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Exosomes derived from mineralizing osteoblasts promote ST2 cell osteogenic differentiation by alteration of microRNA expression

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Mineralizing osteoblasts (MOBs) can release exosomes, although the functional significance remains unclear. In the present study, we demonstrate that exosomes derived from mineralizing pre-osteoblast MC3T3-E1 cells can promote bone marrow stromal cell (ST2) differentiation to osteoblasts. We reveal that MOB-derived exosomes significantly influence miRNA profiles in recipient ST2 cells, and these changes tend to activate the Wnt signaling pathway by inhibiting Axin1 expression and increasing β-catenin expression. We also suggest that MOB derived-exosomes partly induce the variation in miRNA expression in recipient ST2 cells by exosomal miRNA transfer. These findings suggest an exosome-mediated mode of cell-to-cell communication in the osteogenic microenvironment, and also indicate the potential of MOB exosomes in bone tissue engineering.

Keywords: cell-to-cell communication; exosomes; microRNA; osteoblast differentiation

The identification of key regulators of osteogenesis is of practical benefit in osteogenesis mechanism research and bone tissue engineering. Mesenchymal stromal stem cells are capable of differentiating into mineralizing osteoblasts (MOBs), which reside in a mineralizing extracellular matrix. MOBs exist in close proximity to the mesenchymal stromal stem cells in bone marrow; however, the role of MOBs in regulating the mesenchymal stromal stem cells is not well established. Several previous studies have demonstrated that co-culture with MOBs or their conditioned media could promote the osteogenesis of mesenchymal stromal stem cells *in vitro* [1–4]. These findings suggest the presence of soluble pro-osteogenic factors released by MOBs; however, the components of the soluble factors still remain unclear. The identification of these factors would help us better understand the mechanism of bone turnover *in vitro* and further improve the potential of bone tissue engineering.

Exosomes are small vesicles, sized 40–100 nm in diameter, derived from the membranes of multivesicular bodies and constitutively released by a wide range of cell types, including osteoblasts [5,6]. Exosome production is energy-dependent and enhanced by environmental stimuli, including differentiation induction. There is increasing evidence available to support exosomes functioning as cell-to-cell communicators in various developmental and biologic processes, as well as in disease progression [7,8]. These exosomes are enriched in bioactive molecules such as proteins,

Abbreviation
MOB, mineralizing osteoblast.

mRNAs, miRNAs and lipids from the cells of origin, which can be transferred between cells, and thus may impact the genetic content and function of recipient cells [9]. Among the exosome components, miRNAs attract much attention in exosome function research for their important regulatory roles. Increasing evidence indicates that exosome cargo miRNAs can impact the function of recipient cells under physiologic and pathologic conditions. Xu *et al.* [5] demonstrated that miRNAs are encapsulated in MOBs. Furthermore, MOBs have a different miRNA expression pattern from bone marrow-derived mesenchymal stem cells.

MOBs can secret pro-osteonenic factors. Although data are emerging related to the component of MOB-derived exosomes, the functional significance of these vesicles is still unknown. Therefore, we hypothesize that exosomes might be one of the pro-osteonenic factors released by MOBs to promote osteogenesis in recipient cells. The emerging evidence supports that exosome cargo miRNAs function as important regulators in recipient cells. Thus, the present study aimed to characterize the function of MOB-derived exosomes and their ability to mediate cellular osteoblast differentiation by miRNAs. We report that MOB-derived exosomes promote osteoblast differentiation when added to bone marrow-derived stromal cells, suggesting a potential amplification loop for enhancing osteogenesis. These data expand our knowledge of exosomes in osteogenesis and bone tissue engineering.

Materials and methods

Cell culture and osteogenesis induction

MC3T3-E1 mouse calvarial pre-osteoblasts were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Mouse bone marrow-derived stromal cell line ST2 was kindly provided by Professor Dongxu Liu (School of Stomatology, Shandong University, Jinan, China). Both MC3T3-E1 and ST2 cells were grown in Dulbecco's modified Eagle's medium with 10% exosome-depleted FBS. For osteogenesis, the medium was changed to an osteogenic medium, a cocktail of L-ascorbic acid, dexamethasone and β-glycerophosphoric acid (MK430; Takara Bio, Shiga, Japan) every 3 days.

Isolation and characterization of exosomes from mineralizing MC3T3-E1 cells (MOBs)

After osteogenesis induction of MC3T3-E1 cells for 21 days, media were first centrifuged at 300 ***g*** for 10 min, followed by centrifugation at 2000 ***g*** for 10 min and 10 000 ***g*** for 30 min. Then, supernatants containing exosomes were concentrated by centrifuging at 1000 ***g*** in a 100 kDa ultracentrifuge tube (Millipore, Bedford, MA, USA) for 30 min to a volume of between 0.5 and 0.8 mL. Then, exosomes were precipitated by adding an Exoquick reagent (System Biosciences, Mountain View, CA, USA) to the concentrated supernatant in accordance with the manufacturer's instructions. After precipitation overnight and centrifuging at 3000 ***g*** for 2 h, the supernatant was discarded, and the exosome pellets were re-suspended in PBS. Exosomes were quantified with a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Exosomes from mineralizing MC3T3-E1 cells were characterized by scanning electron microscopy. Total RNA was isolated from exosomes by using TRIzol (Invitrogen, Carlsbad, CA, USA) and detected by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

miRNA microarray analysis of MOB-derived exosomes

Total RNA was harvested from mineralizing MC3T3-E1 exosomes ($n = 2$) using a TRIzol and miRNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. For the low yield of exosomes RNA for microarray analysis, we first linearly amplified the total miRNA of exosomes using a Global microRNA amplification kit (System Bioscience) as described previously [10]. Amplified miRNA samples were then labeled using the miRCURY™ Hy3/Hy5 Power labeling kit and hybridized on the miRCURY™ LNA Array (version 18.0) (Exiqon, Vedbaek, Denmark). Following the washing steps, the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA). Scanned images were then imported into GENEPIX PRO, version 6.0 (Axon Instruments) for grid alignment and data extraction. miRNAs with a two-fold change in foreground signal intensity above background signal at least in one of the exosome samples were considered as expressed, and miRNAs expressed at 10-fold above background were considered as highly expressed.

Exosomes labeling and uptake by recipient cells

In total, 100 μg of exosomes derived from mineralizing MC3T3-E1 cells were labeled PKH-67 green fluorescent dye. After incubation for 5 min, exosomes were washed using 100 kDa filter (Microcon YM-100; Millipore) to remove excess dye. The labeled exosomes were then incubated with ST2 cells for 72 h. After incubation, ST2 cells were fixed with 4% para-formaldehyde for 10 min, stained with 4′,6-diamidino-2-phenylindole, and then cells were photographed using laser-scanning confocal microscope.

Real-time PCR quantification of osteogenic differentiation marker genes and Alizarin red staining

MOB-derived exosomes at different concentrations were added to ST2 cells (2×10^5 per 24 well). After 12 days of differentiation, the levels of RUNX2 and ALP mRNA were determined by real-time PCR assay, and cDNA was prepared from total RNA samples using TRIzol reagent and a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). Real-time PCR was performed (LightCycler 480 Thermocycler; Roche Applied Science, Mannheim, Germany) using a SYBR Green qPCR Kit (Toyobo) in accordance with an optimized protocol and primer sets: 5′-AAG TGC GGT GCA AAC TTT CT-3′ and 5′-TCT CGG TGG CTG GTA GTG A-3′ for Runx2; 5′-TGG CTC TGC CTT TAT TCC CTA GT-3′ and 5′-AAA TAA GGT GCT TTG GGA ATC TGT-3′ for ALP. After 21 days of differentiation, cells were fixed with ice-cold 70% ethanol and stained with Alizarin red to detect mineralization.

miRNA profiles of ST2 cells after transfer of MOB-derived exosomes by microarray and bioinformatic analysis

In total, 3 μg of MOB-derived exosomes were added to ST2 cells (2×10^5 per 24 well). After incubation for 72 h, RNA was extracted from ST2 cells using TRIzol, purified with RNeasy Mini Kit (Qiagen), and subsequently labeled a with FlashTag® Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA, USA) and hybridized to the GeneChip miRNA 1.0 Array (Affymetrix) containing 1255 mouse microRNAs in accordance with the manufacturer's instructions. Microarrays were scanned with a GeneChip Scanner 3000 G7 (Affymetrix), and extracted with GeneChip operating software (GCOS, version 1.4; Affymetrix).

Gene expression profile raw data were processed by RMA method. We performed the normalized unscaled standard error method to assess the quality of the arrays. Differently expressed genes between groups were analyzed by the Q value method (Q was set at 0.001 and fold as 1.5). Target genes of differently regulated miRNAs were analyzed in the context of gene ontology annotations and the KEGG database (http://www.genome.jp/kegg) to identify significantly affected functional categories related to osteoblast differentiation (both the P value and false discovery rate cut-off value < 0.001) and signaling pathway. The miRNA target genes interaction network and pathway relation networks were built to identify the 'hub' regulatory genes and pathways directly and systemically as described previously [11–13].

Western blot analysis

From each sample, a total of 40 μg of protein lysates was subjected to 12% SDS/PAGE gels and transferred to a poly(vinylidene difluoride) membrane. After 1 h of blocking, the membrane was incubated overnight anti-Axin1 antibody (dilution 1 : 1000; Proteintech, Chicago, IL, USA), anti-β-catenin (dilution 1 : 1000; Proteintech) and anti-β-actin (dilution 1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by enhanced chemoluminescence detection. The western blots were imaged with enhanced chemiluminescence (Millipore) and exposed to X-ray film.

Statistical analysis

Quantitative data are reported as the mean ± SD and statistical significance was analyzed using Student's t-test for experiments involving two groups. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the PRISM, version 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Identification and miRNA profiles of exosomes derived from the mineralizing MC3T3-E1 osteoblasts

Exosomes were isolated from the conditioned media of cells (after 21 days of osteogenesis induction) using Exoquick, a widely used polymeric exosome precipitation reagent. Electron microscopy images confirmed that the exosome preparations were enriched from 40 nm to 200 nm in diameter. To determine the exosomal miRNA content of MOB-derived exosomes, we analyzed the miRNA expression profiles by microarray technology (Fig. 1). Among the 1135 microRNA investigated, 547 miRNAs were defined as expressed in MOB-derived exosomes, and 43 miRNAs, including some functionally confirmed Osteo-miRNAs (miR-1192, miR-680 and miR-302a), were highly expressed (more than 10-fold signal over background).

MOB-derived exosomes can be taken up by the recipient ST2 cells and promote its osteoblast differentiation

To tested the functionality of MOB-derived exosomes in intercellular communication, we used mouse bone marrow-derived stromal cell line ST2 as the recipient cells. ST2 cells could differentiate into mature osteoblasts that produce extracellular matrix and deposit calcium when stimulated with L-ascorbic acid, dexamethasone and β-glycerophosphoric acid. We observed that MOB-derived exosomes were significantly incorporated into recipient ST2 cells after 3 days of co-

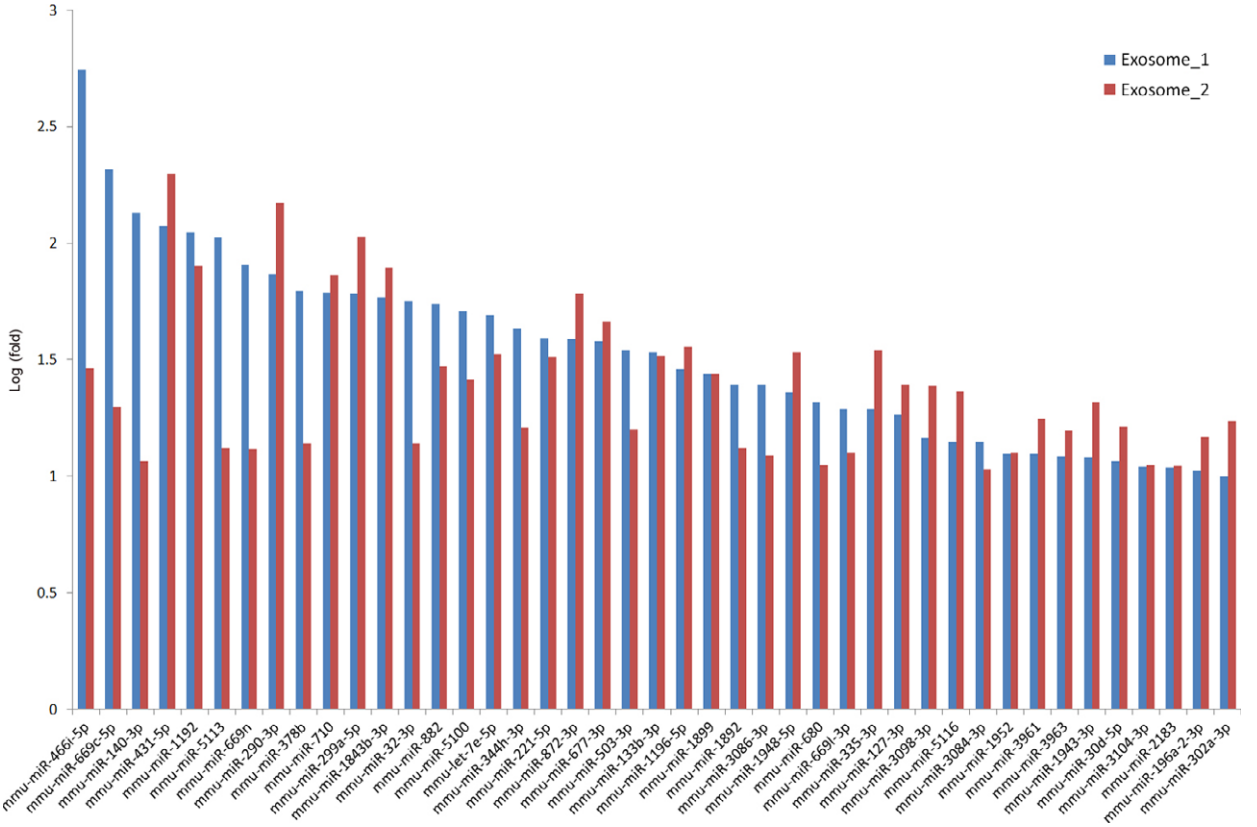

Fig. 1. Characterization of miRNA components in mineralized MC3T3-E1 exosomes. Forty-three miRNAs were highly expressed in mineralized MC3T3-E1 exosomes.

culture (Fig. 2A). Then, we examined the effect of MOB-derived exosomes on ST2 cell osteoblast differentiation. As shown in Fig. 2B,C, mineralizing MC3T3-E1 exosomes had a significant promotion effect on ST2 cell osteogenic differentiation, as manifested by up-regulated expression of osteogenic marker genes RUNX2 and ALP, as well as enhanced matrix mineralization. Furthermore, this promotion effect of MC3T3-E1 on osteogenesis was dose-dependent.

Mineralizing MC3T3-E1 exosomes altered the miRNA expression of recipient ST2 cells, which tended to activated Wnt/β-catenin pathway in ST2 cells

We investigated the impact of MOB-derived exosomes on intracellular miRNA concentrations of ST2 cells. The results shown in Fig. 3A and Table S1 demonstrate that 91 miRNAs were up-regulated and 182 miRNAs were down-regulated in ST2 cells after co-culture of MOB-derived exosomes. Among the 91 overexpressed miRNAs, 18 were detected in MOB-derived exosomes, including four highly expressed miRNAs (miR-3084-3p, miR-680, miR-677-3p and miR-5100).

We then *in silico* analyzed the miRNA targeted gene and pathway networks induced by MOBs exosomes. Gene ontology and pathway network analyses revealed that the targets of varied miRNA profiles were significantly enriched in osteoblast differentiation associated biological process (Table S2). These altered miRNAs cooperatively regulated a network of important pathways that play pivotal roles in the differentiation (Wnt signaling pathway, insulin signaling pathway and TGF-β signaling pathway) and function (calcium signaling pathway) of osteoblasts (Fig. 3B).

Multiple signaling pathways can converge on transcription factors to regulate osteoblast differentiation. We next performed a gene interaction analysis on the altered miRNAs and found that the β-catenin encoding gene *Ctnnb1* was up-regulated and serves as a centred hub gene within this miRNA target gene network (Fig. 3C).

We next analysized the co-target of up-regulated miRNAs induced by MOB exosomes, and found that five up-regulated miRNAs (miR-667-3p, miR-6769b-5p,

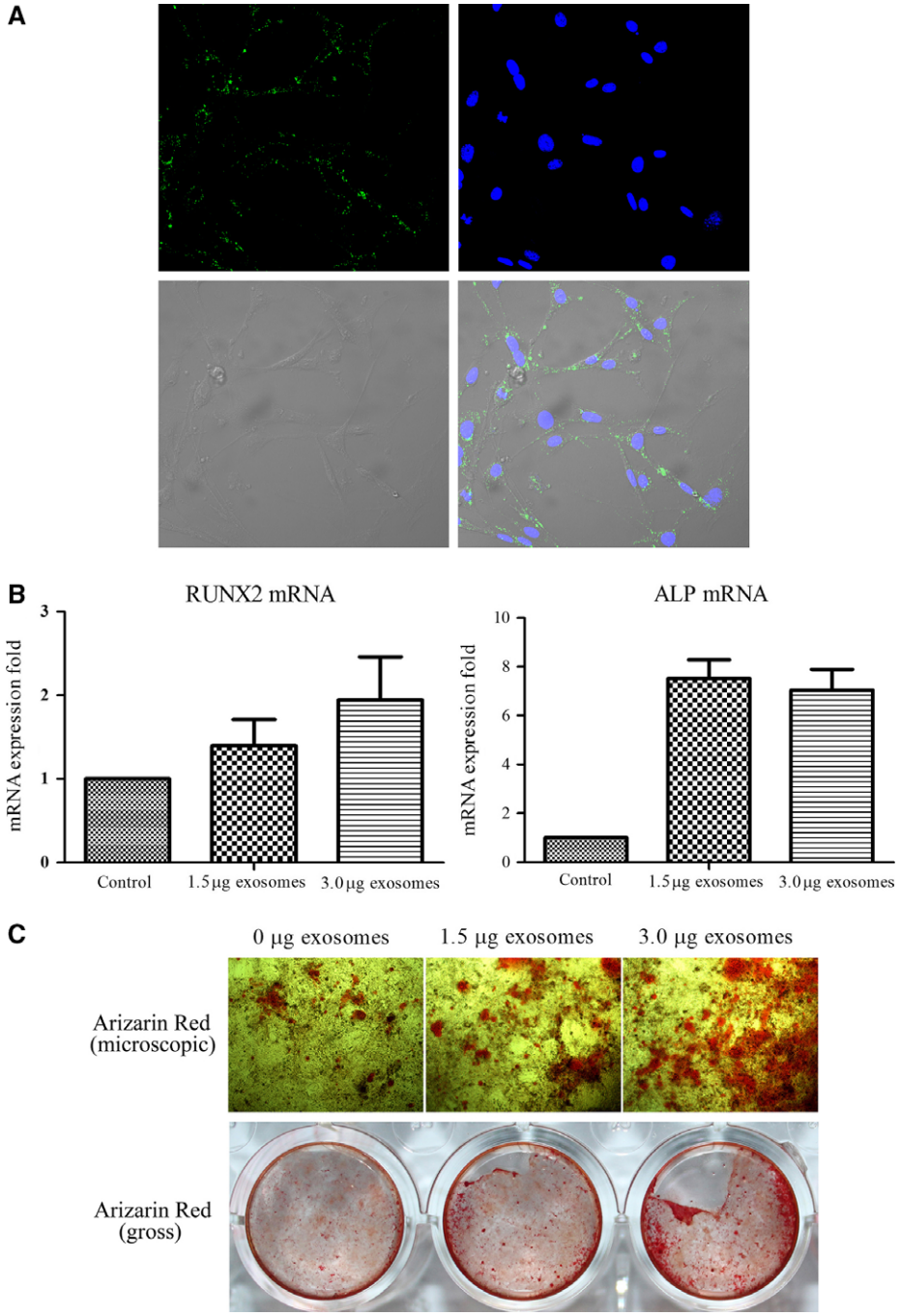

Fig. 2. Exosomes derived from mineralized MC3T3-E1 osteoblasts (MOB exosomes) that entered ST2 stromal cells and promoted osteoblast differention. Labeling for MOB exosomes entering targeted ST cells: green, PKH-67-labeled exosomes; blue, 4′,6-diamidino-2-phenylindole-labeled nuclei (A). MOB exosomes can promote bone morrow stromal ST2 cells towards osteoblast differentiation as manifested by the up-regulation of differentiation markers RUNX2 and ALP detected by real-time PCR (B) and enhanced matrix mineralization (C) in a dose-dependent manner.

miR-7044-5p, miR-7668-3p and miR-874-3p) co-target Axin1, an important negative regulator of Wnt sign aling pathway. As shown in Fig. 3D, we confirmed that Axin1 expression was repressed and β-catenin expression was enhanced after transfer of MOB exosomes. These findings suggest that MOB-derived

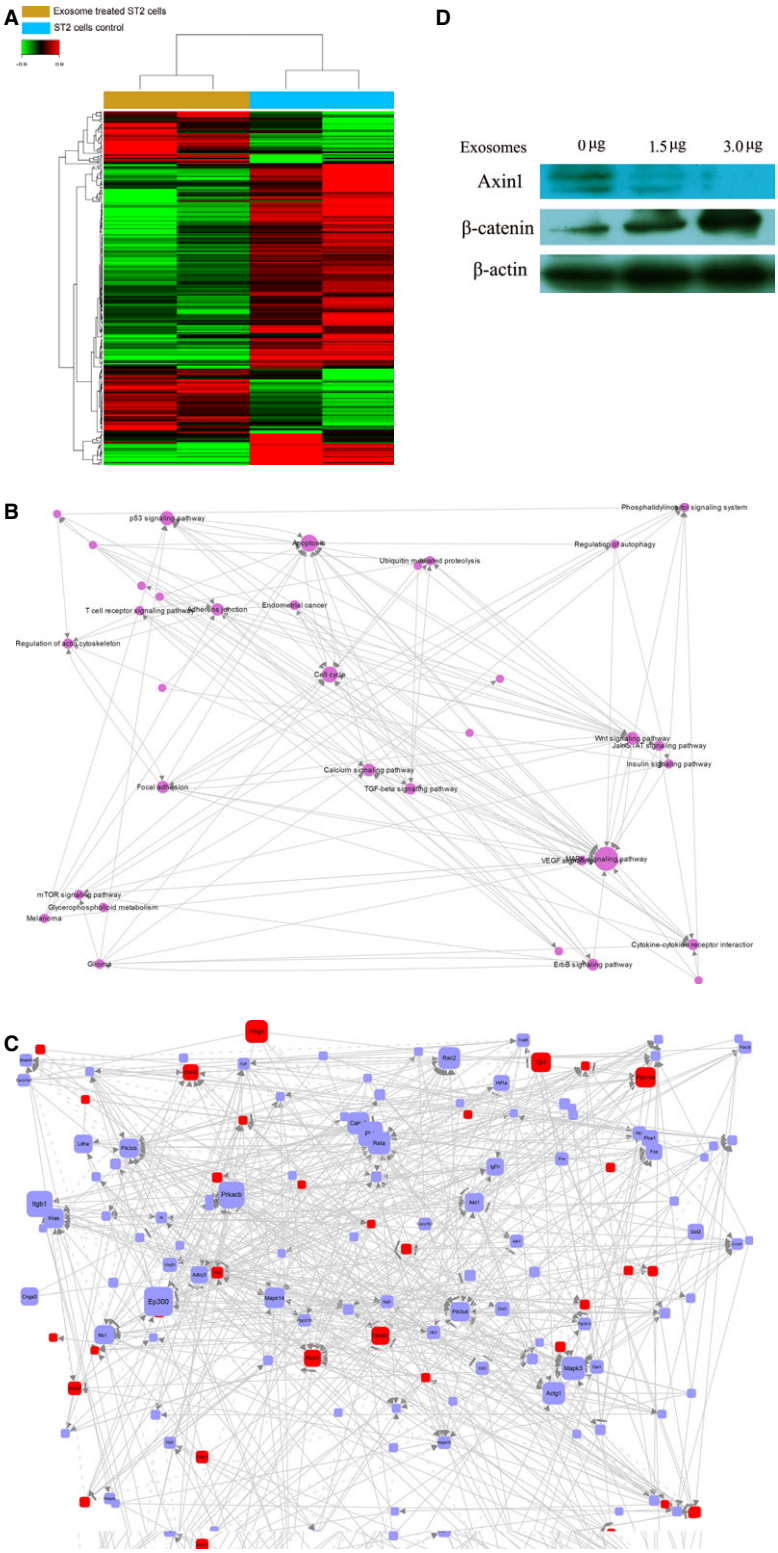

Fig. 3. Mechanism of mineralized osteoblast derived exosomes (MOB exosomes) in promoting recipient ST2 cell osteogenesis. (A) Microarray and clustering analyses on the miRNA expression profiles of ST2 cells with and without transfer of MOB exosomes. (B) Pathway network of miRNA targets. (C) miRNA target genes interaction network. (D) Axin1 expression was inhibited and β-catenin expression was increased in ST2 cells after transfer of MOB exosomes.

exosomes probably promoted ST2 cell osteogenic differentiation via the Wnt signaling pathway by affecting Axin1 and β-catenin expression.

Discussion

MOB-derived exosomes have been described previously [5]; however, whether or how these vesicles participate in osteogenesis remains to be clarified. In the present study, we showed that exosomes released from MOBs can enter into bone marrow stromal cells and accelerate osteoblast differentiation in the recipient cells. Thus, this reflects MOB-derived exosomes functioning in line with their parent cells.

Conditioned media from MOBs have previously been described to have a pro-osteogenic effect, which is probably a result of osteoblast-secreted enzymes or growth factors [1–4]. Our results suggest that, except for secreted growth factors and cytokines, the exosomes may also account for the pro-osteogenic effect of the conditioned media from MOBs. Therefore, within the local bone marrow microenvironment, there appears to be an exosome-mediated mode of communication between stromal stem cells and MOBs. After bone marrow stromal stem cells differentiate into MOBs, MOBs in turn induce stromal stem cells towards osteoblast differentiation by exosomes, thus forming a positive-feedback loop mechanism during osteogenesis.

Among the components of exosomes, miRNAs attract more attention because of their pivotal role in gene expression regulation, and it is estimated that > 30% of protein coding genes are predicted to be regulated by miRNAs. Many studies suggest that miRNA transfer is an important mechanism by which exosomes function [14–16]. In the present study, we found a significant alteration of miRNA profiling in recipient ST2 cells after transfer of MOB-derived exosomes. We then predicted that alteration of these miRNAs may affect multiple important pathways regulating osteoblast differentiation and function, such as the Wnt signaling pathway, insulin signaling pathway, TGF-β signaling pathway and calcium signaling pathway. We further identified that this exosome-mediated gene and pathway network converge on β-catenin encoding gene *Ctnnb1*, an essential transcription factor for osteoblast differentiation. Furthermore, the altered microRNAs in ST2 cells are predicted to cooperatively inhibit one key β-catenin inhibitor Axin1 [17]. As expected mechanically, we demonstrated that MOB-derived exosomes inhibited Axin1 expression and increased β-catenin expression in ST2 cells. Thus, our results indicate that the pro-osteogenetic effect of MOB-derived exosomes may rely on variation with respect to miRNA expression profiles in recipient ST2 cells, and these altered miRNAs cooperatively inhibit Axin1 expression and subsequently up-regulate β-catenin, resulting in the stimulation of osteogenic differentiation.

Exosome cargo miRNAs transfer can alter recipient cell functions by inhibiting exosome RNA targets and it is hypothesized that miRNA expression of the recipient cells might be changed directly by exosome miRNA transfer. However, in a recent quantitative and stoichiometric analysis of the microRNA content of exosomes, Chevillet *et al.* [18] showed that even abundant miRNAs are present at far less than one copy per exosome. In the present study, we found that miRNA alteration in the recipient and miRNA abundance in the donor exosomes are not well matched. Approximately 20% of up-regulated miRNAs in recipient ST2 cells were contained in MOBs exosomes, suggesting that miRNA transfer for MOB exosomes only contributes partly to the altered miRNA expression in recipient ST2 cells. Other MOB exosome components besides miRNAs, such as proteins and lipids, may also contribute to the alteration of miRNA profile changes in recipient ST2 cells.

Interestingly, we also identified many miRNAs that were down-regulated by MOB exosomes. We analyzed the target pathways of these down-regulated miRNAs and found that their co-target genes are enriched in the insulin signaling pathway and also the mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt pathways, which play pivotal roles in osteoblast differentiation. This finding suggests that transfer of exosomes can also inhibit a panel of miRNAs and subsequently activate several important osteogenesis pathways in recipient cells. The reduction cannot be explained by miRNA transfer from exosomes, although it may be the result of a response to the exosome and its enclosed RNA, protein and lipids; however, the exact mechanisms remain to be clarified.

In summary, we have demonstrated *in vitro* that MOB exosomes are capable of entering into bone marrow stromal cells and promoting them toward osteoblast differentiation and that this effect is partly a result of the up-regulation of β-catenin by miRNA alliteration in recipient cells. These findings not only help us to better understand the significance of osteoblast exosomes, but also emphasize a positive-feedback loop mechanism between MOBs and stromal stem cells during osteogenesis. Furthermore, given that the use of exosomes is being studied as a potential therapeutic strategy, the possible utility of exosomes as a pro-osteogenetic factor in bone tissue engineering is also worthy of further evaluation.

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Author contributions

Yazhou Cui and Jinxiang Han were responsible for the study design and writing of the manuscript. Yazhou Cui and Jing Luan were responsible for most of the experiments and the data analysis. Haiying Li and Xiaoyan Zhou participated in the cell culture and western blot experiments.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:
Table S1. Differently expressed miRNAs with and without transfer of mineralized osteoblast derived exosomes.
Table S2. Gene ontology analysis of exosome miRNA target genes.