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

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SUMMARY

The effective osteogenic commitment of human bone marrow mesenchymal stem cells (hBMSCs) is critical for bone regenerative therapies. Extracellular vesicles (EVs) derived from hBMSCs have a regenerative potential that has been increasingly recognized. Herein, the osteoinductive potential of osteogenically induced hBMSC-EVs was examined. hBMSCs secreted negatively charged nanosized vesicles (~35 nm) with EV-related surface markers. The yield of EVs over 7 days was dependent on an osteogenic stimulus (standard chemical cocktail or *RUNX2* cationic-lipid transfection). These EVs were used to sequentially stimulate homotypic uncommitted cells during 7 days, matching the seeding density of EV parent cells, culture time, and stimuli. Osteogenically committed hBMSC-EVs induced an osteogenic phenotype characterized by marked early induction of *BMP2*, *SP7*, *SPP1*, *BGLAP*/*IBSP*, and alkaline phosphatase. Both EV groups outperformed the currently used osteoinductive strategies. These data show that naturally secreted EVs can guide the osteogenic commitment of hBMSCs in the absence of other chemical or genetic osteoinductors.

INTRODUCTION

Human bone marrow mesenchymal stem cells (hBMSCs) are attractive candidates for advanced cell therapies, including bone regeneration (Costa-Pinto et al., 2012). Satisfactory treatments are hampered by the difficulty in obtaining a well-defined population of terminally differentiated cells. This causes heterogeneity of hBMSCs, enhances the possibility of spontaneous differentiation into other lineages (Nombela-Arrieta et al., 2011), and may shorten the cells' engraftment-activation time (Tsubota et al., 1999). Therefore, approaches beyond the standard chemical cocktails have been investigated (Heng et al., 2004) such as genetic modulation through the overexpression of genes (e.g., runt-related transcription factor 2, *RUNX2*) (Karsenty et al., 2009; Monteiro et al., 2014), and the use of synthetic/recombinant factors such as bone morphogenetic protein 4 or cell-derived conditioned medium (CM). Indeed, CM contains an array of growth factors, cytokines, proteins (Makridakis et al., 2013), and the recently highlighted extracellular vesicles (EVs) (Collino et al., 2010). EVs are nanosized particles (exosomes, 30–100 nm; microvesicles, 50–2000 nm) carrying lipids, proteins, and nucleic acids (Akers et al., 2013). It has been suggested that hBMSC-secreted EVs include differentiation cues (miRNA, Collino et al., 2010; Baglio et al., 2015; tRNA, Baglio et al., 2015; and proteins, Kim et al., 2012), even upon osteogenic induction (Xu et al., 2014). The regenerative potential of MSC-EVs is supported by preclinical studies showing the improvement of at least one clinical outcome associated with acute kidney/liver/lung injury, myocardial infarction, or hindlimb ischemia (Akyurekli et al., 2015). Furthermore, the literature shows that hBMSCs undergo osteogenic differentiation induced by EVs derived from monocytes (Ekstrom et al., 2013) or platelet lysate (Torreggiani et al., 2014). These data enable us to hypothesize that EVs may be vehicles of communication toward tissue regeneration. The knowledge on the bone regenerative potential of hBMSC-EVs is scarce. Therefore, the purpose of this study is to validate the functionality of hBMSC-EVs in the osteoinduction of hBMSCs. We hypothesized that if EVs mirror the content and fate of parent cells, then EVs derived from osteogenically committed hBMSCs will induce the osteogenic commitment of homotypic cells without further supplementation.

RESULTS

hBMSCs Exposed to Osteogenic Stimuli Secrete EVs during Culture

hBMSCs were induced into the osteogenic lineage over 7 days by continuous chemical stimuli provided by standard osteogenic medium (OM) (Jaiswal et al., 1997) or by a single genetic stimulus in basal medium (RUNX-2). We next isolated EVs from the CM of chemically and genetically induced hBMSCs (OM-EVs and RUNX2-EVs, respectively) at specific time points using polymeric precipitation.

EVs showed a polydisperse size distribution by dynamic light scattering (Figure 1A), with polydispersity indexes between 0.15 and 0.6 (min and max), independently of the culture conditions and time. The main peak corresponded to ≥65% of the population (65.2%–88%, 68.7%–73.5%, 76.2%–84%, min-max for OM-, RUNX2-, basal medium [BM]-EVs, respectively). Within this population, the diameter of EVs was very homogeneous (31.9–40.2 nm), irrespective of culture time or stimulus (Figure 1B). In contrast, the size of EVs within the second peak (Figure 1A) ranged between 45 and 348.6 nm. These findings were corroborated by atomic force microscopy, which showed a population of intact rounded structures with estimated desiccated diameters of 32, 40, and 24 nm (Figure 1C, a–c). On the other hand, as assessed by laser Doppler micro-electrophoresis, the surface charge of OM-, RUNX2-, and BM-EVs averaged at −5.2, −6.4, and −5.9 mV, respectively, and was similar within the different groups (Figure 1B).

To shed light into the biochemical profile of EVs, we first used flow cytometry (Figure 1D) to detect the tetraspanins CD9/63/81, expected to be enriched in EVs fractions (Lötvall et al., 2014). These analyses showed that EVs labeled for each of the markers show a distinct positive shift of the fluorescence signal beyond the non-labeled controls. Furthermore, the data suggested abundant CD81-positive and weak CD9-positive EVs populations, despite the culture conditions (Figure 1D). The presence of CD63-positive EVs was further confirmed using ELISA (Figure 1E).

In addition, we aimed at estimating the release of EVs during culture, based on the normalized number of CD63 particles and total EV protein (Figure 1E). An osteogenic stimulus-dependent release was observed, differing from that observed in control conditions. Specifically, while for OM cultured cells the release of EVs remained at a steady level over time, for RUNX2 stimulated cells the secretion of EVs, in terms of total protein, increased over time peaking from the fifth day of culture onward (day 3 versus 5 or versus 7, $p < 0.01$; Figure 1E).

Collectively, these results show that hBMSCs secrete populations of nanosized particles with physical and biochemical features of EVs, and the yield is regulated by the osteogenic stimulus provided.

hBMSCs Osteogenically Derived EVs Outperform Current Strategies to Elicit hBMSCs Osteogenic Commitment

To test our hypothesis, OM- and RUNX2-EVs were isolated at set time points during culture (Figure 1E) and used to sequentially feed uncommitted homotypic hBMSCs, matching seeding density, culture time, and stimuli without further supplementation. Therefore, the concentration of EVs added to culture mimicked the specific release of parent cells into the CM.

We first assessed whether EVs could interact and deliver nucleic acids to homotypic recipient cells. RUNX2-EVs isolated from 1-day cultured cells expressing *RUNX2-GFP* tag, were added to uncommitted hBMSCs. After 1 day, the *GFP* expression was on average 233-fold greater versus BM in cells exposed to RUNX2-EVs (6.8–803, min-max), although at lower levels than that observed for parent cells transfected by lipofection (average 1.6×10^6 fold change, 4×10^4–7×10^6, min-max). This suggests that EVs are able to interact with the recipient cells and transfer functional plasmid DNA.

The ability of OM- and RUNX2-EVs to promote the onset of osteogenesis was examined and compared with currently available strategies. The differentiation process is known to impair cell proliferation, due to an increase in the length of the cell cycle (Roccio et al., 2013), and to induce changes in the protein synthesis rate (Kristensen et al., 2013). Therefore, we evaluated the profile of recipient hBMSC proliferation (Figure 2A) and total protein (Figure 2B). Notably, a high seeding density was used to ensure high transfection efficiency. Under these conditions, the proliferation capacity of OM-EV-treated cells decreased after day 3 versus BM and OM (Figure 2A), in parallel with a net protein increase (day 5, versus BM) (Figure 2B). Furthermore, RUNX2-EV treatment did not impair cell proliferation versus BM, in contrast to parent cells (RUNX2), attaining proliferation levels higher than those of parent and OM-EV-exposed cells at the seventh day (Figure 2A), without major changes in total protein (Figure 2B).

The osteoinductive potential of EVs was then evaluated in terms of the expression of the extracellular matrix marker alkaline phosphatase (ALP) (Figure 2C). OM- and RUNX-2 EVs induced early activation of ALP, in contrast to that exhibited by the parent cells. Notably, this was observed in the absence of the mineralization inductors dexamethasone and β-glycerophosphate (Langenbach and Handschel, 2013). To better define the osteogenic commitment of these cells, the temporal gene expression of osteogenic markers was investigated (Figure 3). OM-EV-cultured hBMSCs showed early overexpression of the activator bone morphogenetic protein 2 (*BMP2*) (Figure 3C) by ~3- to 6-fold versus BM and OM, and a transient increase in the expression of the Sp7 transcription factor (osterix) (*SP7*) (Figure 3B) by 6-fold versus BM at day 3. Indeed, osteoblastic differentiation is determined by the overexpression of *SP7* (Sinha and Zhou, 2013) through the BMP-2 signaling cascade (Ulsamer et al., 2008). Herein, the data suggest early activation of the cascade independent of the upstream factor *RUNX2*. In addition, at early culture times, the downstream factors secreted phosphoprotein 1 (osteopontin) (*SPP1*) (Figure 3D) and