EXOSOMES: NOVEL EFFECTORS OF HUMAN PLATELET LYSATE ACTIVITY

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Abstract

Despite the popularity of platelet-rich plasma (PRP) and platelet lysate (PL) in orthopaedic practice, the mechanism of action and the effectiveness of these therapeutic tools are still controversial. So far, the activity of PRP and PL has been associated with different growth factors (GF) released during platelet degranulation. This study, for the first time, identifies exosomes, nanosized vesicles released in the extracellular compartment by a number of elements, including platelets, as one of the effectors of PL activity. Exosomes were isolated from human PL by differential ultracentrifugation, and analysed by electron microscopy and Western blotting. Bone marrow stromal cells (MSC) treated with three different exosome concentrations (0.6 μg, 5 μg and 50 μg) showed a significant, dose-dependent increase in cell proliferation and migration compared to the control. In addition, osteogenic differentiation assays demonstrated that exosome concentration differently affected the ability of MSC to deposit mineralised matrix. Finally, the analysis of exosome protein content revealed a higher amount of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB) and transforming growth factor beta 1 (TGF-β1) as compared to PL. In regards to RNA content, an enrichment of small RNAs in exosomes as compared to donor platelets has been found. These results suggest that exosomes consistently contribute to PL activity and could represent an advantageous nanodelivery system for cell-free regeneration therapies.

Keywords: Exosomes, platelet lysate, platelet rich-plasma, bone marrow stromal cells, growth factors, RNA, nanodelivery system, cell-free regeneration therapies.

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Introduction

Despite the remarkable ability of bone to undergo extensive remodelling and regeneration, prompt healing is sometimes impaired if the amount of bone loss is excessive or the local or systemic conditions are unfavourable. In order to increase the chances of cure in the context of regenerative medicine techniques as applied to orthopaedic conditions, new approaches based on platelet derivatives, such as platelet-rich plasma (PRP) and platelet lysate (PL), have attracted the attention of several investigators. PRP and PL contain a high concentration of growth factors (GF), cytokines and molecules that actively contribute to the tissue repairing process. In non-union fractures, characterised by a significant reduction of endogenous GF levels (Gandhi *et al*., 2005), platelet derivatives provide a carrier to deliver supra-physiologic concentrations of GF to the injury site, eventually improving tissue regeneration. This potential benefit has led to an extensive use of PRP and PL in the orthopaedic practice (Savarino *et al*., 2006; Dallari *et al*., 2007; Lane *et al*., 2013; Ruggiu *et al*., 2013; Zhu *et al*., 2013) as well as in different clinical applications such as musculoskeletal injury (Foster *et al*., 2009; Nguyen *et al*., 2011; Lee 2013), maxillofacial surgery (Fennis *et al*., 2002; Froum *et al*., 2002), dentistry (Soffer *et al*., 2003; Govindasamy *et al*., 2011; Chen *et al*., 2012; Malik *et al*., 2012), dermatology (Cho *et al*., 2012), soft tissue injuries (Carter *et al*., 2011; Liao *et al*., 2014), in addition to it being a medium supplement for *in vitro* cell culture systems (Warnke *et al*., 2013). However, despite the popularity of PRP treatments in bone healing, studies that analyse the effectiveness of such therapeutic tools are still controversial (Hall *et al*., 2013; Lee *et al*., 2013; van Bergen *et al*., 2013). Up to now, the regenerative potential of PRP and PL has been mainly attributed to their GF content that is released as a result of platelet degranulation. This differs depending on the preparation technique, which may influence platelet content and, consequently, GF availability (Perut *et al*., 2013; Passaretti *et al*., 2014).

PL activity might also be due to the efficient cell to cell transport system of GF and other bioactive molecules that is mediated by their encapsulation into nm-sized (30-100 nm) vesicles, called exosomes. They derived from specialised intracellular compartments, known as late endosomes or multivesicular bodies (MVB), and released by cells under physiological and pathological conditions (Baglio *et al*., 2012; Kharaziha *et al*., 2012; Urbanelli *et al*., 2013). Exosome secretion has been described for most cells, both constitutively and upon activation signals (Raposo *et al*., 1996; Théry *et al*., 1999; de Jong *et al*., 2012). Recently, exosomes have also been successfully

purified from many body fluids such as urine (Gonzales *et al*., 2010), blood (Caby *et al*., 2005; Grant *et al*., 2011), amniotic fluid (Keller *et al*., 2011) and pleural effusions (Bard *et al*., 2004). The presence of a wide range of functional proteins, mRNAs, microRNAs, and lipids within exosomes (Mathivanan *et al*., 2010; Record *et al*., 2011) has suggested their role as biological nanovectors mediating cell communication.

In this study, for the first time, we investigated the role of exosomes as effectors of PL activity in order to explore their therapeutic potential for cell-free therapies in bone regeneration. For this purpose, we isolated exosomes from different samples of human PL, and evaluated their content in terms of total RNA and growth factors expression. Finally, we demonstrated their effectiveness to influence MSC osteogenic differentiation and to promote cell proliferation and migration.

Materials and Methods

Generation of human platelet lysate (PL)

Platelet units were collected after their expiration date (5 days after harvesting). They were generated by a pool of 3-5 buffy coats and comprise an average of 3.07 x 10^{11} platelets in 350 mL of plasma. According to the method Lohmann *et al*. (Lohmann *et al*., 2012), aliquots of 40 mL were frozen at -80 °C, thawed at 37 °C, and centrifuged at 2,600 x *g* for 30 min at room temperature (F34-6-38 rotor, Eppendorf 5810 R, Eppendorf, Milan, Italy). The supernatant (human platelet lysate, PL) was transferred into new tubes, and 2 U/mL heparin (Epsoclar, Mayne Pharma, Naples, Italy) was added before exosome isolation. A volume of 15 mL of the thawed solution was aliquoted and stored at -80 °C. PL was centrifuged at 2,000 x *g* for 10 min at room temperature to remove membrane fragments and 2 U/mL heparin was added as an anticoagulant before addition to culture medium to avoid PL gel formation.

Exosome isolation from PL

Exosomes were isolated by serial low speed centrifugation followed by ultracentrifugation from 6 samples of PL. According to the method of Théry *et al*. (Théry *et al*., 2006), PL was centrifuged at 500 x *g* for 10 min (two times), 2,000 x *g* for 15 min (two times) and 10,000 x *g* for 30 min (two times) (F34-6-38 rotor, Eppendorf 5810 R, Eppendorf) at 4 °C to remove cellular debris. The supernatant was then ultracentrifuged at 30,000 rpm for 1 h at 4 °C (Ti 45 rotor, Beckman Coulter, Rome, Italy). The exosome pellet was washed once in a large volume of phosphate buffered saline (PBS) and centrifuged at 30,000 rpm for 1 h at 4 °C (Ti 70 rotor, Beckman Coulter, Milan, Italy). The exosome pellet was re-suspended in sterile PBS and stored at -80 °C until use. Exosome quantity was determined by the Bradford method (Bio-rad, Milan, Italy).

Isolation of human platelets

Approximately 12 mL of venous blood was drawn from healthy volunteers and collected in a collection tube supplemented with 2 U/mL heparin. Whole blood was centrifuged immediately at 1,000 rpm for 5 min at room temperature to obtain platelet-rich plasma (PRP). Then, PRP was transferred in a new tube and centrifuged at 1,900 rpm for 15 min. After removal of the supernatant, the resulting platelet pellet was used for Western blot and RNA analysis.

Electron microscopy of PL-derived exosomes

Exosomes derived from PL were resuspended in 2 % paraformaldehyde (PFA) and loaded onto Formvar-carbon-coated grids. Next, exosomes were fixed in 1 % glutaraldehyde, washed, and contrasted with a solution of uranyl oxalate, pH 7, embedded in a mixture of 4 % uranyl acetate and 2 % methyl cellulose before observation with a Zeiss EM 109 electron microscope (Zeiss, Jena, Germany).

Western blotting

Purified exosomes or platelets were treated with RIPA lysis buffer (1 % Triton X-100, 10 % Na-deoxycholate, 5 M NaCl, 1 M Tris-HCl, pH 7.4, 0.5 M EGTA, pH 8, 1 M NaF) and protease inhibitors (Roche, Milan, Italy) for 20 min at 4 °C. Nuclei and cell debris were removed by centrifugation. Samples solubilised in lysis buffer were quantified by the Bradford assay. Thirty µg of each sample was electrophoresed on a 10 % SDS-PAGE and proteins were transferred to a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was blocked with 5 % bovine serum albumin (BSA) (Sigma-Aldrich, Milan, Italy) in T-TBS (0.1 M Tris-HCl pH 8, 1.5 M NaCl and 1 % Tween 20) for 1 h at room temperature, and subsequently incubated with anti-CD63 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD41 (1:1,000) (BioLegend, San Diego, CA, USA) antibodies overnight at 4 °C. After vigorous washing in T-TBS, the membrane was incubated with the secondary antibody for 1 h at room temperature. Anti-rabbit antibody (1:1,000) for CD63 and anti-mouse antibody (1:1,000) for CD41, both conjugated to horseradish peroxidase (GE Healthcare, Milan, Italy) were diluted in T-TBS contained 5 % BSA and used as secondary antibodies. Immunocomplexes were detected with the ECL Western blotting analysis system (Amersham Pharmacia, Piscataway, NJ, USA).

Bone mesenchymal stem cell (MSC) culture

After informed consent was obtained, bone marrow samples derived from the medullary cavity of the femur of 4 patients undergoing total hip replacement were flushed with PBS. Subsequently, mononuclear cells were isolated by the Ficoll Histopaque density gradient method (Sigma-Aldrich). Cells were maintained in 5 % CO_2 humidified atmosphere at 37 °C, in α-minimum essential medium (αMEM) supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin plus 100 µmol/L ascorbic acid-2 phosphate (Sigma-Aldrich), and 10 % foetal bovine serum (FBS) (Lonza, Milan, Italy). After 4 days of culture, cells adherent to culture plastic were considered as mesenchymal stem cells (MSC) and grown in αMEM supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 % FBS (basal culture conditions). Passage 2-3 MSC were used in all experiments. To avoid contamination from FBS-

derived exosomes, heat-inactivated FBS was centrifuged at 30,000 rpm (rotor Ti 45, Beckman) overnight at 4 °C. The pellet was discarded and the supernatant (exosome-free FBS, EFF) was passed through a 0.2 μm filter and stored at -20 °C until use for proliferation and differentiation assays.

Labelling and uptake of PL-derived exosomes

Exosome pellets were resuspended in PBS and stained with PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich), according to the manufacturer's instructions with minor modifications. Briefly, exosomes (2 μg) were diluted in PBS before 200 μL of Diluent C was added. As a control, 200 μL of Diluent C with the same volume of PBS was used. 0.8 μL of PKH26 dye was added to 200 μL of Diluent C before being added to the exosomes and the control. Samples were mixed gently for 5 min before 400 μL of αMEM supplemented with 10 % EFF was added to bind the excess dye. The samples were then centrifuged at 30,000 rpm (rotor Ti 45, Beckman) for 1 h at 4 °C and passed through a 0.2 μm filter.

Two μg of the PKH26 labelled exosomes, or the same volume of the PKH26-PBS control, were resuspended in αMEM supplemented with 10 % EFF and added to 1 x 10^4 MSC maintained at 37 °C in a humidified atmosphere with 5 % CO_2. After 4 and 20 h of incubation, uptake was stopped by washing and fixation in 3.7 % paraformaldehyde (PFA) for 10 min. Cells were then stained with a fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma-Aldrich) and visualised with a Nikon Eclipse E800M fluorescence microscope (Nikon, Tokyo, Japan). All cells per high power-field were counted, and the percentage of PKH26 positively stained cells was determined. Five representative high power-fields per sample were evaluated.

Proliferation assay

Cell proliferation was assessed by the Alamar Blue test (Invitrogen, Monza, Italy). MSC were seeded in duplicate at a density of 1.5 x 10^4 cells per well (12 well plate) in αMEM with 10 % EFF alone (control) or supplemented with two different exosome concentrations (5 μg and 50 μg) or with the corresponding quantities of PL. 10 % PL, containing an average amount of 0.6 μg of exosomes plus several growth factors released from platelets, was used as a positive control. Cell proliferation was also evaluated by incubating MSC with the same amount of exosomes founded in 10 % PL (0.6 μg). At day 3 and 6 after seeding, cells were exposed to 10 % Alamar Blue, and after 4 h the fluorescence was read at a wavelength of 535-590 nm using a microplate-reader (Tecan Infinite F200pro, Tecan, Milan, Italy). The results were expressed as mean of the relative fluorescence units (RFU) measured in two wells using four different MSC samples.

Migration assay

Cell migration was analysed by the Boyden chamber assay, using transwell inserts with 8-μm pore membrane filters (Costar, Sigma-Aldrich). MSC were grown to subconfluence (70 %) prior to harvesting by trypsinisation. Then, MSC were seeded in duplicate (1.5 x 10^4 cells per well in 200 μL of serum-free αMEM) into the upper chamber of the Transwell system, and 600 μL of serum-free αMEM supplemented with two different exosome concentrations (5 μg and 50 μg) or with the corresponding quantities of PL were applied to the lower chamber of the Transwell. 10 % PL was used as a positive control. After 20 h of incubation at 37 °C, the upper sides of the filters were carefully washed with PBS and non-migrating cells were removed with a cotton swab. Migrated cells were fixed and stained on the lower side of the filter using 0.5 % crystal violet dissolved in methanol for 30 min. Chambers were rinsed with water, dried, and examined under a Nikon Eclipse-TE 2000-S microscope (Nikon). Migrated cells were quantified by counting.

Osteogenic induction and mineralisation assay

MSC were seeded in duplicate at a density of 1 x 10^4/cm^2 in basal culture conditions supplemented with 50 μg/mL L-ascorbic acid 2-phosphate and 10^{-8} M dexamethasone (Sigma-Aldrich). At cell confluence, cells were incubated in αMEM supplemented with 10 % EFF, 50 μg/mL ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich), alone (control) or in presence of 5 μg or 50 μg of exosomes, or with the corresponding quantities of PL. The differentiation medium added to 10 % PL was used as an additional control of osteogenic induction. Medium was changed twice-a-week. After 17 days, cells were fixed in 3.7 % PFA (Sigma-Aldrich) for 20 min, and calcium deposits were stained with 1 % Alizarin Red S (pH 4.2) (Sigma-Aldrich) for 1 h at room temperature. The stained mineralised matrix was observed using a Nikon Eclipse-TE 2000-S microscope (Nikon).

For quantification of staining, 500 μL of 10 % cetylpyridinium chloride (CPC) (p/v) (Sigma-Aldrich) was added to each well, and the plate was incubated at room temperature for 30 min with shaking. The dye was then removed and 200 μL aliquots were read in duplicate at 570 nm in 96-well format using a microplate-reader (Tecan Infinite F200pro, Tecan, Milan, Italy).

Quantification of growth factors in PL and PL-derived exosomes by ELISA

Exosome samples were lysed in RIPA lysis buffer, as previously described. PL samples were thawed and centrifuged to remove membrane fragments. The concentration of human basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB) and transforming growth factor beta 1 (TGF-β1) within PL and PL-derived exosomes was measured using commercially available reagents, based on a sandwich enzyme immunoassay technique (DuoSet ELISA; R&D Systems, Minneapolis, MN, USA), and following the manufacturer's protocol. Standard reference curves were prepared using recombinant human bFGF, between 0 and 1,000 pg/mL, and recombinant human VEGF, PDGF-BB and TGF-β1, between 0 and 2,000 pg/mL. Briefly, 100 μL of standards and samples were pipetted into a 96-well microplate pre-coated with mouse anti-human bFGF, VEGF, PDGF-BB and TGF-β1 antibodies. Biotinylated detection antibodies against human bFGF, VEGF, PDGF-BB and TGF-β1 were

used to detect growth factors bound by the immobilised antibodies. Following washing to remove any unbound antibody-enzyme reagents, a tetramethylbenzidine substrate solution was added to the wells. The reaction was stopped with 2 N H_2SO_4, and the optical density was read using a microplate reader set to 450 nm (Tecan Infinite F200pro). The concentrations of bFGF, VEGF, PDGF-BB and TGF-β1 were extrapolated by means of a dedicated software.

RNA isolation and detection

RNA was extracted from platelets and exosomes (n = 3) using NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) and Trizol® (Invitrogen), respectively, according to the manufacturer's protocols. The RNA quality, yield, and size of exosomal and cellular total RNA were evaluated with the RNA 6000 Nano Kit using the Agilent 2100 Bioanalyser (Agilent Technologies, Milan, Italy). Electropherograms were analysed using the Agilent 2100 Expert B.02.07 software that includes data collection, presentation, and interpretation functions.

Statistical analysis

Statistical analysis was performed by StatView5.01 software (SAS Institute Inc., Cary, NC, USA). Quantitative results were expressed as arithmetic mean plus or minus the standard deviation of the mean. The Wilcoxon signed rank test was used in a paired analysis to evaluate the effects of exosomes and PL on cell migration and mineralisation. The Mann-Whitney U-test was applied to assess the effects of exosomes and PL on cell proliferation and to detect differences in bFGF, VEGF, PDGF-BB and TGF-β1 content in exosomes and PL as unpaired comparison for two independent variables. All p values < 0.05 were considered as statistically significant.

Results

Identification of PL-derived exosomes

Exosomes were isolated from six human platelet lysate samples through a series of low speed centrifugations followed by ultracentrifugations. In particular, an average of 2.9 x 10^3 μg ± 2.1 x 10^3 exosomes were isolated from platelet units, with an average volume of 350 mL and containing an amount of 3.07 x 10^{11} ± 0.83 x 10^{11} platelets. As extensively described in literature (Lehmann *et al*., 2008; Lasser *et al*., 2011; Sokolova *et al*., 2011; Baglio *et al*., 2012; Mineo *et al*., 2012; Salomon *et al*., 2013; Tomasoni *et al*., 2013), the presence of exosomes was determined by electron microscopy. Exosomes with a characteristic rounded, cup-shaped morphology, and a typical size range of 30-100 nm are shown in Fig. 1a. Exosome purity was validated by Western blot for the expression of CD63, a specific exosomal marker (Théry *et al*., 2002; Mathivanan *et al*., 2010), and CD41, a heterodimeric integral membrane protein expressed in platelets and megakaryocytes (Heijnen *et al*., 1999; Italiano *et al*., 2010) (Fig. 1b). The results revealed that PL-derived exosomes were positive for the tetraspanins CD63, while no amount of CD41 was detected, suggesting that the exosome preparations obtained were pure and devoid of micro-vesicle contamination (Heijnen *et al*., 1999).

Exosome uptake by MSC

To examine whether exosomes from human platelet lysate can be taken up by MSC, PKH26 labelled exosomes (red) were incubated with MSC at two different time points and examined using fluorescence microscopy. After 4 h of incubation, a few exosomes were taken up by MSC with a localisation mainly in the cytoplasm (data not shown). After 20 h, exosomes were taken up by 98 % ± 1.8 % of MSC and accumulated especially in the perinuclear region (Fig. 2). As expected, no fluorescent signal was detected in the control.

Effect of exosomes on MSC functions

Since MSC were shown to internalise PL-derived exosomes, we aimed to determine whether exosomes are able to influence MSC activities, such as proliferation, migration, and osteogenic differentiation.

First, MSC proliferation was measured in the presence of three different concentrations of exosomes (0.6 μg, 5 μg and 50 μg) or with 5 μg and 50 μg of PL at day 3 and 6 after seeding. As expected, the proliferation of MSC in every culture condition increased from the seeding to the end-point with a log phase. In particular, exosomes were capable to induce a significant increase in the MSC proliferation rate compared to control medium (αMEM with 10 % EFF) (Fig. 3). At day 6, the amount of 0.6 μg

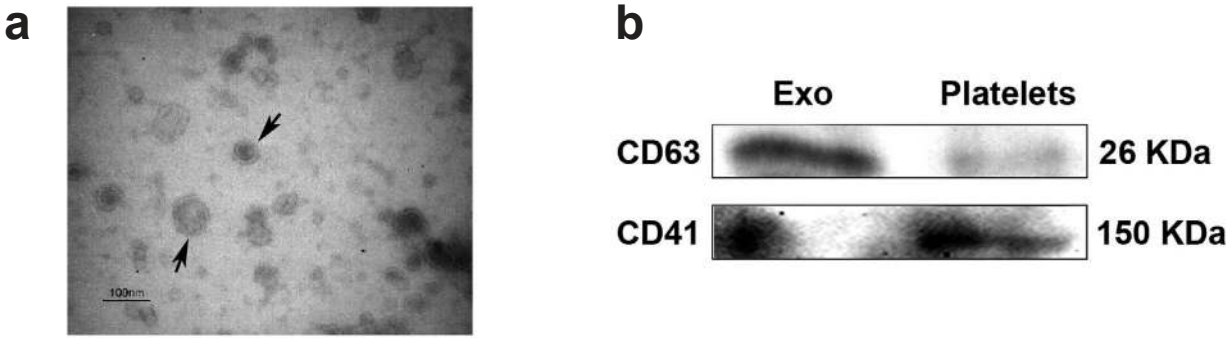

Fig. 1. Characterisation of PL-derived exosomes. Exosomes isolated from platelet lysate were examined by electron microscopy. Scale bar = 100 nm (**a**). Exosome purity was assessed also by Western blotting for the expression of the specific exosomal marker CD63 and for the expression of CD41, a glycoprotein expressed in platelets (**b**).

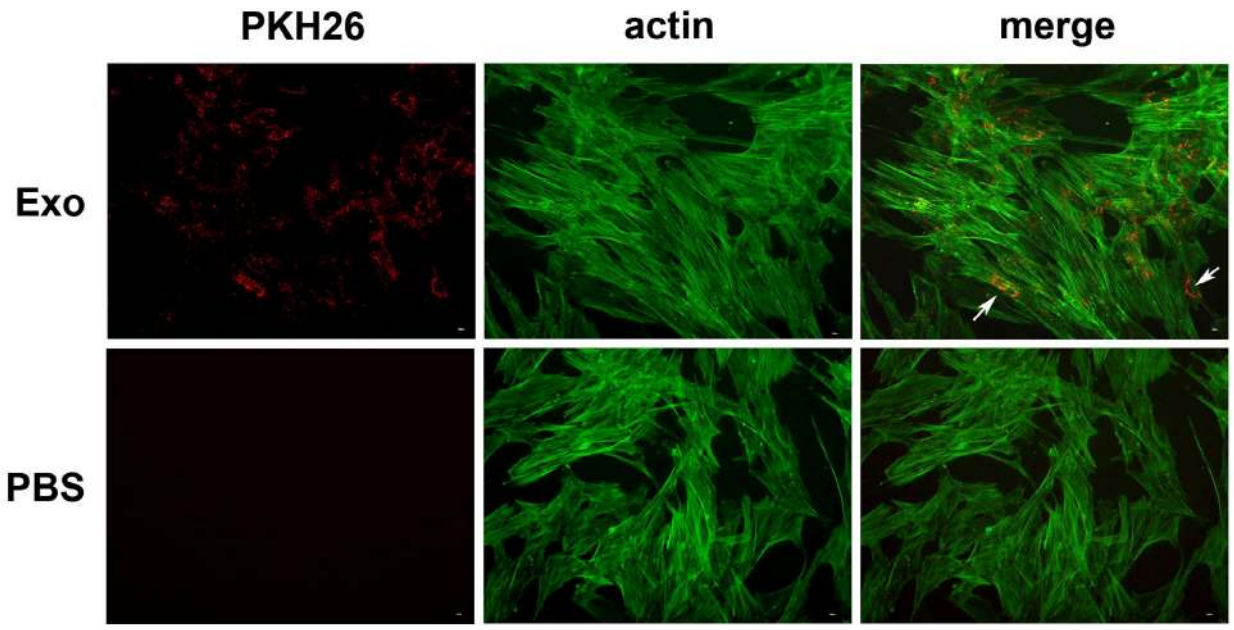

Fig. 2. Uptake of PL-derived exosomes by MSC. 2 μg of the PKH26-labelled PL exosomes (red) (upper panel) or a PKH26-PBS control (lower panel) were added to 1 x 10^4 MSC and incubated at 37 °C for 20 h. The cell uptake of the fluorescently labelled PL-derived exosomes was detected by fluorescence microscopy. Cells were fixed and actin filaments were stained with a FITC-conjugated phalloidin (green). Arrows indicate exosome localisation. Scale bar = 10 μm.

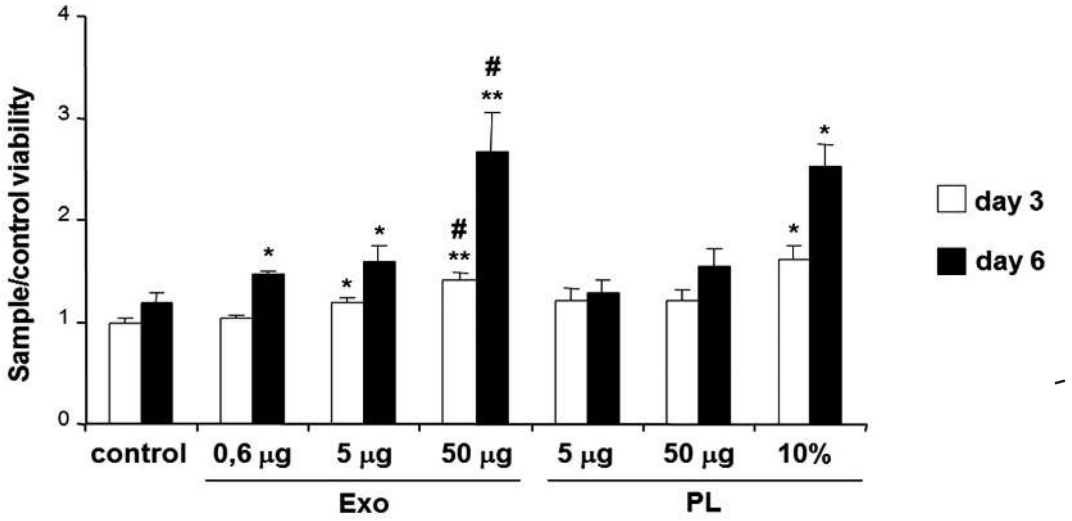

Fig. 3. Effect of exosomes on MSC proliferation. MSC were exposed to αMEM with 10 % EFF alone (control), or supplemented with 0.6 μg, 5 μg and 50 μg of PL-derived exosomes or PL. 10 % PL served as a positive control. Cell proliferation was assessed at different time points by Alamar blue assay and quantified by a fluorescence microplate reader. The results were expressed as mean of viability rate in respect to cells in control medium (EFF) at day 3. Data derived from six independent experiments. Significant difference between control *vs*. 0.6 μg of exosomes at day 6, 5 μg of exosomes and 10 % PL both at day 3 and day 6 (*) ($p < 0.05$); highly significant difference between control *vs*. 50 μg of exosomes both at day 3 and day 6 (**) ($p < 0.05$); significant change between 5 μg *vs*. 50 μg of exosomes both at day 3 and day 6 (#) ($p < 0.05$).

of exosomes was sufficient to induce a significant 1.2-fold increase in MSC proliferation (1.47 ± 0.038), as compared to control condition (1.19 ± 0.104). Since day 3, a similar trend was also observed when cells were treated with both 5 μg and 50 μg of exosomes. In addition, the proliferation rate induced by 50 μg of exosomes was much more relevant to that observed with 0.6 μg and 5 μg exosome concentration during the whole cell culture, demonstrating a dose-dependent increase in cell growth. The culture of MSC with 50 μg of exosomes also showed a proliferation rate similar to that obtained with 10 % PL, containing an average amount of 0.6 μg of exosomes plus several free growth factors present in PL preparations, and used as a positive control.

Next, to investigate the potential chemotactic effect of PL-derived exosomes and their influence on MSC migration, we performed a migration assay (Fig. 4). After an incubation time of 20 h, a total number of 7.87 ± 0.72

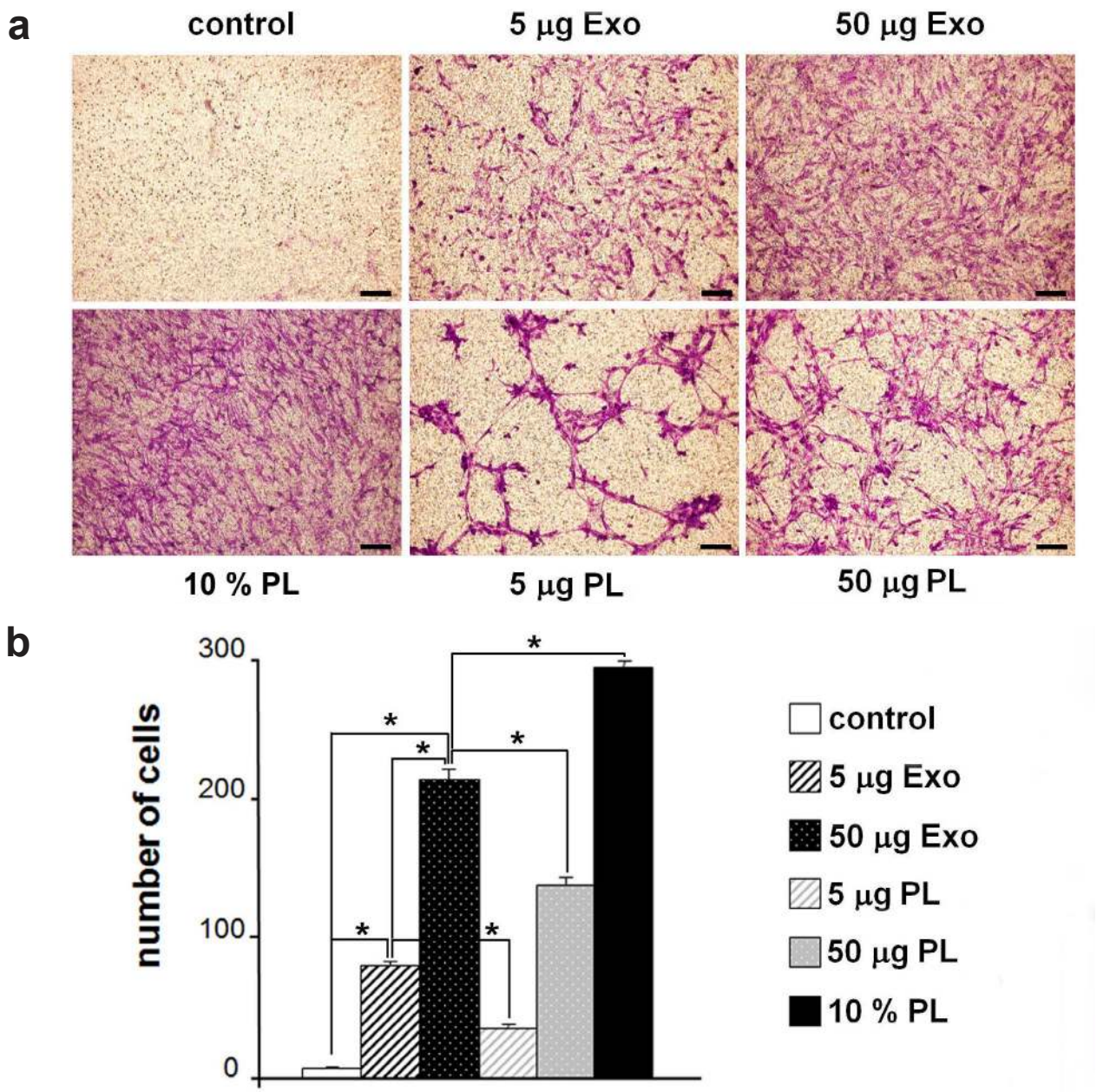

Fig. 4. Effect of exosomes on MSC migration. MSC migration as analysed by Boyden chamber assay. Representative images of MSC localised on the lower site of the Boyden filter after migration through the membrane in response to 5 µg and 50 µg of PL-derived exosomes or the same concentrations of PL or to serum free which served as a negative control. 10 % PL was used as a positive control of cell migration. Scale bar = 50 µm. (**a**). Data represent the number of cells migrated in response to different culture conditions. Values are the mean ± standard deviation of the number of cells per high-power field that migrated through the membrane pores relative to the negative control. Results derived from three independent experiments performed in triplicate (* $p < 0.05$) (**b**). Significant difference between control *vs*. 5 µg and 50 µg of PL and 10 % PL ($p < 0.05$); Significant change between 5 µg *vs*. 50 µg of PL ($p < 0.05$); Significant difference between 50 µg of exosomes or PL *vs*. 10 % PL ($p < 0.05$).

cells migrated through the membrane without the addition of any stimulus (negative control). Both exosomes and PL conditions induced a significant increase in migration. In particular, a 10.3- and a 27.1-fold increased migration were detected by using 5 µg or 50 µg of exosomes, respectively, demonstrating a dose-dependent effect, as previously observed for MSC proliferation. Moreover, cell mobilisation induced by exosomes was significantly higher than that promoted by PL at the same concentrations (Fig. 4b).

We subsequently assessed if the osteogenic differentiation potential of MSC was influenced by PL-derived exosomes. Cells were cultured with specific osteogenic factors with or without exosomes or PL for 17 days, and osteogenic differentiation was evaluated by staining of calcium phosphate precipitates with Alizarin S Red. As shown in Fig. 5a, PL induced a MSC differentiation comparable to that observed in the control, without any substantial difference for the concentration used. The two concentrations of exosomes differently

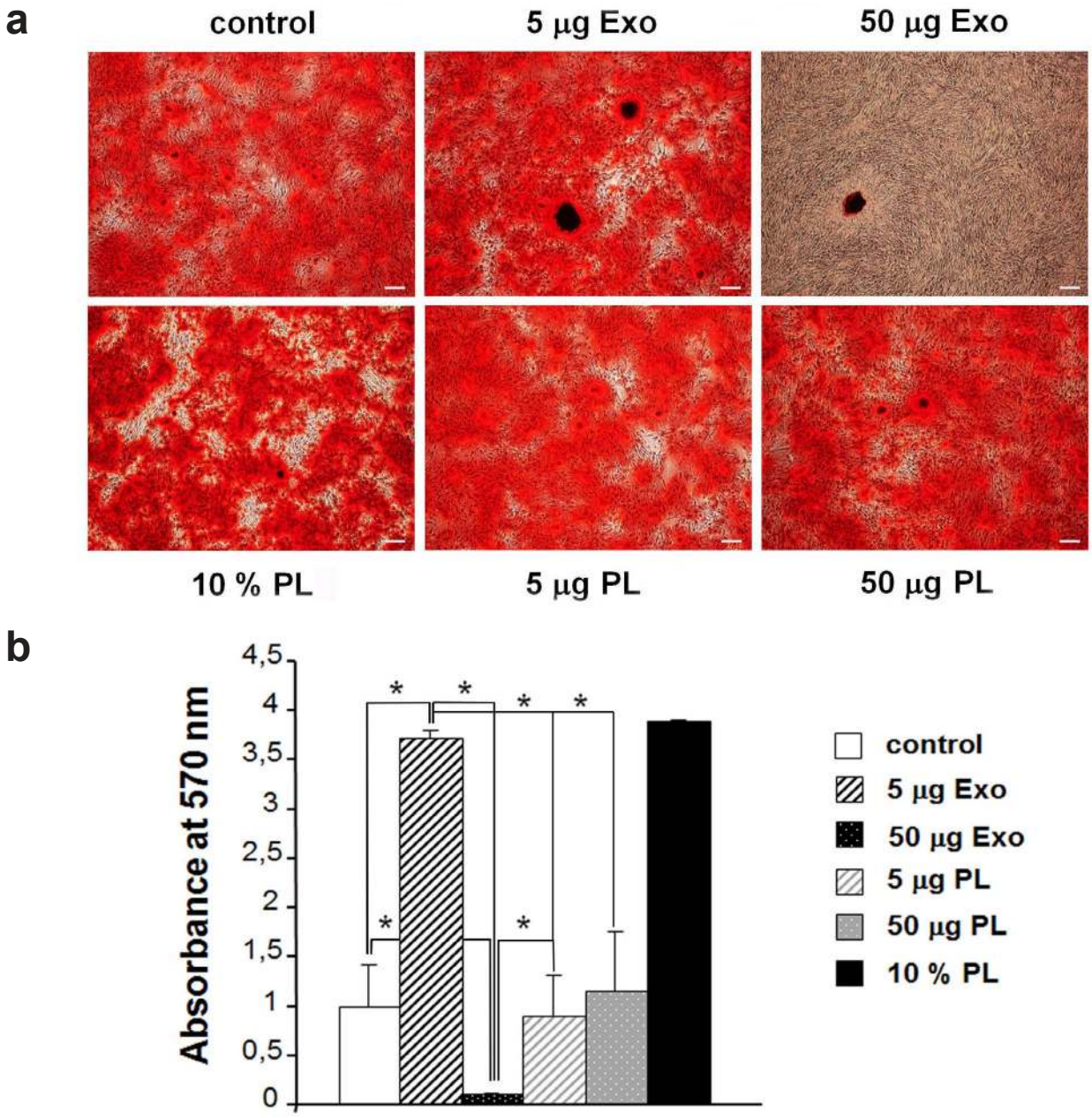

Fig. 5. Osteogenic differentiation capacity of MSC in relation to PL and PL-derived exosomes. MSC were cultured in αMEM with 10 % EFF and specific osteogenic factors alone (control) or in the presence of 5 µg and 50 µg of exosomes or PL. The differentiation medium added to 10 % PL was used as an additional control of osteogenic induction. Osteogenic differentiation was analysed after 17 days by staining of calcium phosphate precipitates with Alizarin Red S. Scale bar = 200 µm (**a**). Quantification of Alizarin Red staining by extraction with 10 % CPC. The results were expressed as mean of the absorbance values ± standard deviation derived from three independent experiments (* $p < 0.05$) (**b**).

affected osteogenic differentiation. MSC cultured with 5 µg of exosomes resulted in a substantial osteogenesis, characterised by the presence of several mineralised nodules larger than those found in control and PL cultures, whereas the higher exosome concentration significantly decreased the ability of MSC to deposit mineralised matrix. These results were confirmed quantifying the amount of calcium-containing mineral deposition by CPC assay (Fig. 5b).

PL-derived exosomes express bFGF, VEGF, PDF-BB and TGF-β1

In order to investigate whether potential factors are involved in the observed modulation of MSC functions, bFGF, VEGF, PDF-BB and TGF-β1 were evaluated in PL and PL-derived exosomes. ELISA assays revealed an enrichment of all these growth factors in exosomes compared to PL. In particular, the expression of bFGF,

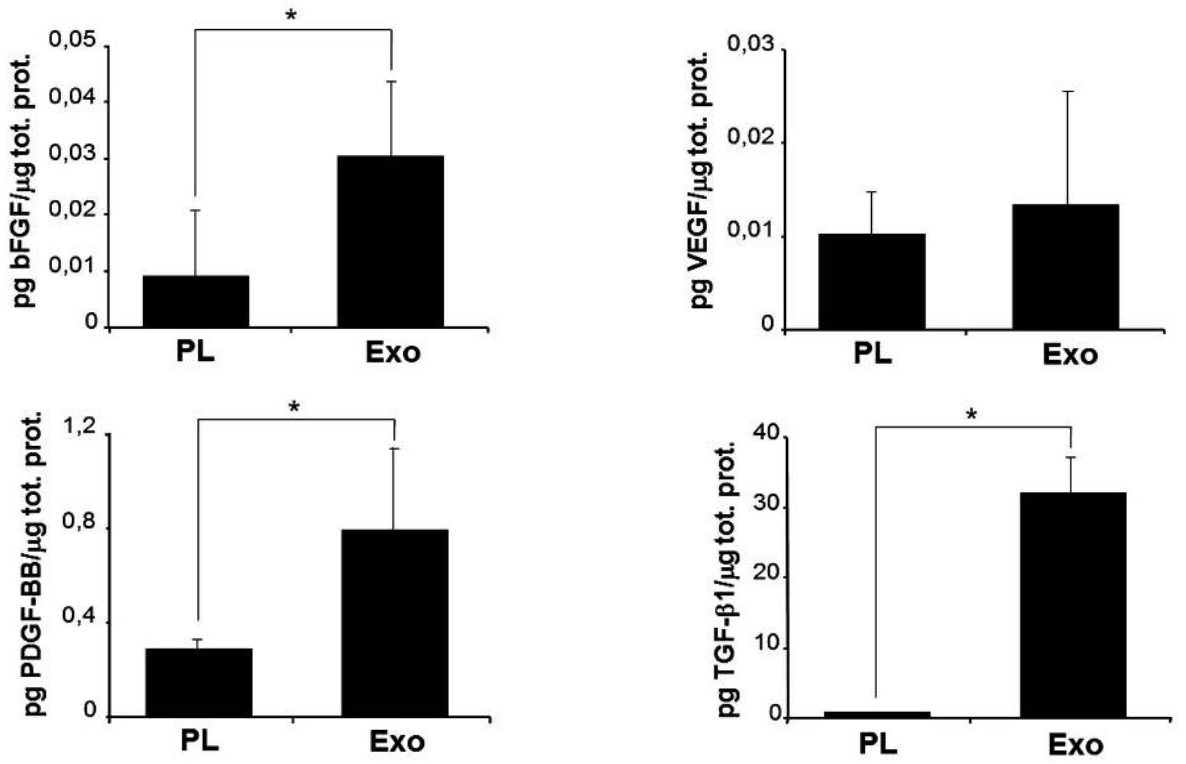

Fig. 6. Evaluation of growth factors in PL and PL-derived exosomes. To determine bFGF, VEGF, PDGF-BB and TGF-β1 concentration, 100 µL of PL or PL-derived exosomes were subjected to ELISA analysis. Data were normalised for the total protein content and showed the presence of bFGF, VEGF, PDGF-BB and TGF-β1 in PL-derived exosomes at a higher amount compared to PL (* $p < 0.05$). Results represent the mean ± standard deviation obtained from $n = 3$ exosome samples and $n = 3$ PL analysed in triplicate.

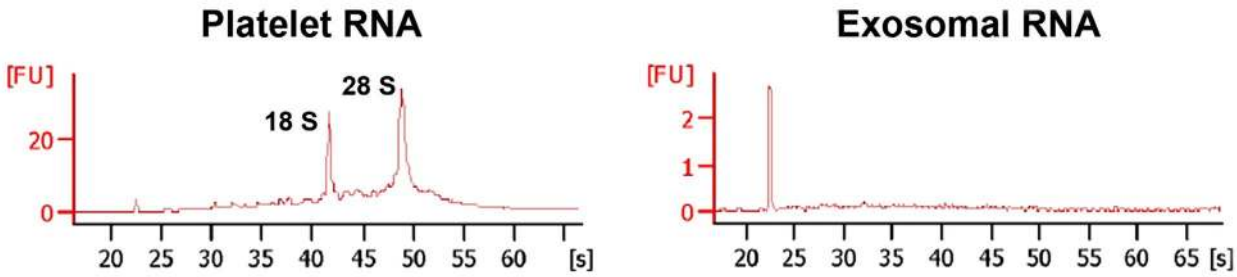

Fig. 7. Bioanalyser analysis of cellular and exosomal total RNA. Cellular and exosomal RNA was analysed with the RNA Nano 6000 Kit using an Agilent 2100 Bioanalyzer. The electropherograms show the peak distribution in seconds migration time (s) and fluorescence intensity (FU) of total RNA in platelets and in exosomes. The electropherograms are representative of $n = 3$.

PDGF-BB and TGF-β1 in the nanovescicles was significantly higher compared to PL (3.3-fold increased expression for bFGF; 2.7-fold for PDGF-BB and 35.5-fold for TGF-β1) (Fig. 6).

PL-derived exosomes contain RNA

To assess the RNA profiling of both exosomes and cells, total RNA was analysed with the RNA 6000 Nano Kit using an Agilent 2100 Bioanalyzer. As shown in Fig. 7, the bioanalyser electropherogram of exosomes was different with regard to the pattern of cellular RNA. In platelets the most dominant peaks were 18S and 28S rRNA. Otherwise, exosomal RNA differed in their profile as exosomes lacked the two rRNA peaks and were rich in short RNA, as represented by the peak between 20 and 25 s.

Discussion

The need to achieve prompt and adequate bone regeneration in cranial, oral, maxillofacial, and orthopaedic surgery is a key issue in regenerative medicine, resulting in the development of several techniques (Fleming *et al*., 2000). Among these, platelet derivatives such as PRP and PL

have been employed as special sources of GF mimicking the physiological mechanisms of bone healing. Several studies have shown their beneficial potential for a variety of indications (Hee *et al*., 2003; Sanchez *et al*., 2003; Franchini *et al*., 2005). However, despite the hype, an active debate on the clinical benefits of these solutions is still ongoing. Indeed, even if PRP and PL are being suggested as cost-effective adjuvants with low morbidity and low associated complications, a clear connection between their use, the underlying molecular mechanisms, and a positive clinical outcome is still lacking. Up to now, the effect of platelet concentrates on bone cells has been attributed to their GF released during platelet degranulation. However, PL activity might also be due to the efficient intercellular communication of bioactive molecules, mediated by nanosized vesicles, identified as exosomes.

Based on these considerations, the aim of this study was to investigate if exosomes can be considered as relevant effectors of PL activity.

We isolated exosomes from human PL (PL) by ultracentrifugation and evaluated their potential effect on MSC functionality as compared to PL. Since PL-derived exosomes were shown to interact with MSC, we further examined whether exosomes would affect cell proliferation, migration, and osteogenic differentiation. Based on literature data (Bu *et al*., 2006; Yang *et al*., 2011), we treated cells with different exosome concentrations. 10 % PL, containing an average amount of 0.6 μg of exosomes plus growth factors released from platelets, was included as a positive control to confirm the cell-system model, since it represents the percentage of PL mostly used as effective supplement for MSC cultivation *in vitro* (Schallmoser *et al*., 2007; Schallmoser *et al*., 2008; Bieback *et al*., 2009) and it has been demonstrated to induce MSC proliferation and osteogenic differentiation (Fekete *et al*., 2012; Lohmann *et al*., 2012; Jonsdottir-Buch *et al*., 2013). MSC cultured with exosomes showed a significant, dose-dependent increase in cell proliferation and migration as compared to the EFF control, suggesting a potential enrichment of proteins or other bioactive molecules related to these processes, within exosomes. In particular, the amount of 0.6 μg of exosomes contained in 10 % PL, is sufficient to induce a significant increase of MSC growth. However, the proliferation rate observed with this concentration is significantly lower than that obtained with 10 % PL, since the effect of PL is also mediated by free growth factors released from platelets. This hypothesis is supported by Hemeda *et al.* (Hemeda *et al*., 2014) who recently reported the importance of several factors (*i.e.*, growth factors, metabolites, *etc*.) to be considered in PL activity.

Interestingly, the proliferation rate of MSC cultured in 50 μg of exosomes both at day 3 and day 6, was comparable to that induced by 10 % PL, suggesting that, at this concentration, exosomes possess the same potential to stimulate MSC growth as that provided by 10 % PL. Similar exosome effects have also been observed in tumour (O'Brien *et al*., 2013; Roccaro *et al*., 2013) and normal cell-system models (Zhang *et al*., 2012b; Salomon *et al*., 2013), indicating that a cocktail of factors contained in exosomes can boost cell proliferation and migration. PL-derived exosomes showed to affect also the osteogenic differentiation potential of MSC. In particular, we observed that osteogenically stimulated confluent cells did not lose their lineage differentiation ability in the presence of 5 μg of exosomes or PL. Indeed, MSC treated with the lower dose of exosomes showed a comparable pattern of osteogenesis as that obtained by culturing cells with 10 % PL, suggesting that exosomes have the same potential to steer MSC toward the osteogenic differentiation as that provided by 10 % PL. On the other hand, the ability of MSC to deposit mineralised matrix was significantly decreased after exposure to 50 μg of exosomes. The lower effect of 50 μg of exosomes, compared with the other culture conditions, might be attributed to the higher proliferation rate observed in the presence of this exosome concentration.

In this study, we demonstrated that a substantial part of proliferation, migration and osteogenic effects of the PL is mediated by exosomes. However, the exact nature of the signals involved in these processes and delivered by PL-derived exosomes, as well as the mechanism of action, is still unknown. The involvement of several soluble mediators in the activity of PL, such as coagulation, vasoactive, and growth factors is well documented (Slater *et al*., 1995; Rendu and Brohard-Bohn., 2001). Among these factors, PDGFs, IGFs, VEGF, TGF-β, and bFGF have the closest association with bone healing, as they are involved in bone regeneration and are potential regulators of osteoblastic migration, proliferation, and differentiation (Lieberman *et al*., 1998; Lind, 1998; Schmidt *et al*., 1998; Gerber *et al*., 1999; Schliephake, 2002).

Recent studies have described the expression of growth factors, or other usually soluble mediators, in association with the exosome membrane (Zhang *et al*., 2006; Sanderson *et al*., 2008; Seelenmeyer *et al*., 2008). Based on this evidence, we evaluated if bFGF, VEGF, PDGF-BB and TGF-β1 of PL were also associated with exosomes. We chose to test these growth factors, because they have been extensively described as actively involved in osteogenesis and angiogenesis (Montero *et al*., 2000; Kilian *et al*., 2004; Presta *et al*., 2006; Graham *et al*., 2009; Kempen *et al*., 2010; Caplan and Correa., 2011; Malhotra *et al*., 2013). Moreover, recombinant bFGF, PDGF-BB and VEGF have been shown to favour fracture healing (Eckardt *et al*., 2005; Moore *et al*., 2009; Kawaguchi *et al*., 2010). Our results showed a significant enrichment of bFGF, PDGF-BB and TGF-β1 expression in exosomes compared to PL, suggesting a possible physiological role for PL-derived exosomes as a means of delivering these growth factors or other mediators though the extracellular space.

Up to now, GF present in biologic fluids have been accounted as free molecules. Our discovery, as well as similar observations reported by others (Clayton *et al*., 2007; Clayton *et al*., 2008; Wang *et al*., 2008), showed that GF can exist also encapsulated into exosomes. This evidence should be deeply considered every time that a specific factor is evaluated, in order to avoid potential false positives. For instance, we previously demonstrated that a high serum level of FGF-2 in children with orthopaedic

diseases was correlated with a good bone healing, suggesting that FGF-2 may be a sentinel factor that may predict the outcome of a severe bone lesion. However, our test showed a consistent number of false positives, where FGF-2 levels lower than the threshold value were also detected in some subjects with a good clinical outcome (Granchi *et al*., 2013). This result, apparently inconsistent, may be explained evaluating the expression of FGF-2 inside serum-derived exosomes which, as demonstrated in the present study, may be enriched of this osteoinductive factor, thus leading to bone healing. The analysis of GF or other bioactive molecules both free and encapsulated may help in the development of more reliable predictive assays to be used in regenerative approaches.

Another possible mechanism involved in mediating exosome effects could be *via* the exosomal delivery of RNA. Several reports have revealed the presence of mRNA and microRNA in exosomes derived from different cell sources (Camacho *et al*., 2013; Lv *et al*., 2013; Moldovan *et al*., 2013). In this study, we demonstrated that also PL-derived exosomes contain short RNAs, suggesting that these vesicles can be considered as biological nanovectors, which may transfer specific signals from platelets to other cells involved in bone healing. Recently, Plè *et al*. have demonstrated the presence of more than 492 different mature microRNA in human platelets (Plé *et al*., 2012), some of which, are implicated in proliferation (Zhang *et al*., 2012a), migration (Zhao *et al*., 2014), and osteogenic differentiation (Trompeter *et al*., 2013). Therefore, it would be fundamental to investigate if these micro-RNAs are expressed in PL-derived exosomes as well as to evaluate the presence of other RNA selectively expressed in exosomes, which might mediate specific effects. Previous studies, indeed, have shown that exosomes from mast cells contain RNA and that the RNA profile differs between exosomes and their donor cells (Valadi *et al*., 2007).

A full characterisation of PL-derived exosome content, both at the RNA and protein levels, may provide detailed information on the molecular mechanisms whereby platelet derivatives interact with cells during bone regeneration, and may help to explain the discrepancies seen in the translation from preclinical studies to clinical use of platelet derivatives. One of the most plausible hypotheses is that platelets induce tissue repair and angiogenesis through a paracrine effect mediated by exosomes (Brill *et al*., 2004; Rhee *et al*., 2004; Brill *et al*., 2005). An increasing number of studies, indeed, demonstrated that these nanovescicles play an important role in intercellular communication and represent one of the primary mediators of paracrine effect of their donor cells (Lai *et al*., 2004). Sahoo *et al*. showed that exosomes from human CD34+ stem cells mediate their proangiogenic paracrine activity both *in vitro* and *in vivo* (Sahoo *et al*., 2011). Similarly, Lai *et al.* demonstrated that bone marrow-derived MSC mediated its cardioprotective paracrine effect by secreting exosomes, thus reducing myocardial ischemia/reperfusion injury (Lai *et al.*, 2010).

The active paracrine trophic potential of exosomes introduces a radically different dimension to the therapeutic applications of MSC in regenerative medicine. Indeed, by replacing transplantation of MSC with a cell-free approach based on the administration of MSC secreted exosomes, many of the safety concerns and limitations associated with the transplantation of viable replicating cells could be mitigated.

In addition, exosomes have the capacity to carry a large cargo load, to protect the contents from degradative enzymes or chemicals and, because of low immunogenicity and stability, they represent optimal carriers for nanodelivery treatments. For instance, since the clinical application of PL is limited because of its short half-life and the variability of GF profiles among different donors (Marx, 2004; Pietramaggiori *et al*., 2008; Sugimori *et al*., 2006), the establishment of a PL-derived exosome factory may be envisaged for the delivery of adequate amounts of desired biological factors for effective bone regeneration strategies.

However, a complete knowledge of exosome content is an essential requirement before any exosome application. As mentioned above, exosomes contain proteins and RNA but they have also been implicated as a vehicle for viral and bacterial infection (Silverman and Reiner, 2011), including the assembly and release of HIV (Gould *et al*., 2003; Nguyen *et al*., 2003; von Schwedler *et al*., 2003) and intercellular spreading of infectious prions (Ritchie *et al*., 2013; Schneider and Simons, 2013). In addition, recently, it was reported that tumour cells (Balaj *et al*., 2011) and cardiomyocytes (Waldenström *et al*., 2012) release exosomes carrying DNA. Although the role of exosomal DNA is still unknown, it has been suggested that it could reach the cytosol of the target cells and be imported into the nucleus, where it may integrate into the genome, potentially leading to mutations, deletions, rearrangements and changes in gene expression. This aspect should be explicitly considered every time that cell culture supernatants or biological fluids, such as platelet derivatives, are applied both *in vitro* and *in vivo* studies. For instance, the analysis of exosomal content is a basic prerequisite, when pooled human platelet lysate (pPL) is used as medium supplement for *ex vivo* large-scale MSC isolation and expansion. In this situation, indeed, pPL-exosomes deriving from different patients may possess diverse exosomal content and transport molecules, which may positively affect cell behaviour or compromise cell physiological functions.

In conclusion, our discovery of exosomes as additional effectors of PL activity provides valuable data that could result into a more focused application of platelet concentrates into the clinical setting.

Moreover, this study gives new insights into the biology of exosomes, highlighting some of their positive and negative features that need to be considered to develop new therapeutic nanodelivery systems.

Conclusions

In the present study we demonstrate, for the first time, that exosomes can be successfully isolated and purified from human PL. PL-derived exosomes increase MSC proliferation and migration at a higher extent than PL. In addition, exosome concentration affects the osteogenic differentiation of MSC. Preliminary analysis of exosome content revealed that PL-derived exosomes express bFGF, VEGF, PDGF-BB and TGF-β1 and are enriched in short

RNA. Our results provide significant evidence of exosomes as new additional effectors of PL, clarifying its activity on bone regeneration. In addition, our study highlights the importance of exosomes as a potential nanodelivery system for cell-free regeneration therapies.

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Discussion with Reviewers

M. Alini: The concentration of exosomes in PL is 10-times less than that used to evaluate MSC proliferation. To prove exosomes activity (In PL) towards MSC proliferation, the authors should use the concentration measured in PL. Please comment.
Authors: According to the editor's suggestions, we performed the proliferation assay by using the concentration of exosomes contained into 10 % PL (0.6 μg).

The effect of exosomes is dose dependent. The amount of 0.6 μg is sufficient to induce a significant increase of MSC proliferation (see Fig. 3). However, the proliferation rate observed with 0.6 μg is significantly lower than that observed with 10 % PL, since the effect of PL is mediated also by free growth factors contained into PL preparation. This hypothesis is supported also by Hemeda *et al*. (Hemeda *et al*., 2014, text reference) who recently reported the importance of several factors (*i.e.*, growth factors, metabolites, *etc*.) to be considered in PL activity.

M. Herrmann: The findings in your study are new – are they clinically applicable? Is there a possibility to modify exosomes to ultimately boost their effect?
Authors: Up to now there are only few studies in literature where exosomes have been used in clinical trials (Dai, 2008; Escudier *et al*., 2005; Morse *et al*., 2005, additional references). Exosomes represent a challenging opportunity for new effective therapeutic applications. Indeed, they may be modified to become well-suited biologically active vectors able to deliver a broad variety of cargos (Sun *et al*., 2010; Alvarez-Erviti *et al*, 2011: Lai *et al*., 2013; Tian *et al*., 2014, additional references). However, for translation of exosomes to the clinic, exosome isolation and characterisation methods should be standardised and large-scale production must be developed.

S. Lippross: Uptake experiments are preliminary and solely based on microscopy. A proof of internalisation of exosomes is missing. Please comment.
Authors: As demonstrated by other papers (Mineo *et al*., 2012, text reference; Camacho *et al*., 2013; Roccaro *et al*., 2013; Riches *et al*., 2014, additional references), analysis performed by microscopy represents one of the main methods used to assess exosome uptake.

S. Lippross: The quantification results of the Alizarin Red staining do not correspond to the pictures shown in Fig. 5a. Please comment.

Authors: The quantification of Alizarin Red staining is a mean derived from three independent experiments, while images are only representative. The trend is virtually the same; moreover, the quantification assay is more sensitive, thus able to reveal different levels of mineralisation not appreciable using optical microscopy.

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