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Transplanted MSCs promote alveolar bone repair via hypoxia-induced extracellular vesicle secretion

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Abstract

Object: Mesenchymal stem cell (MSC) therapy is a potential strategy for promoting alveolar bone regeneration. This study evaluated the effects and mechanisms of transplanted MSCs on alveolar bone repair.

Methods: Mouse alveolar bone defect model was treated using mouse bone marrow mesenchymal stem cell (BMSC) transplantation. The bone repair was evaluated by micro-CT and Masson staining. The conditioned medium of hypoxia-treated BMSCs was co-cultured with normal BMSCs in vitro to detect the regulatory effect of transplanted MSCs on the chemotactic and migratory functions of host cells. The mechanisms were investigated using Becn siRNA transfection and western blotting.

Results: BMSC transplantation promoted bone defect regeneration. The hypoxic microenvironment induces BMSCs to release multiple extracellular vesicle (EV)-mediated regulatory proteins that promote the migration of host stem cells. Protein array analysis, western blotting, GFP-LC3 detection, and Becn siRNA transfection confirmed that autophagy activation in BMSCs plays a key role during this process.

Conclusion: The local hypoxic microenvironment induces transplanted MSCs to secrete a large number of EV-mediated regulatory proteins, thereby upregulating the migration function of the host stem cells and promoting alveolar bone defect regeneration. This process depends on the autophagy-related mechanism of the transplanted MSCs.

KEYWORDS
alveolar bone, bone marrow mesenchymal stem cells (BMSCs), stem cell transplantation, tissue engineering, tissue regeneration

1 | INTRODUCTION

The repair and regeneration of alveolar bone defects are always a challenge for many clinical diseases, including tumors, trauma, tooth extraction, and periodontitis (Gu et al., 2022; Guo et al., 2023; Song et al., 2023; Wong et al., 2022). Multiple strategies have been developed to address these problems, including the implantation of scaffold material after infection control (Geão et al., 2019; Gu et al., 2022). However, even though inflammation control is an important basis for tissue remodeling and regeneration, the dysfunction of local host cells in the microenvironment is increasingly observed to be difficult to reverse. In particular, the number of local stem cells is insufficient and their functional decline makes seed cells for tissue regeneration scarce (Hu et al., 2018; Tang et al., 2016; Zhang et al., 2020). Strategies for resolving such situations require further investigation.

Considering that the status and function of local stem cells are key factors for tissue repair and regeneration, stem cell transplantation has become a hot topic in alveolar bone regeneration technology in recent years, and mesenchymal stem cell (MSC) transplantation has been shown to promote bone repair (Sun et al., 2014). However, the specific molecular mechanism by which transplanted MSCs promote tissue regeneration remains unclear, thus limiting the clinical application and development of stem cell therapy (Liu et al., 2008; Sun et al., 2014). Studies have shown that the surrounding microenvironment of tissue-engineered MSCs changes dramatically before and after transplantation from a 21% oxygen-rich culture environment to an extremely hypoxic environment in vivo (Yu et al., 2015). Such drastic environmental changes lead to the rapid apoptosis of MSCs after transplantation. Therefore, only a small number of transplanted MSCs can directly differentiate into new tissues as seed cells, while most of them release "signals" to regulate the host's cell function and indirectly promote tissue repair (Pang et al., 2021). However, the regulatory mechanisms of transplanted MSCs and the key regulatory factors have not been fully verified.

This study aimed to explore the promoting effect of transplanted MSCs on alveolar bone repair and investigate the therapeutic mechanism to provide a theoretical basis for the application of clinical stem cell therapy or stem cell-related biomaterial design. Cell sheet transplantation of mouse bone marrow mesenchymal stem cells (BMSCs) was used to treat a mouse alveolar bone defect model in vivo. The conditioned medium of hypoxia-treated BMSCs was co-cultured with normal BMSCs in vitro to detect the regulatory effect of transplanted MSCs on the chemotactic and migratory functions of host cells. Furthermore, a protein array was used to explore the key factors and mechanisms regulating the transplanted MSCs. These results provide a theoretical basis for the molecular mechanisms by which stem cell therapy promotes alveolar bone regeneration.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of BMSCs

Male C57BL/6 mice aged 6–8 weeks were purchased from Vital River (Beijing, China). All animal experiments were approved by the Animal Ethics Committee of the Beijing Stomatological Hospital (KQYY-202008-005).

Approximately, 6–8 weeks C57BL/6 mice were sacrificed and placed in a 75% alcohol container for 1 min. The femurs were collected with sterile instruments on a clean table and washed repeatedly with phosphate-buffered saline (PBS). Thereafter, both ends of the femur were cut off with sterile scissors, and the BMSCs were collected by washing out the internal bone marrow tissue and passing through a 70 μm cell strainer. The cells were then centrifuged at 1100 rpm for 6 min. The single-cell suspension was re-suspended with the prepared primary medium, which is composed of α-MEM medium (Invitrogen, Carlsbad, CA, USA) with 20% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX, USA), 5% penicillin/Strep (Invitrogen), and 5% glutamine (Invitrogen). The cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere, and the medium was changed every 3 days. The cells were passaged when they reached 70%–80% confluency. All the cells used in this study were obtained from passage 2 (P2). The cell identification results for the BMSCs used in this study are shown in Figure S1.

2.2 | In vitro hypoxia induction

After passing through P2, BMSCs were seeded at a density of 2×10^6 cells/well in a medium dish. After 24 h, the cells were changed into serum-free culture medium and cultured in a normal oxygen environment (control group) or in a hypoxic environment (hypoxia group) using AneroPack C-1 and a 2.5 L sealed culture tank C-31 (Mitsubishi Gas Chemical Company, Niigata, Japan) for another 24 h. The conditioned media of the two groups were collected, and cell samples of the two groups were collected for subsequent detection.

2.3 | Cell scratch migration assays

After passage to P2, the BMSCs were seeded at a density of 2×10^5 cells/well in a 6-well plate. After 24 h, the medium was discarded, and the 200 μL sterile pipette tip was used to perform scratches. The scratched cells were washed with PBS and photographed under the microscope (0 h). The conditioned media of control group or hypoxic group were added and cultured at 37°C with 5% CO_2 in a humidified atmosphere, and observations were performed at 0, 12, 24, and 48 h.

2.4 | Real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) after induction. cDNA was further synthesized according to the kit instructions, and real-time PCR reaction was performed on *Cxcl12*, *Ccl5*, *Bfgf*, *Egf*, *Becn*, *Hif1a*, and *β-actin* primers (Table S1) of each group using Power SYBR®Green PCR Master Mix kit (Life Technologies, Warrington, UK).

2.5 | Western blotting

After induction, the cells were washed twice with pre-cooled PBS and total protein was extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA). The electrophoresis was performed at constant voltage of 80V using 25 μg protein samples. The transmembrane reaction was performed at 250 mA for 1.5 h. The membranes were then incubated with 5% skim milk at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies, including Beclin-1 (Cell Signaling Technology, CST, #3495, Boston, MA, USA), LC3 (CST, #4599), β-actin (CST, #3700), and H90 (CST, #4877), overnight at 4°C. The next day, a diluted secondary antibody (Pierce, Malibu, CA, USA) was added and incubated at room temperature for 1 h, followed by the addition of ECL (Pplygen, Beijing, China) was added for exposure.

2.6 | Extracellular vesicle (EV) collection

Centrifugation (110,000×g, 2 h, Beckman, Optima MAX-XP, CA, USA) was applied to the conditioned medium to isolate the EVs. The size, diameter, and morphology of the collected EVs were observed using a transmission electron microscope (TEM; JEOL Co., Ltd., JEM-1400Flash, Tokyo, Japan). The content of EVs was quantified using a BCA protein assay kit (Pplygen).

2.7 | Protein array

Conditioned media of normoxic-cultured BMSCs, hypoxic-cultured BMSCs, and hypoxic-cultured Becn siRNA-transfected BMSCs were collected and sent to RayBiotech, Inc. (Guangzhou, China) for protein array analysis. Data analyses were performed, including normalization of the original data, differential protein screening, differential protein clustering, and GO/KEGG pathway enrichment analysis.

2.8 | Mouse alveolar bone defect model establishment

A total of 10 mice were used for in vivo study, which were randomly divided into a blank control group (control group) and a stem cell transplantation group (BMSC trans group), with five animals in each group.

After routine disinfection and anesthesia, the flap was opened at the first molar of the upper jaw and a spherical defect with a diameter of 1 mm was prepared at the root fork of the buccal molar using a dental turbine and ball drill. BMSC cell sheets with 2×10^5 cells were inserted into the defect in the BMSC trans group, whereas the blank control group was not treated, and then tightly sutured. The animals in both groups were sacrificed 1 month after the operation. The degree of alveolar bone defect repair was analyzed by micro computed tomography (micro-CT) scan (scan thickness 14.93 μm). The defect healing ratio was calculated as the ratio of the new bone area 1 month after surgery to the initial bone defect immediately after surgery (baseline). The bone mineral density was obtained from a 0.05 mm^2 oval region of interest on the sagittal image of the bone defect in the micro-CT image. In addition, the degree of local alveolar bone damage and new bone volume were observed and evaluated using hematoxylin and eosin (H&E) and Masson's trichrome staining.

2.9 | Immunohistochemistry

CD146 immunohistochemical staining was used to evaluate MSC homing and osteocalcin (OCN) immunohistochemical staining was used to evaluate bone formation in the operative area. Sections (5 μm thick) were cut and blocked with 10% goat serum for 1 h. Then, the samples were incubated overnight with CD146 antibody or OCN antibody at 4°C according to the manufacturer's protocols. The next day, the second antibody was added and incubated in 37°C for 30 min; then, the 3,3′-diaminobenzidine was added for visualization. For statistical analysis, a researcher who was blinded to the grouping selected five non-overlapping visual fields under a 40× microscope and counted the number of positive cells in the visual field by the manual counting method.

2.10 | Statistical analysis

All experiments were independently repeated at least thrice, and SPSS 19.0 (SPSS Co., Ltd. Inc., Chicago, IL, USA) was used for the statistical analysis. An independent samples *t*-test was used to compare two groups of measurement data, and a one-way analysis of variance was used to compare multiple groups. For samples with heteroscedasticity, the Kruskal–Wallis and Mann–Whitney *U*-tests were used to assess differences. Statistical significance was set at $p < 0.05$ was statistically significant.

3 | RESULTS

3.1 | BMSC transplantation significantly promoted the alveolar bone defect repair

BMSCs were cultured to form cell sheets in vitro and used for transplantation of alveolar bone defects in vivo (Figure 1a). The morphological results 1 month after surgery showed that the
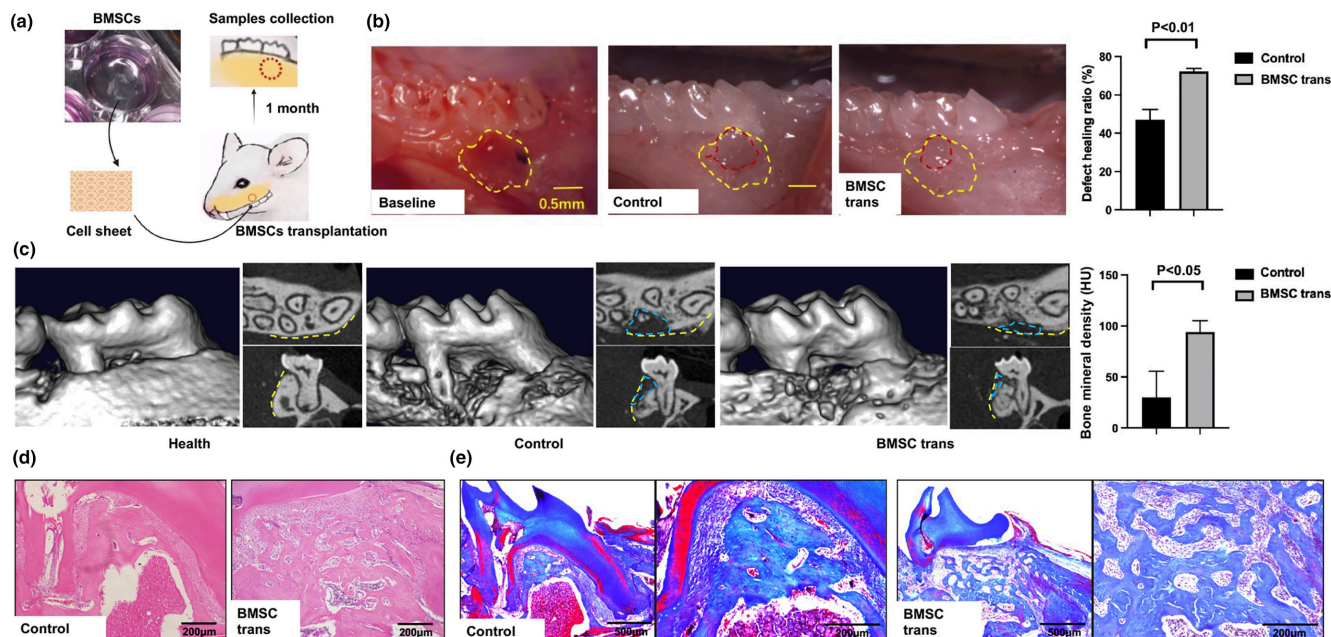
FIGURE 1 Local BMSC transplantation significantly promoted alveolar bone defect repair. (a) Schematic illustration of modeling process for in vivo experiments. (b) Morphological results at 1 month after operation showing a significantly higher healing rate of alveolar bone defect of BMSC trans group (72.21 ± 1.63%) than that of Control group (47.16 ± 5.19%). (c) The micro-CT results showing the volume of bone defect in the operative area (the yellow and blue dashed line indicates the preoperative baseline and local bone defect, respectively, at 1 month after the surgery). (d) H&E staining showing a large amount of vascularized new bone was regenerated in the bone defect area after BMSC transplantation. (e) Confirmation of the repair and regeneration of bone tissue (the new bones were stained a light blue) promoted by BMSC transplantation via Masson staining. All results are representative of at least three independent experiments. Data are presented as mean ± SD.

healing rate of the alveolar bone defect in the BMSC trans group was significantly higher than that in the control group (Figure 1b; the baseline results showed the initial size of the postoperative bone defect). The micro-CT results showed the volume of bone defect and local new bone volume in the operative area more intuitively, which was consistent with the morphological results (Figure 1c, the health group results showed the location of the normal alveolar bone boundary before surgery). In addition, H&E staining showed a large amount of vascularized new bone was regenerated in the bone defect area after BMSC transplantation (Figure 1d). Masson staining further confirmed that BMSC transplantation promoted the repair and regeneration of bone tissue (Figure 1e, the new bones were stained a light blue). These results indicate that MSC transplantation can promote the healing and regeneration of alveolar bone defects.

3.2 | Hypoxia-induced BMSC conditioned medium promoted the migration and chemotactic function of BMSCs in the normoxic microenvironment

To explore the mechanism by which transplanted stem cells regulate bone regeneration, BMSCs were implanted subcutaneously with a gelatin sponge, and cell survival was observed at different time points. The results showed that BMSCs rapidly underwent apoptosis after transplantation, with almost no surviving cells at 48 h

(Figure 2a). In addition, real-time PCR results detecting of *Hif1a* indicated the microenvironment in the bone defect area was hypoxic (Figure S2). Therefore, BMSCs were cultured in a hypoxic microenvironment and apoptosis was detected using flow cytometry (Figure 2b). The results were similar, showing that the apoptosis rate of cells was significantly increased after 48 h of culture. These results indicate that transplanted BMSCs may play a regulatory role in local cells rather than directly differentiating into the regenerated tissue. Furthermore, to verify the regulatory effect of hypoxia-induced BMSCs on other cells, conditioned media of normoxic-cultured BMSCs and hypoxic-cultured BMSCs were used to intervene with BMSCs cultured under normoxic conditions. The results showed that hypoxic-conditioned medium significantly promoted the migration function of normal BMSCs (Figure 2c) and upregulated the expression levels of chemokine (Figure 2d,e) and growth factor genes (Figure 2f,g) in the cells. Moreover, these results confirmed that the conditioned medium of the 24 h hypoxic culture of BMSCs (24 h CM) had the most significant regulatory effect.

3.3 | Hypoxia induces BMSCs to release extracellular vesicles (EVs) to regulate other cell function

Since the hypoxic-conditioned medium showed obvious regulatory functions in vitro, we used the BCA kit to detect the protein
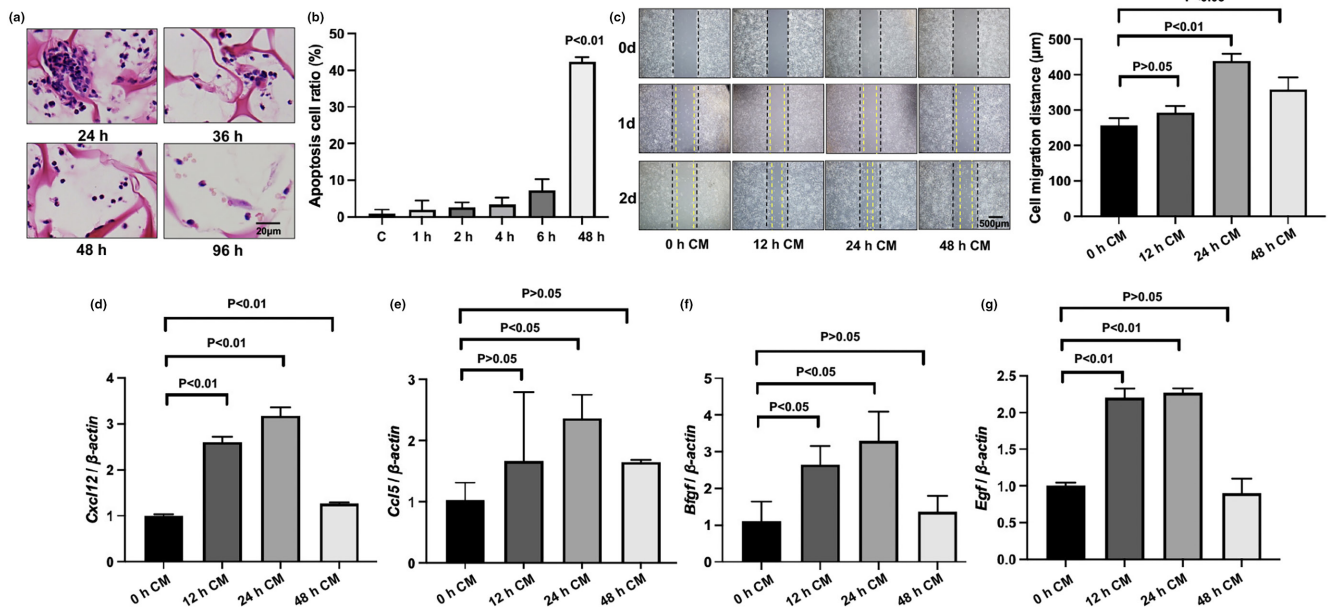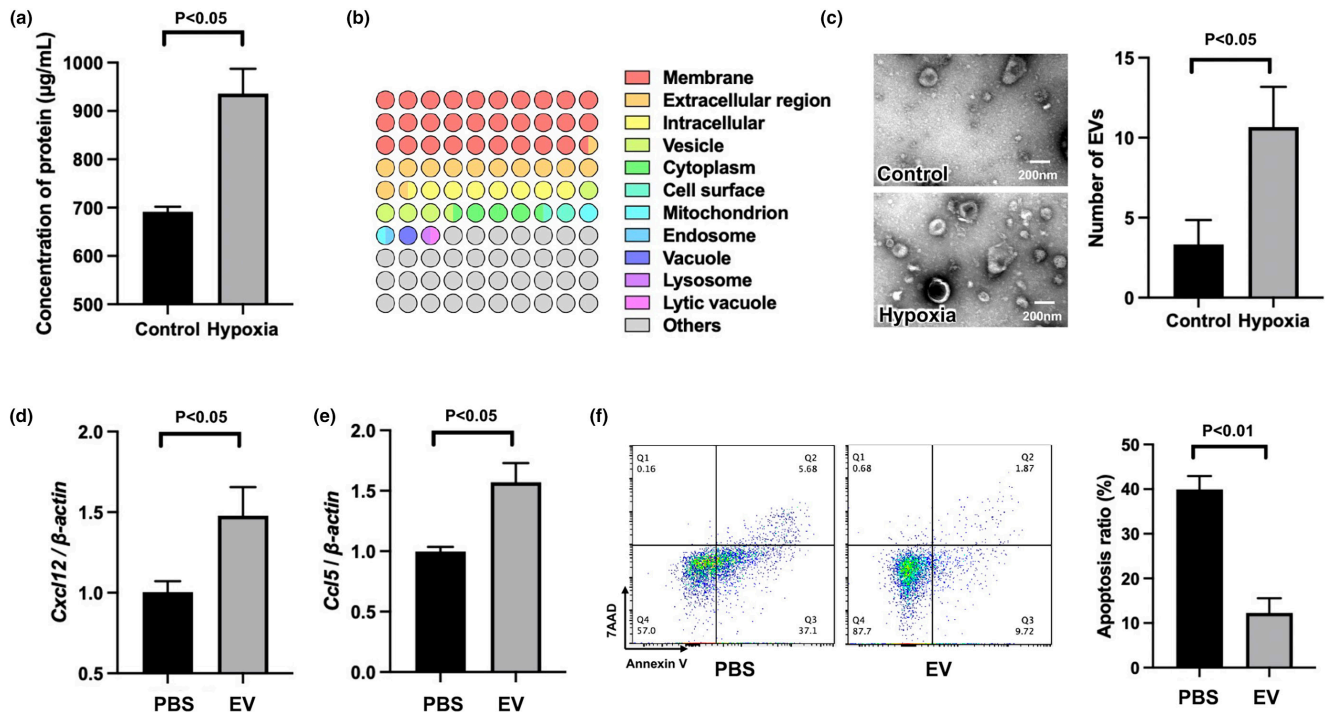
FIGURE 2 Hypoxia-induced BMSC conditioned medium promoted the migration and chemotactic function of normoxic cultured BMSCs. (a) Gelatin sponge-carried BMSC transplantation showing rapid apoptosis of BMSCs after transplantation, with almost no surviving cells at 48h. (b) Flow cytometry showing a significantly upregulated apoptosis rate of cells after 48h of hypoxic culture ($0.90 \pm 1.13\%$ in control group, $42.37 \pm 1.17\%$ in 48h group). (c) Cell scratch experiment showing that 24h hypoxic cultured conditioned medium (24h CM) significantly promoted the migration of BMSCs under normal oxygen condition. (d, e) Real-time PCR results showing that 24h hypoxic cultured conditioned medium (24h CM) upregulated the gene expression levels of chemokine in the normoxic cultured BMSCs, including *Cxcl12* and *Ccl5*. (f, g) Upregulation of the gene expression levels of growth factor in the normoxic cultured BMSCs, including *Bfgf* and *Egf* when subjected to 24h hypoxic cultured conditioned medium (24h CM). All results are representative of at least three independent experiments. Data are presented as mean $\pm$ SD. CM, conditioned medium.

concentration in these two conditioned medium, and the results showed that the protein concentration in the hypoxic-conditioned medium was significantly higher than that in the normoxic one, indicating that BMSCs in the hypoxic microenvironment released a large number of regulatory proteins (Figure 3a). Protein arrays were performed to detect the different proteins in these two conditioned media. Enrichment analysis of the cell components showed that most of these proteins were localized to EV-associated regions, including the membrane, extracellular region, vesicle, and cell surface. We visualized the proportion of protein distribution in the protein array results, with each dot representing 1%. The results are shown in Figure 3b. In addition, transmission electron microscopy further confirmed that the hypoxia-conditioned medium contained a large number of EVs (Figure 3c). Interestingly, by extracting these EVs and intervening with normal BMSCs, we demonstrated that these EVs can achieve regulatory effects similar to those of the hypoxic-conditioned medium, such as the upregulation of chemokine gene expression in normal BMSCs (Figure 3d,e). Moreover, these EVs can also improve the resistance of cells to a hypoxic microenvironment, resulting in a decrease in the apoptotic rate of cells in a hypoxic environment (Figure 3f). These results further confirmed that hypoxia-induced BMSC-EVs play an important role in regulating host cell functions.

3.4 | Hypoxia-induced BMSC-EV release is regulated by an autophagy-dependent pathway

To further explore the mechanism of BMSC-EV expression in hypoxic environments, autophagy, a typical hypoxia-induced functional change, was examined. Western blotting showed that the expression levels of the autophagy-related proteins Beclin-1 and LC3II/LC3I were significantly increased after hypoxic induction (Figure 4a), confirming that autophagy occurred in BMSCs in the hypoxic microenvironment. Transfection results of the GFP-LC3 plasmid confirmed this conclusion (Figure 4b). However, the current results are insufficient to prove that autophagy is a direct regulator of hypoxia-induced BMSC-EV expression. Therefore, Becn siRNA transfection was used to inhibit autophagy in BMSCs in a hypoxic environment, and the inhibition efficiency was verified using real-time PCR (Figure 4c) and western blotting (Figure 4d). Subsequently, quantitative detection of BCA protein was repeated, and the results showed that with the inhibition of autophagy, the concentration of proteins secreted by hypoxia-induced BMSCs was also downregulated (Figure 4e). In addition, transmission electron microscopy results showed that the inhibition of autophagy resulted in a significant decrease in the number of BMSC-EVs in the hypoxic-conditioned medium (Figure 4f), which preliminarily indicated that hypoxia-induced BMSC-EV release was dependent on the autophagic function of cells.

FIGURE 3 Extracellular vesicles (EVs) are the important factors in hypoxia-induced BMSC regulation. (a) The protein concentration in the hypoxic conditioned medium ($936.07 \pm 51.22\,\mu g/mL$) was significantly higher than that in the normoxic one ($691.59 \pm 10.48\,\mu g/mL$), indicating that BMSCs in the hypoxic microenvironment released a large number of regulatory proteins. (b) Cell component enrichment analysis of the protein array showing that most of the regulatory proteins were localized to EV-associated regions. (c) Transmission electron microscopy confirming that the hypoxic-conditioned medium contained a large number of EVs. (d, e) Upregulation of the gene expression levels of chemokine in the normoxic cultured BMSCs, including *Cxcl12* and *Ccl5* via the intervention of the EVs derived from hypoxia-induced BMSCs. (f) Improvement in the resistance of cells to hypoxic microenvironment, resulting in a decrease in apoptosis rate of cells in hypoxic environment due to the intervention of the EVs derived from hypoxia-induced BMSCs ($39.93 \pm 3.00\%$ in PBS group, $12.25 \pm 3.31\%$ in EV group). All results are representative of at least three independent experiments. Data are presented as mean $\pm$ SD.

## 3.5 | Autophagy mediated the secretion of various chemokines in hypoxia-induced BMSCs

The conditioned medium of hypoxia-cultured BMSCs and hypoxia-cultured Becn siRNA-transfected BMSCs was further used for protein array analysis to explore the role of autophagy in BMSC regulatory functions. By analyzing the differential proteins, we found that when autophagy function of BMSCs was inhibited, the expression of a variety of proteins related to stem cell chemotaxis and angiogenic cytokines was downregulated, including insulin-like growth factor binding protein (IGFBP)-3 (Yen et al., 2015), IGFBP-6 (Tran et al., 2022), IGFBP-5 (Han et al., 2017), vascular cell adhesion molecule (VCAM)-1 (Du et al., 2016), epidermal growth factor (Li et al., 2022), and vascular endothelial growth factor (Ma et al., 2022) (Figure 5a). Cellular component enrichment analysis of these different proteins showed that these proteins were localized in EVs, extracellular organelles, and exosomes (Figure 5b). Molecular functional enrichment analysis of the differentially expressed proteins revealed that these proteins were mainly involved in chemotactic-related functions, including C-X-C chemokine receptor (CXCR) binding, chemokine activity, and C-C chemokine receptor (CCR) binding (Figure 5c). These results were consistent with our previous in vitro studies showing that BMSCs release EVs in a

hypoxic microenvironment, thus promoting chemotaxis of other cells; this regulatory effect depends on hypoxia-induced activation of autophagy in BMSCs. We treated normal BMSCs with the conditioned medium of normoxic-cultured BMSCs, hypoxic-cultured BMSCs, and hypoxic-cultured Becn siRNA-transfected BMSCs, then detected the cell migration and chemokine gene expression levels. As expected, the conditioned medium of hypoxic-cultured Becn siRNA-transfected BMSCs had less effect on the migration and chemotactic ability of other cells, demonstrating the key role of autophagy in hypoxia-induced BMSC regulation (Figure 5d–f).

For the in vivo verification, CD146 immunohistochemical staining showed an increase in the number of local MSC in the operative tissue of the BMSC trans group, demonstrating an increase in host stem cell homing (Figure 6a). Moreover, by labeling osteoblasts with OCN, we demonstrated that BMSC transplantation promoted local osteoblast differentiation in the bone defect areas (Figure 6b), and these results confirmed the conclusions of in vitro studies.

4 | DISCUSSION

The crucial role of MSC functions in alveolar bone regeneration has been recognized (Liu et al., 2022; Xiao et al., 2021). Since local cell
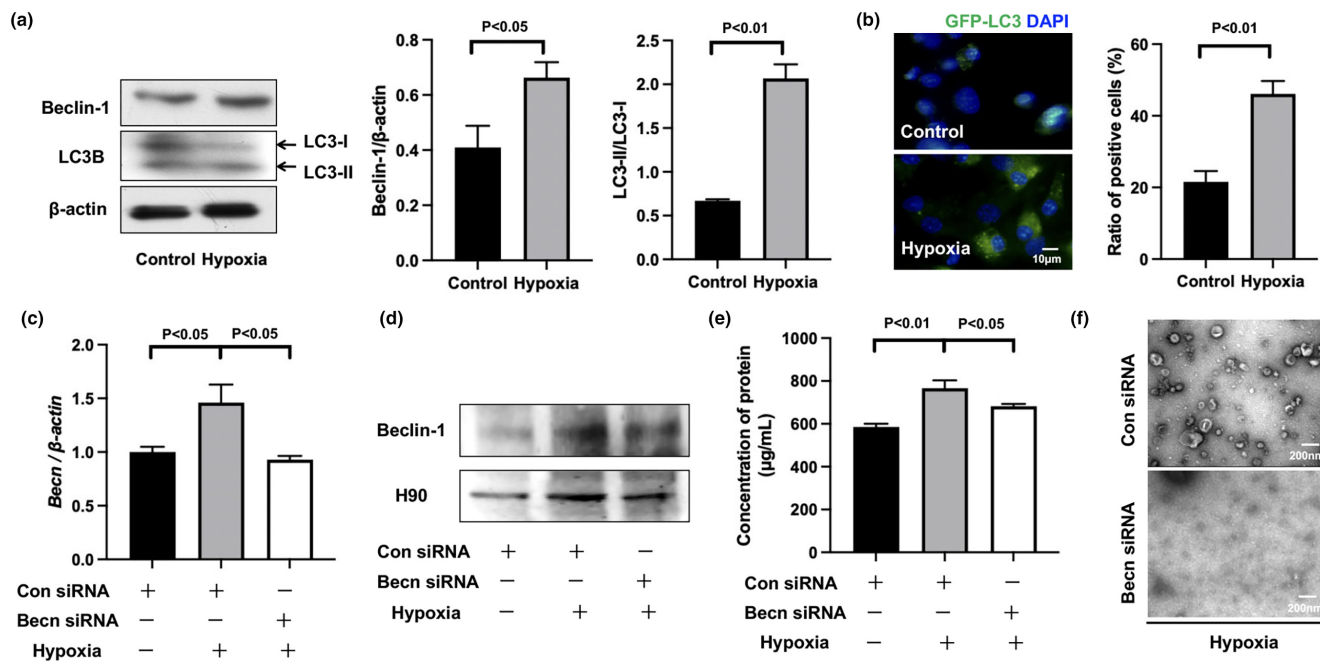
FIGURE 4 Hypoxia-induced BMSC-EV release is regulated by an autophagy-dependent pathway. (a) Western blotting showing that the expression levels of autophagy-related proteins Beclin-1 and LC3II/LC3I in BMSCs were significantly increased after hypoxic induction. (b) Transfection results of GFP-LC3 plasmid confirming the autophagy activation in hypoxia-induced BMSCs. (c) Real-time PCR confirming the inhibition effect of Becn siRNA transfection on *Becn* gene upregulation induced by hypoxia in BMSCs. (d) Western blotting confirming the inhibition effect of Becn siRNA transfection on Beclin-1 protein upregulation induced by hypoxia in BMSCs. (e) BCA protein quantitative detection showing the downregulation of the concentration of protein secreted by hypoxia-induced BMSCs with the inhibition of autophagy ($585.40 \pm 14.84\,\mu g/mL$ in normoxic cultured BMSC conditioned medium, $766.27 \pm 36.62\,\mu g/mL$ in hypoxic cultured BMSC conditioned medium, $681.80 \pm 11.34\,\mu g/mL$ in hypoxic cultured Becn siRNA transfection BMSC conditioned medium). (f) Transmission electron microscopy results showing a significant decrease in the number of BMSC-EVs in hypoxic-conditioned medium due to the inhibition of autophagy. All results are representative of at least three independent experiments. Data are presented as mean $\pm$ SD.

dysfunction caused by inflammation leads to difficulties in tissue repair (Lin et al., 2023; Tang et al., 2015), microenvironment homeostasis remodeling and host cell mobilization have become mainstream tissue engineering regulatory strategies. Despite the remarkable effect of stem cell therapy in promoting bone regeneration, the survival rate of transplanted stem cells is low due to the hypoxic surrounding microenvironment (Yu et al., 2015). Therefore, most of the transplanted cells promote tissue regeneration by releasing "signals" through paracrine function to regulate the host's own cell functions (Pang et al., 2021), while the mechanism remains unclear.

Autophagy is a key adaptive feature of cells in hypoxic environments that maintains the overall homeostatic balance of cell function and the microenvironment by removing damaged organelles, regulating the production of reactive oxygen species, and cell apoptosis (Li et al., 2021). With the deepening of research on autophagy, a consensus has been reached that autophagy can be divided into three morphological categories: macroautophagy, microautophagy, and chaperon-mediated autophagy (Xie & Klionsky, 2007). For decades, a large number of studies related to autophagy have mainly focused on macroautophagy (hereafter referred to as autophagy), which is the focus of this study. Mechanisms underlying hypoxia-induced autophagy are complex. Hypoxia-induced oxidative stress is a key factor in autophagy. Superoxide radicals produced by

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can trigger autophagy, which may be regulated by the activation of the AMP-activated protein kinase (AMPK) pathway (Popov et al., 2023). In addition, Hypoxia-inducible factors (HIF) is a key transcription factor, which regulates gene transcription in response to hypoxia (Fang et al., 2015). HIFs can induce the BH3-only protein BNIP3, disrupt the inhibitory interaction between Beclin1 and Bcl-2 and activate mitochondrial autophagy (Fang et al., 2015). In addition, the mammalian target of rapamycin complex 1 (mTORC1), an inhibitor of cell autophagy, is negatively regulated by HIF-1/BNIP3 (Li et al., 2023). As a proapoptotic protein, HIF-induced BNIP3 initiates cell apoptosis (Greijer & van der Wall, 2004) and excessive autophagy will also lead to excessive degradation of cell contents, resulting in autophagy death (Wirawan et al., 2012). Therefore, the fate of apoptosis in transplanted cells under the action of multiple factors seems inevitable; however, their functional changes and regulatory significance before apoptosis should also be investigated.

A moderate level of autophagy in such an adverse environment is conducive to maintaining the stability of cell function and state (Ren et al., 2022), and is also involved in protein secretion and regulatory functions of the cells (Buratta et al., 2020). However, the roles of autophagy in EV release remain controversial. In the classical concept, the release of EVs is caused by the maturation of early
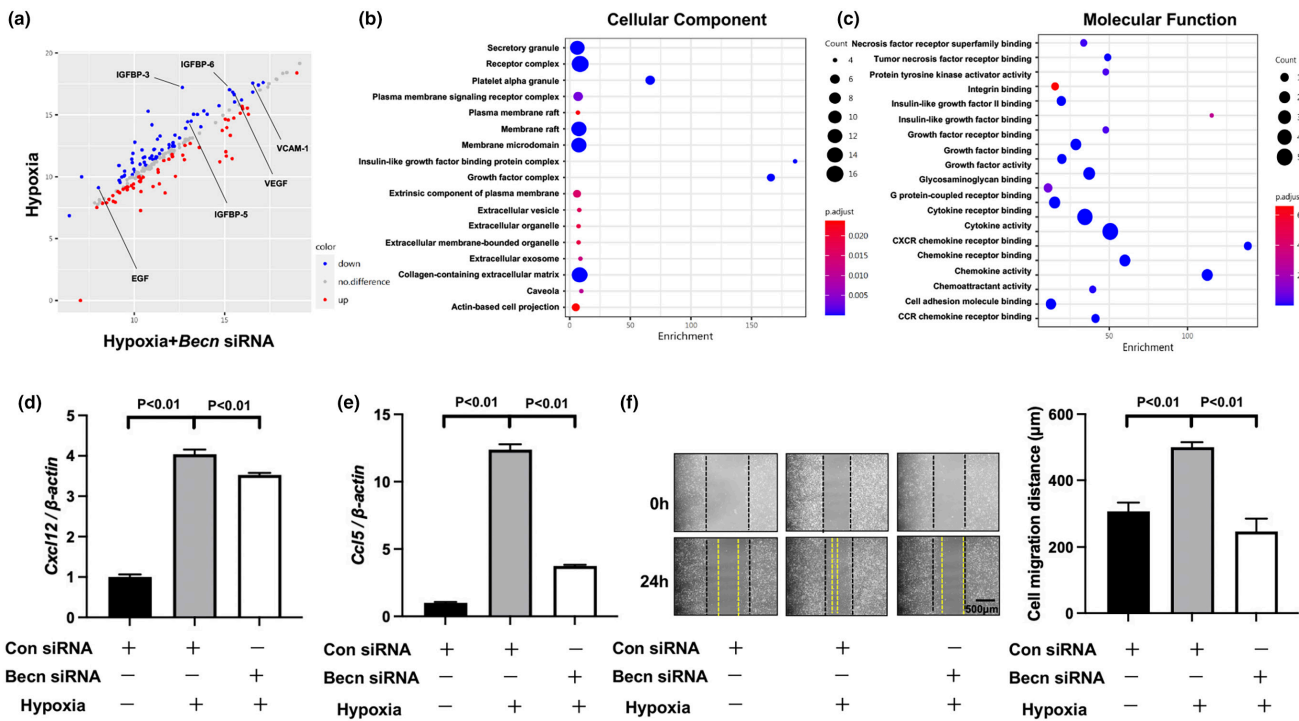
FIGURE 5 Protein array results revealed that autophagy mediated the secretion of various chemokines in hypoxia-induced BMSCs. (a) Protein array showing downregulation of the expression of a variety of proteins related to cell chemotaxis due to inhibition of the autophagy function of BMSCs. (b) The cellular component enrichment analysis showed that these different proteins were localized in EVs, extracellular organelles, and exosomes. (c) The molecular functional enrichment analysis revealed that these different proteins were mainly involved in chemotactic-related functions. (d, e) Real-time PCR results showing that compared to the hypoxic cultured BMSC conditioned medium, the conditioned medium of hypoxic-cultured Becn siRNA transfection BMSCs had less promotion effect on the gene expression levels of chemokine in the normoxic cultured BMSCs, including *Cxcl12* and *Ccl5*. (f) The results of cell scratch experiment showing the attenuation of the promotion effect of hypoxic-conditioned medium on cell migration with the inhibition of autophagy. All results are representative of at least three independent experiments. Data are presented as mean ± SD.

endosomes to produce multivesicular bodies (MVBs), which further fuses with the plasma membrane (Hessvik & Llorente, 2018). On the one hand, the autophagosomes produced by autophagy can directly fuse with the plasma membrane to promote autophagosome mediated secretion; on the other hand, they can also fuse with MVBs to form the interaction or even antagonism with EV secretion (Buratta et al., 2020). Several studies reported that autophagy is one of the mechanisms that inhibit exosome expression (Hu et al., 2023; Ojha et al., 2017). However, multiple studies have found evidence of the mechanisms by which autophagy promotes EV release. Several autophagy proteins have been proved to play key roles in the assembly and transport of MVBs, including autophagy-related gene (ATG)12–ATG3 complex (Hessvik & Llorente, 2018), and ATG9 (Bader et al., 2015). In addition, the related protein Beclin-1 (Becn1) is activated under hypoxic conditions and promotes the secretion of inflammatory factors and chemokines by macrophages through the glutamine/glutathione-related pathway, thereby promoting immune responses and intercellular interactions (Xu et al., 2023). Becn1 also regulates the activation of the downstream AMPK pathway, promotes the transcription of cell-related factors, and enhances the transfer and secretion of EV-mediated regulatory factors (Kuramoto et al., 2021). Inhibition of Beclin-1 through either siRNA transfection or Spautin-1 treatment reduces both exosome

release and autophagy flux in chronic myeloid leukemia cells (Liu et al., 2016). In summary, autophagy has been revealed to act as an unconventional secretion process known as the secretory autophagy (Yan et al., 2022). However, the role of autophagy in the paracrine processes of transplanted MSCs, which affect host cell function, remains unclear.

In this study, we found that BMSC transplantation significantly promoted the repair of alveolar bone defects and new bone formation. Both H&E staining and Masson staining demonstrated the effect of local vascularized bone regeneration after BMSC therapy. Through subcutaneous transplantation of BMSCs on a gelatin sponge and in vitro flow cytometry detection for cell apoptosis, we found that most transplanted BMSCs were apoptotic due to the local hypoxic microenvironment, but their conditioned medium promoted the migration function and the gene expression of chemokines of other BMSCs. These results indicate that transplanted stem cells promote tissue regeneration by promoting chemotaxis and homing of host stem cells via paracrine functions. To explore the key factors of transplanted BMSC regulatory function, we used BCA protein quantitative detection to explore the protein concentration in hypoxic-conditioned medium and found that the protein concentration in the hypoxic-conditioned medium was significantly higher than that in the normoxic medium, indicating that
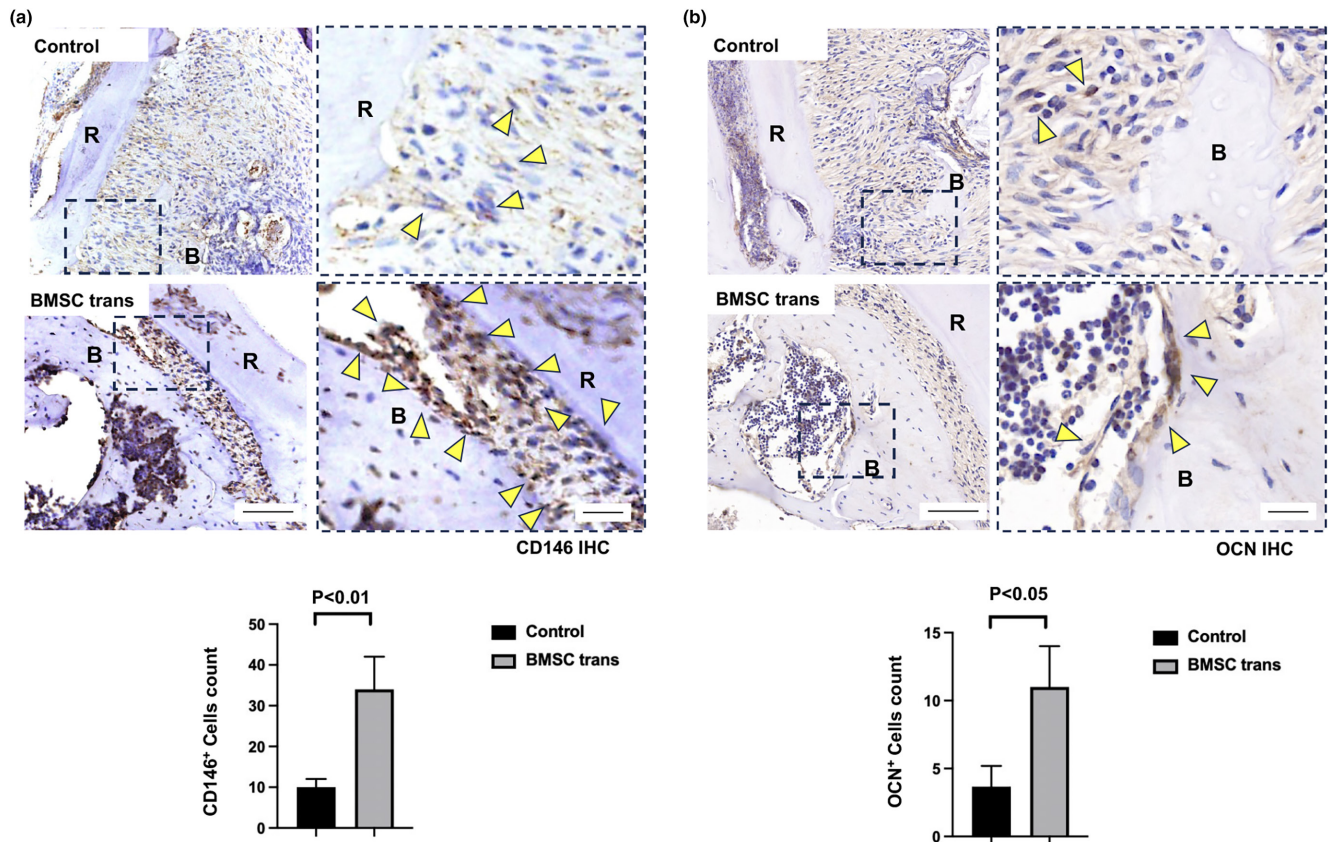
FIGURE 6 BMSC transplantation promoted host stem cell homing and osteogenic differentiation in vivo. (a) CD146 immunohistochemical staining showing an increase in host stem cell homing in bone defect areas owing to BMSC transplantation. (b) OCN immunohistochemical staining showing the promotion of local osteoblast differentiation in bone defect areas owing to BMSC transplantation. All results are representative of at least three independent experiments. Data are presented as mean ± SD. Scale bar = 100 μm in low power visual field and 20 μm in high-power visual field. In (a) and (b), R = root, and B = bone.

BMSCs in the hypoxic microenvironment released a large number of regulatory proteins. Through protein array enrichment analysis of secreted proteins and transmission electron microscopy, it was found that hypoxia-induced BMSCs released a large number of EVs, which promoted the chemotactic function and hypoxia resistance of other cells. Considering that autophagy is an important response to hypoxia, we verified the significant activation of the autophagy pathway in BMSCs under hypoxia by western blotting and GFP-LC3 plasmid transfection. Moreover, Becn siRNA transfection was applied to inhibit the autophagy of hypoxia-induced BMSCs, which resulted in the cancellation of the promoting effect of hypoxia-induced BMSCs on chemotaxis and migration to other cells. Additional protein array results and enrichment analysis confirmed our hypothesis that BMSCs release EVs in a hypoxic microenvironment; thus promoting chemotaxis of other cells, this regulatory effect depends on hypoxia-induced activation of autophagy in BMSCs.

In future studies, the key protein components of the transplanted stem cells should be clarified. In addition, whether transplanted MSCs have an impact on the functions of host immune cells, as well as their impact on the proliferation and differentiation functions of host stem cells, requires further exploration.

5 | CONCLUSION

In summary, this study showed that the local hypoxic microenvironment induced transplanted MSCs to secrete a large number of EV-mediated regulatory proteins, thereby upregulating the chemotactic and migration functions of the host stem cells and promoting the repair and regeneration of alveolar bone defects. This process depends on the autophagy-related mechanism of the transplanted MSCs. The results of this study further elucidate the regulatory mechanism of stem cell therapy to promote tissue regeneration and provide new regulatory targets and new drug development ideas for bone defect treatment.

AUTHOR CONTRIBUTIONS

Yi Liu: Validation; investigation; funding acquisition; writing – original draft; supervision; funding acquisition; writing – review and editing.

CONFLICT OF INTEREST STATEMENT

None of the authors disclosed any potential conflicts of interests.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared upon reasonable request to the corresponding author.

PATIENT CONSENT

The patient reported in this manuscript provided written informed consent for the publication of the case details.

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SUPPORTING INFORMATION

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