs total protein (corresponding to high, 200% and medium, 100 % amounts) were added to 5 ml of BM and mixed with ExoQuick solution (Figure S2B). EVs were isolated as described above. The pellets were resuspended in 500 μl of PBS and the EVs enrichment was evaluated in terms of total protein recovered that can give a rough indication of the EVs amount (Webber and Clayton, 2013). Total protein (in μg) was calculated as the protein concentration × volume after processing. Percentage recovery was calculated as the protein amount recovered divided by the protein amount spiked × 100.

Characterization of osteogenically induced hBMSCs-EVs

Size and charge

Size was evaluated by dynamic light scattering (DLS) in 1 cm polystyrene cells and ζ- potential by Laser Doppler micro-electrophoresis in folded capillary cells using a Malvern Nano ZS (He–Ne laser source; λ= 633 nm; Malvern, Portugal) under a fixed angle of 173°. DLS measures the changes in the light scattered from a laser due to the Brownian motion of EVs populations (ranging from 0.6 nm and 6 μm) in solution along time. This assumes that the particles are spherical and depends on the solvent, temperature, refractive index of the EVs and of the solvent and EVs size. The relative size distribution is obtained through the equipment software algorithm that correlates light intensity fluctuations with particle size. It also provides an estimate of the width of the size distribution- the polydispersity index-

that ranges from 0 (monodisperse) to 1 (very broad distribution) (van der Pol et al., 2010). The zeta potential is measured using Laser Doppler Electrophoresis, which measures light scattered by EVs (ranging from 5 nm and 10 μm) when an electric field is applied to the solution. All the measurements were performed using filtered solutions composed of EVs (15 μg of total protein) in 1 ml of PBS at 37°C.

Morphology
EVs (15 μg of total protein/ ml of PBS) were adsorbed for 30 min into coverslips (Sarsted, Germany), rinsed with ultrapure water twice and air-dried. The samples were analyzed by atomic force microscopy (Dimension Icon, Bruker**,** Japan) using the ScanAsyst Imaging Mode, in air.

Total protein
The quantification of EVs' total protein was performed using the Micro BCA Protein Assay kit (Fisher Scientific, Portugal). The samples were diluted in PBS. The protein concentration was calculated using a standard curve relating the concentration of bovine serum albumin (BSA; ranging from 2 to 40 μg/mL) to the absorbance measured at 562 nm on a microplate reader (Synergie HT, Bio-Tek, Alfagene). The values recorded for the total protein in each sample were adjusted for the CM volume and standardized against the cell number of progenitor cells and days in culture.

Surface markers
First, the evaluation of EVs' CD9/CD63/CD81 markers was performed by flow cytometry. CD63-beads were prepared by overnight incubation at room temperature of 4% (w/v) 4 μm beads aldehyde/sulfate latex (Molecular Probes, Alfagene) with anti-human CD63 (BD Biosciences) in 2-(*N*-morpholino)ethanesulfonic acid (Sigma) buffer pH6. EVs were incubated with PBS washed CD63-beads (150 μg EVs/ 5×10^5 CD63-beads) overnight at 4°C. After glycine (Sigma) block (final concentration 100 μM) and wash with 0.5% BSA (w/v) in PBS the bead-sample complexes were incubated with CD9- fluorescein isothiocyanate (FITC, 0.06 μg), CD63 allophycocyanin (APC, 0.22 μg), or CD81 phycoerythrin (PE, 0.08 μg) (all from BD Biosciences, Enzifarma, Portugal) for 40 min at 4°C under gentle agitation in 0.5% BSA in PBS. After washing, the samples were acquired on a FACsCalibur Flow Cytometer (BD Biosciences) at low flow rate. Offline data analysis was performed on FlowJo. From the dot plot representation of forward and side scatter, single EVs-CD63-beads were gated for fluorescence analysis (10,000 events per gated population). This population was compared to unlabeled controls.
Second, the quantification of CD63 positive EVs was performed using the EXOEL CD63 kit (System Biosciences). This is an ELISA based on the colorimetric detection of the specific interaction between enzyme labeled CD63 and EVs immobilized on a microtiter plate. The quantitative results (number of EVs particles) were obtained using a protein standard curve relating the amount of EVs (ranging from 1.35×10^{10} to 2.1×10^8 particles) to the absorbance measured at 450 nm on a microplate reader (Synergie HT). The values recorded for the number of EVs particles in each sample were adjusted for the CM volume and standardized against the cell number of progenitor cells and days in culture.

Gene expression analyses
RNA was extracted from Tri-reagent derived cell lysates according to manufacturer's instructions. The RNA pellets were solubilized in water RNase/DNase free (VWR, Portugal). The RNA concentration was determined by microspectrophotometry (NanoDrop 1000). The cDNA synthesis was performed using the qScript cDNA synthesis kit (Quanta BioSciences, VWR) with 100 ng RNA template in a final volume of 20 μl. Cycling was as follows: 1 cycle at 22°C, 5 min; 1 cycle at 42°C, 30 min; 1 cycle at 85°C, 5 min. The amplification of the target cDNA was performed using the PerfeCTa SYBR Green FastMix (Quanta BioSciences) with 1 μL of cDNA, 125 nM of each primer (Table S1) in a final volume of 20 μl. Real time-PCR cycling was as follows: 1 cycle at 95°C, 2 min; 44 cycles at 95°C, 10 s/ gene annealing temperature (Table S1), 30 s/72 °C, 30 s; followed by dissociation curve analysis. All the reactions were carried out on a PCR cycler Mastercycler Realplex (Hamburg, Germany). The transcripts expression data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in each sample. The quantification was performed according to the Livak method ($2^{-\Delta\Delta Ct}$ method), considering the BM condition for each time point as a calibrator (threshold = 1).

SUPPLEMENTAL REFERENCES
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