

Supplementary Material

Exosomes Derived from Mucoperiosteum Krt14⁺Ctsk⁺ Cells Promote Bone Regeneration by Coupling Enhanced Osteogenesis and Angiogenesis

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






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1 Author Contributions

Conceptualization, B.X., and N.N.; Methodology, H.R. and Z.D.; Software, Z.R., and H.R.; Validation, Z.R., X.Y., and G.L.; Formal Analysis, Z.R., and S.Y.; Investigation, Z.R. and X.Y.; Resources, S.W., S.J, and G.P.; Data Curation, Z.R., and C.X.; Writing – Original Draft, Z.R.; Writing – Review & Editing, N.N., H.R., and B.X.; Visualization, Z.R.; Supervision, B.X. and G.P.; Project Administration, B.X. and G.P.; Funding Acquisition, B.X.

We also provided some detailed evidence of the contributions of each author below.

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 Draft 3.12	2024/3/13 12:49	DOCX 文档	3,513 KB
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 Draft 12.27	2023/12/27 9:49	DOCX 文档	3,514 KB
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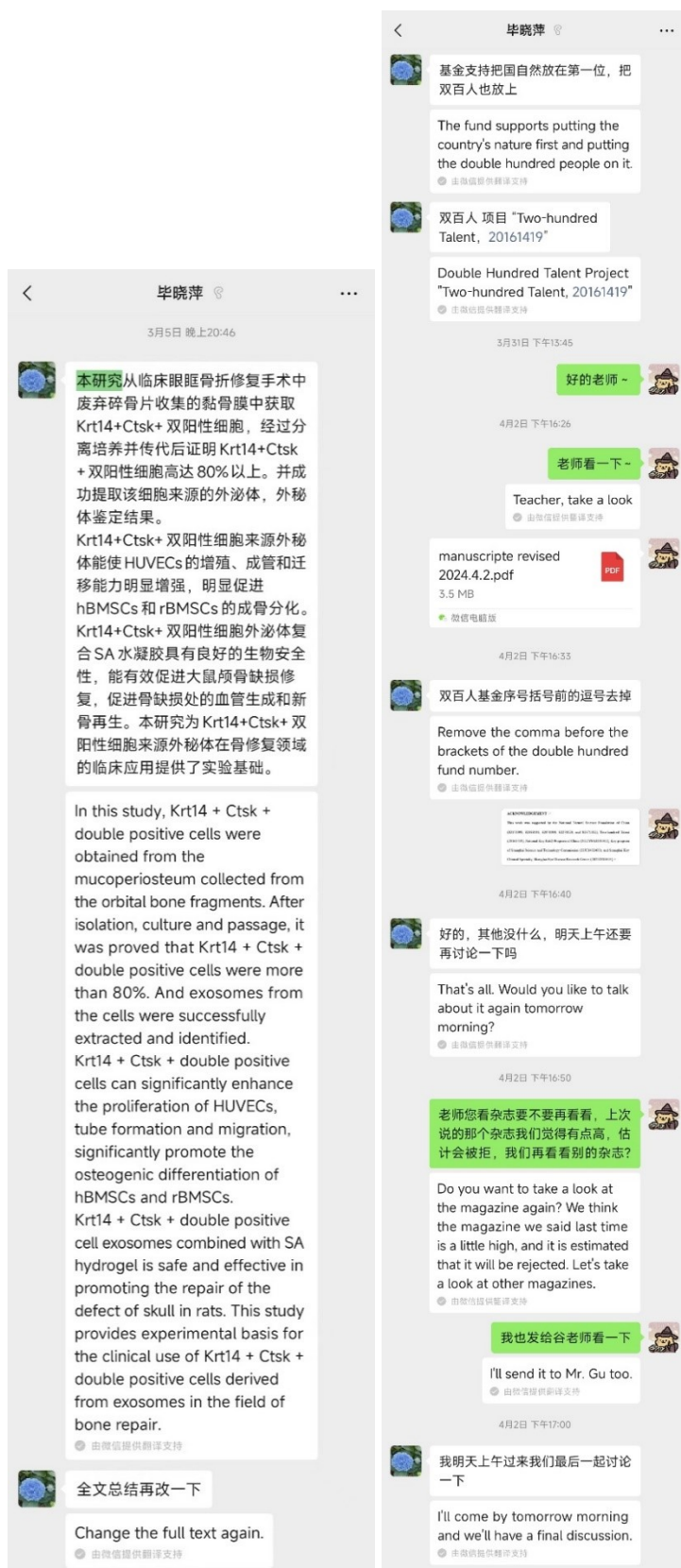
These are different versions of the manuscript written or revised by **Rong Zhou, Rui Huang, and Ni Ni**.

different cells on tissue repair may vary due to diverse genomic or epigenomic profiles ^[21] . For example, exosomes from MSCs of different tissue origins are found to exert distinct effects on neurite outgrowth in neurons ^[21] . In terms of osteogenesis, <i>huc</i> MSC-derived exosomes probably promote the repair of fractures in rats via the <i>Wnt</i> signaling pathway ^[21] . On the contrary, osteoclast-derived exosomes selectively inhibit osteoblast activity ^[26] . These findings underscore the importance of considering cell source and exosome composition when exploring their therapeutic potential. Besides, exosomes derived from both BMSCs and ADSCs facilitate osteogenesis of BMSCs, while BMSCs-exosome exhibit stronger bone regeneration capabilities ^[21] . This phenomenon indicated that exosomes derived from cells with osteogenic potential facilitate osteogenesis, and the extent of osteogenic facilitation varies according to pro-osteogenic ability. ⁴⁷	Rui Huang 翻译了: E Rui Huang 翻译了: could Rui Huang 翻译了: implying Rui Huang 翻译了: through Rui Huang 翻译了: with the osteogenic potential of the cell source Rui Huang 翻译了: pivotal for intramembranous bone formation Zhen Sherry 翻译了: in spite of Zhen Sherry 翻译了: (https://www.nature.com/articles/s41467-018-03124-1) (原文英文链接) Rui Huang 翻译了: Krt14 ⁺ Ctik ⁺ cells present high proliferation and osteogenic capacities Rui Huang 翻译了: Notably, Ctk ⁺ cells exhibit superior osteogenic potential when compared to BMSCs ^[21] .
Periosteal stem cells are stem cells derived from local periosteum that are formed during development by mesenchymal cells and are currently demonstrated to be specifically labeled with Mx1 ⁺ αSMA ⁺ , Prx1, or Ctk ⁺ ^[28-30] , possessing the intrinsic capability to proliferate, self-renew, and differentiate into osteogenic cells. Despite direct osteogenic differentiation, these skeletal stem/progenitor cells have been proved to secrete osteogenic factors during bone healing procedure ^[29,30] . Recently, Weng and colleagues established a maxillary sinus floor lifting (MSFL) murine model, by which they identified a novel Krt14 ⁺ Ctk ⁺ subset of cells that display both epithelial and mesenchymal properties, along with the transcriptional profile of osteoprogenitors. Using dual recombinase-mediated lineage tracing and loss-of-function analyses, they revealed that Krt14 ⁺ Ctk ⁺ cells contribute significantly to both MSFL-induced osteogenesis and physiological bone homeostasis ^[31] . Although Krt14 ⁺ Ctk ⁺ cells are proved to participate in bone repair, it's still obscure whether paracrine patterns (particularly exosomes) play an role in the process. ⁴⁷	Rui Huang 翻译了: c Rui Huang 翻译了: c
In this study, we capitalized on the osteogenic properties of Krt14 ⁺ Ctk ⁺ cells by extracting their exosomes. In cell experiments, we found that these exosomes significantly enhanced angiogenic differentiation of human umbilical vein endothelial cells (HUEVCs) and osteogenic differentiation of BMSCs. As for <i>in vivo</i> experiments, hydrogels loaded with the exosomes were	
Combining <i>in vivo</i> with <i>in vitro</i> experiments, it can be concluded that exosomes promote the formation of new blood vessels in the defect area by enhancing the capabilities of ECs to proliferate, migrate, and tube formation and stimulating secretion of vascular factors. For osteogenesis, exosomes promote bone regeneration and repair of bone defects by modulating the osteogenic differentiation of stem or progenitor cells. These findings may provide plausible explanation for Krt14 ⁺ Ctk ⁺ cells contributing to osteogenesis and physiological bone homeostasis as a coordinator. Since Krt14 ⁺ Ctk ⁺ cells can be easily amplified, it is possible to harvest large quantities of pro-osteogenic and pro-angiogenic exosomes for bone repair. This study enhances our understanding of the role of mucoperiosteal progenitor cells in craniofacial bone repair and proposes a new strategy for efficient bone repair in skeletal diseases. ⁴⁷	Zhen Sherry 翻译了: reveal Rui Huang 翻译了: with low toxicity Zhen Sherry 翻译了: Krt14 ⁺ Ctk ⁺ Rui Huang 翻译了: 格式: 字母-小四-加粗 Rui Huang 设置格式: 缩进: 前行缩进 0 厘米 Zhen Sherry 设置格式: 缩进: 前行缩进 2 字符 Rui Huang 设置格式: 字母-斜体 Zhen Sherry 翻译了: umbilical vein endothelial cells (
Conclusions ⁴⁷	Zhen Sherry 翻译了: 斜体 Rui Huang 设置格式: 字母-斜体 Zhen Sherry 翻译了: Krt14 ⁺ Ctk ⁺ Zhen Sherry 翻译了: Krt14 ⁺ Ctk ⁺ Rui Huang 翻译了: In conclusion, the Krt14 ⁺ Ctk ⁺ cell-derived exosomes played a positive and effective role in bone repair.
Materials and Methods ⁴⁷	

These are several paragraphs revised by Rui Huang and Rong Zhou.

Rat Cranial Defect ⁴⁷	Rui Huang 设置格式: 上标 Zhen Sherry 翻译了: sinus mucosa Zhen Sherry 翻译了: mucosal Zhen Sherry 翻译了: (如参考文献)
Scheme ⁴⁷	Rui Huang 翻译了: Then we isolated this population of cells from the sinus mucosa. Not surprisingly, immunocytochemistry of isolated cells after incubating and passaging to P3 showed 91.81% ± 1.13% of the cells we focused on were Krt14 ⁺ Ctk ⁺ . Rui Huang 翻译了: Then we extracted exosomes from this group of cells. The exosomes were characterized by TEM, NTA, and western blot. TEM provided the morphology of the exosomes, the cup- or round-shaped vesicles of double-concave and double-layer membrane. NTA showed the size distribution of exosomes, the average diameter of which was 185.3 nm. The specific surface markers including CD63 and TRG101 were detected by western blot. Zhen Sherry 设置格式: 缩进: 前行缩进 2 字符 Zhen Sherry 翻译了: group
The mucoperiosteum was collected from adjacent the orbital bone, after being digested and cultured, Krt14 ⁺ Ctk ⁺ cells were obtained. The exosomes were extracted from the conditioned medium of the Krt14 ⁺ Ctk ⁺ cells by ultracentrifuge. The exosomes capuled with hydrogel were implanted in the rat cranial defect to facilitate bone regeneration, in early stage of which blood vessel formation promoted by means of proliferation, migration and tube formation of vascular endothelial cells, while osteogenesis of BMSCs was also enhanced. ⁴⁷	
Results ⁴⁷	
The isolation and characterization of exosomes derived from Krt14⁺Ctk⁺ cells ⁴⁷	
During MSFL surgery and bone homeostasis, a specific cluster of Krt14 ⁺ Ctk ⁺ cells demonstrated osteogenic potential. To precisely identify and localize these cells, we conducted immunofluorescence staining on both tissue sections and cultured cells. Our findings from the immunofluorescence staining of the mucoperiosteum histological section revealed that a subset of the cells exhibited positivity for both Krt14 and Ctk, aligning with previous reports in the literature ^[28] . As anticipated, immunocytochemistry of the isolated cells after incubation and passaging to P3 confirmed that a remarkable 91.81% ± 1.13% of the cells targeted expressed both Krt14 and Ctk markers, further validating their unique identity. ⁴⁷	
After successfully isolating this particular cell population, we proceeded with the identification of exosomes. These exosomes underwent comprehensive characterization utilizing transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and western blot. TEM imaging revealed the distinctive morphology of the exosomes, presenting	
However, the implications of exosomes derived from different cells on tissue repair may vary due to the inherent differences in the cells' capabilities ^[21] . For example, exosomes from MSCs of different tissue origins have been found to exert distinct effects on neurite outgrowth in neurons ^[21] . Furthermore, exosomes from <i>huc</i> rat MSCs show reduced bone healing effects due to the enrichment of miR-128-3p, which inhibits SMAD ^[21] . These findings underscore the importance of considering cell source and exosome composition when exploring their therapeutic potential. ⁴⁷	Zhen Sherry 设置格式: 带格式 Zhen Sherry 设置格式: 带格式 Rui Huang 翻译了: 带格式 Rui Huang 设置格式: 带格式 Zhen Sherry 设置格式: 带格式 Zhen Sherry 设置格式: 带格式 Rui Huang 翻译了: Generally, engineered Rui Huang 翻译了: 带格式 Zhen Sherry 翻译了: α-SMA + Mx1 Zhen Sherry 设置格式: 带格式 Zhen Sherry 翻译了: and Zhen Sherry 设置格式: 带格式 Rui Huang 翻译了: Moreover Zhen Sherry 设置格式: 带格式 Rui Huang 翻译了: et al. and colleagues Zhen Sherry 翻译了: 带格式 Rui Huang 翻译了: features of Zhen Sherry 翻译了: c
Periosteal stem cells are stem cells derived from local periosteum that are formed during development by mesenchymal cells and are currently demonstrated to be specifically labeled with Mx1 ⁺ αSMA ⁺ , Prx1, or Ctk ⁺ ^[28-30] , possessing the intrinsic capability to proliferate, self-renew, and differentiate into osteogenic cells, pivotal for intramembranous bone formation. Notably, Ctk ⁺ cells exhibit superior osteogenic potential when compared to BMSCs ^[21] . Recently, Weng and colleagues established a maxillary sinus floor lifting (MSFL) murine model, by which they identified a novel Krt14 ⁺ Ctk ⁺ subset of cells that display both epithelial and mesenchymal properties, along with the transcriptional profile of osteoprogenitors. Using dual recombinase-mediated lineage tracing and loss-of-function analyses, they revealed that Krt14 ⁺ Ctk ⁺ cells contribute significantly to both MSFL-induced osteogenesis and physiological bone homeostasis. Krt14 ⁺ Ctk ⁺ cells exhibit high proliferation and osteogenic capacities ^[31] . However, the potential role of exosomes extracted from Krt14 ⁺ Ctk ⁺ cells in bone regeneration remains unexplored. ⁴⁷	
In this study, we capitalized on the unique properties of Krt14 ⁺ Ctk ⁺ cells by extracting their exosomes for use in promoting angiogenesis and osteogenesis <i>in vitro</i> . As for experiments <i>in vivo</i> , hydrogel loaded with the exosomes was transplanted to the cranial defect of rats. The biocompatible hydrogel not only provided mechanical support to the defect site but also facilitated vascularization and bone tissue growth through its porous structure ^[31] . We evaluate	

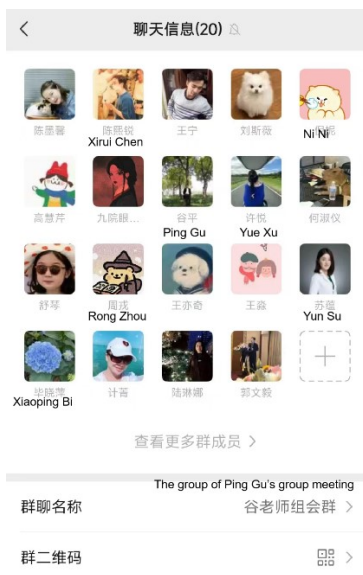
These are several paragraphs revised by Ni Ni and Rong Zhou.



These are the chat application messages of Xiaoping Bi and Rong Zhou, showing the revision of the manuscript and the foundation of the research.



These are chat application messages between **Yue Xu** and **Rong Zhou**, showing the discussion about checking the manuscript. **Yue Xu** also contributed to the animal experiments.



This is the chat group of **Ping Gu**'s group meeting, where **Ping Gu**, **Xiaoping Bi**, **Ni Ni**, **Yun Su**, **Yue Xu**, **Xirui Chen**, and **Rong Zhou** had discussions about the manuscript or figures of the research every Friday. **Ping Gu** is engaged in the research fields of bone repair, contributing to supervision and project administration. **Yun Su** also contributed to the formal analysis. **Xirui Chen** also contributed to the data curation.



These are chat application messages between **Dandan Zhang** and **Rong Zhou**, showing the discussion of the design of the research. **Dandan Zhang** also provided help about methodology.



These are chat application messages between **Li Gu** and **Rong Zhou**, showing the discussion about animal experiments.



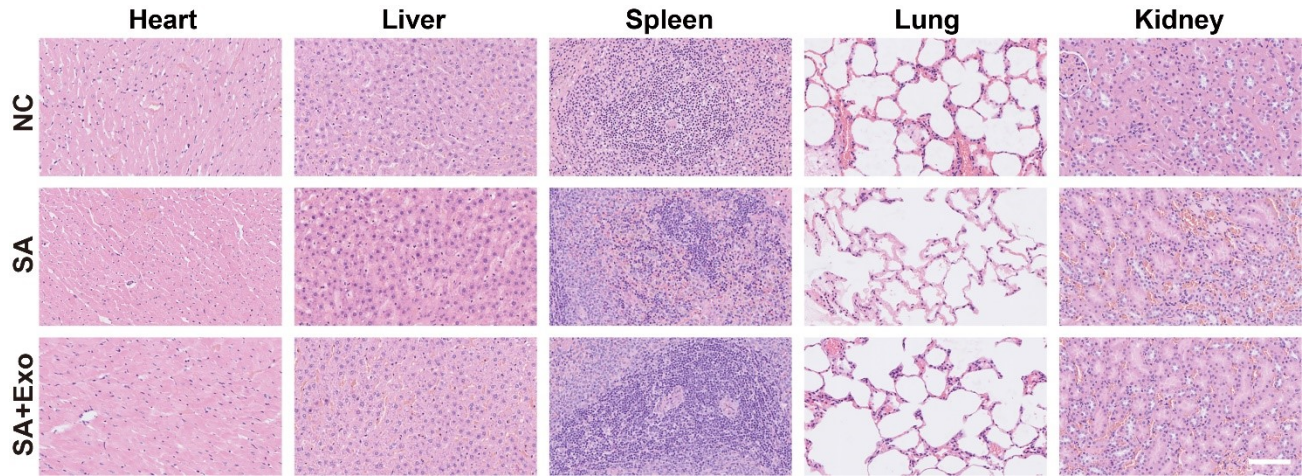
These are chat application messages between **Wodong Shi** and **Rong Zhou**, showing the discussion about the patient samples.



These are chat application messages between **Jing Sun** and **Rong Zhou**, showing the discussion about the patient samples.

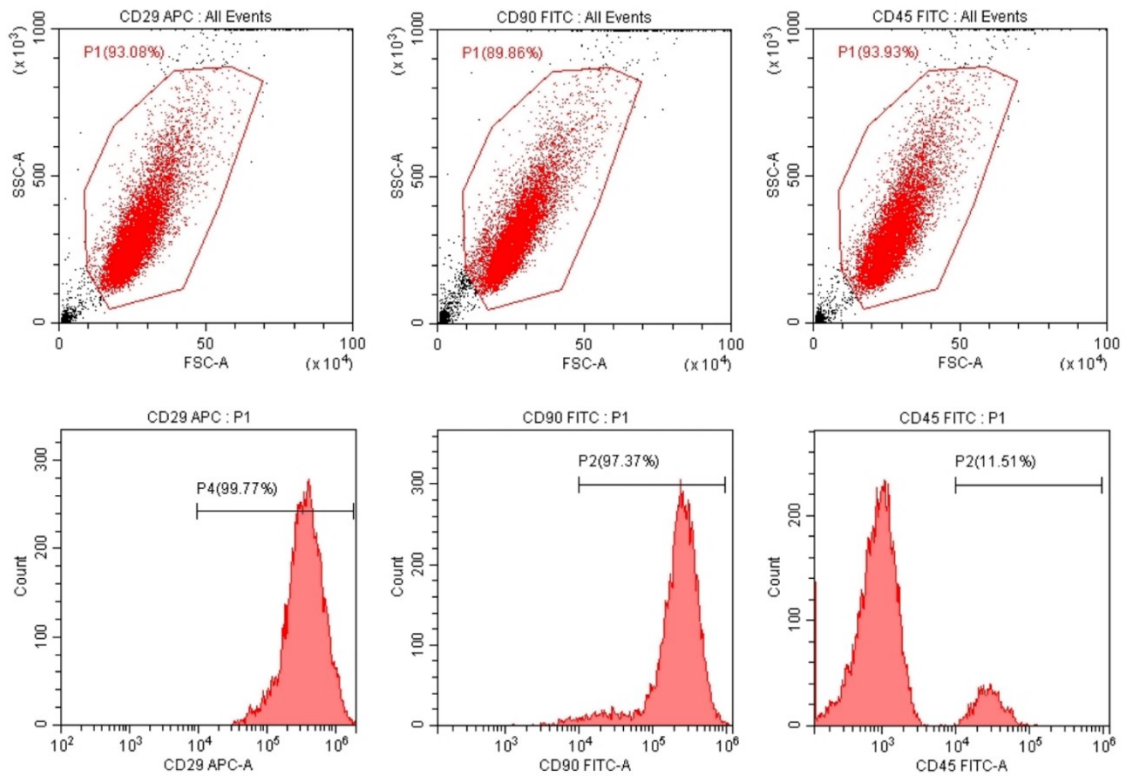
2 Supplementary Figures and Tables

2.1 Supplementary Figure 1



Supplementary Figure 1. H&E staining in the main organs. Scale bar = 100 μ m.

2.2 Supplementary Figure 2



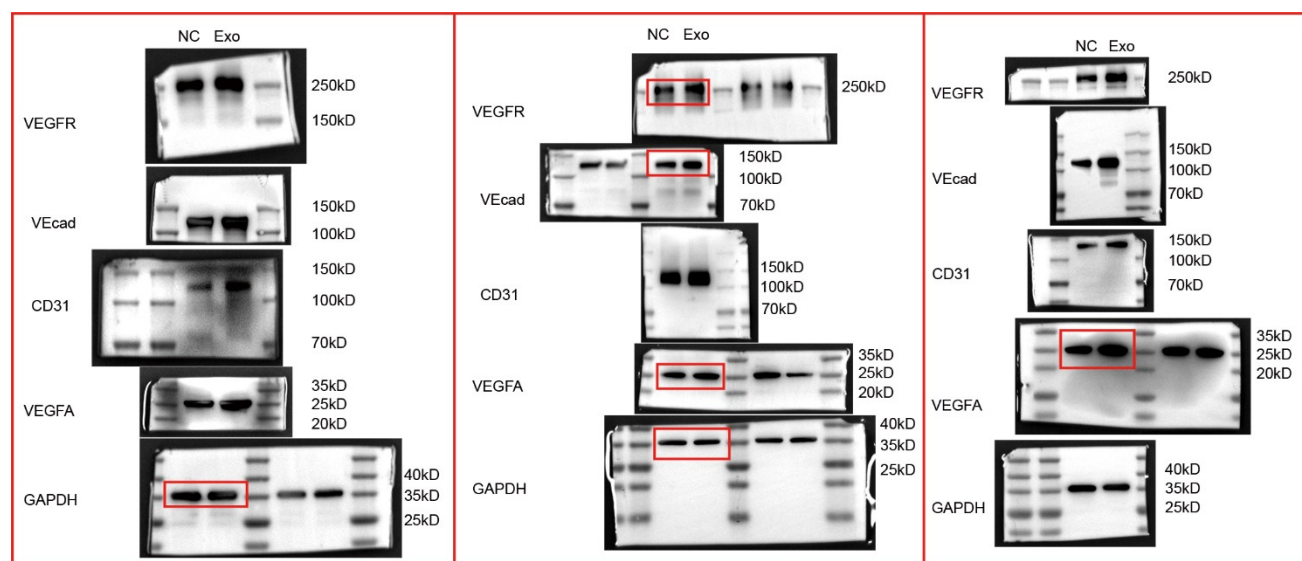
Supplementary Figure 2. The flow cytometry of rBMSCs including CD29, CD90, and CD45.

2.3 Supplementary Table 1

Supplementary Table 1. Primer sequences used in PCR assays.

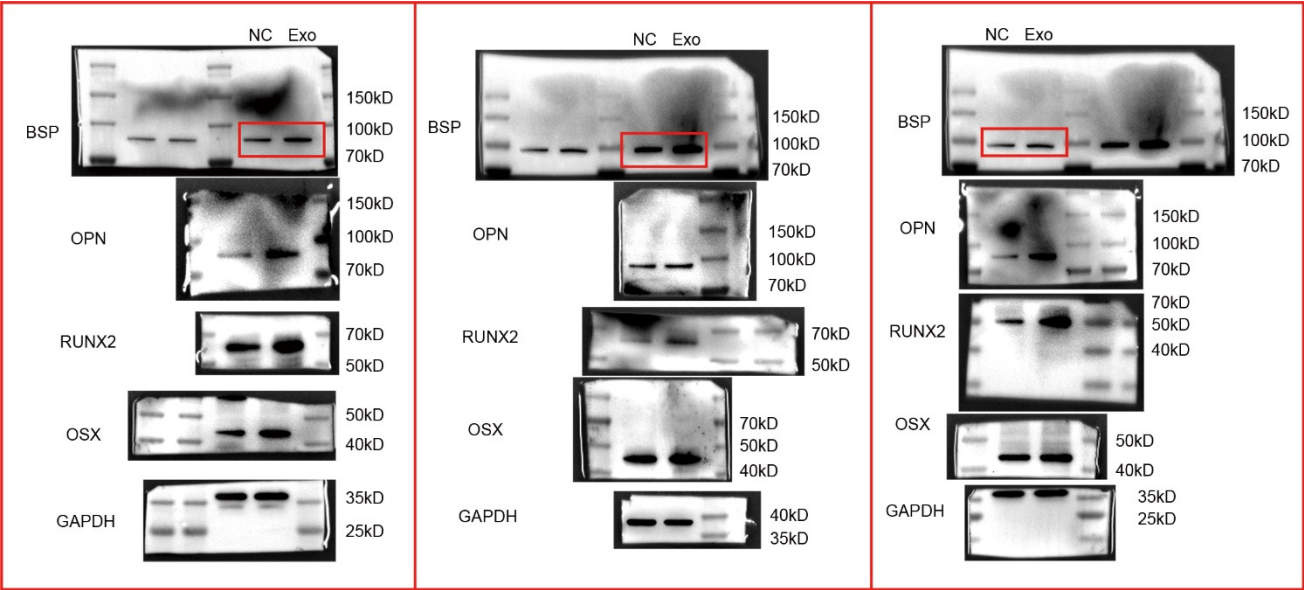
Gene name	F Primer Sequence	R Primer Sequence
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
BSP	CGATTTCAGTTCAGGGCAGTA	CGATTTCAGTTCAGGGCAGTA
OPN	GCAGCTTTACAACAAATACCCAG	CTTACTTGGAAGGGTCTGTGGG
OSX	CCTGAGTGGAACAGGAGTGGA	CCTGAGTGGAACAGGAGTGGA
RUNX2	GCGGTGCAAACCTTTCTCCAG	TGCTTGCAGCCTTAAATGACTC
VEGFA	CAAATGTGAATGCAGACCAAAGA	ATTAGACAGCAGCGGGCAC
CD31	CAGACGTGCAGTACACGGAA	GGAGCCTTCCGTTCTAGAGTATC
VEcadherin	CCCACAGGCACGATCTGTT	TGCCTACATGATGGGGAAGTG

3.1 Western blot



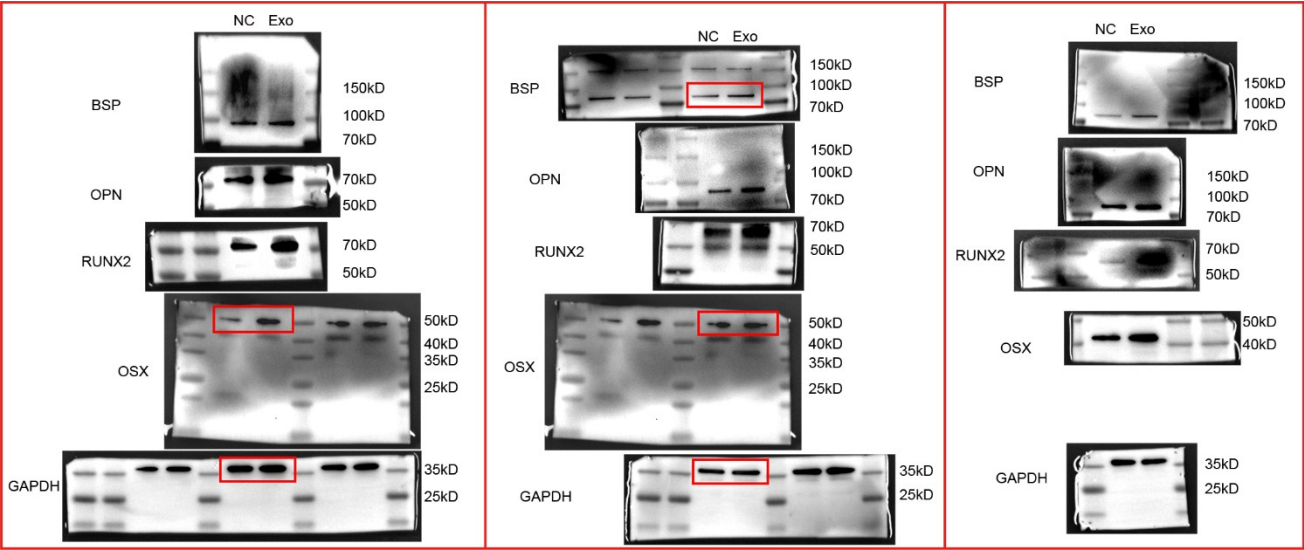
Supplementary western blot figure of **Figure 2**.

The proteins have similar molecular weights, so the bands are displayed on different membranes.



Supplementary western blot figure of **Figure 4**.

The proteins have similar molecular weights, so the bands are displayed on different membranes.



Supplementary western blot figure of **Figure 5**.

The proteins have similar molecular weights, so the bands are displayed on different membranes.