Exosomal let-7f-5p derived from mineralized osteoblasts promotes the angiogenesis of endothelial cells via the DUSP1/Erk1/2 signaling pathway

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Abstract
Blood vessel formation is the prerequisite for the survival and growth of tissue-engineered bone. Mineralized osteoblasts (MOBs) have been shown to regulate angiogenesis through the secretion of exosomes containing various pro-angiogenic factors. However, whether the mineralized osteoblast-derived exosomes (MOB-Exos) containing let-7f-5p can regulate the angiogenesis of endothelial cells (ECs) is still unknown. In this study, the angiogenic capabilities of ECs respectively treated with MOB-Exos, let-7f-5p mimicked MOB-Exos (miR mimic group), and let-7f-5p inhibited MOB-Exos (miR inhibitor group) were compared through in vitro and in vivo studies. Moreover, the potential mechanism of MOB-Exo let-7f-5p regulating angiogenesis was explored by verifying the role of the Erk1/2 signaling pathway and target gene DUSP1. The results showed that MOB-Exos could significantly promote the angiogenesis of ECs, which could be enhanced by mimicked exosomal let-7f-5p and attenuated by inhibited exosomal let-7f-5p. Let-7f-5p could suppress the luciferase activity of wide-type DUSP1, and the mutation of DUSP1 could abrogate the repressive ability of let-7f-5p. Furthermore, the expression of DUSP1 exhibited a reversed trend to that of pErk1/2. The expression of pErk1/2 was significantly higher in the miR mimic group and lower in the miR inhibitor group than that in the MOB-Exos group, while inhibition of pErk1/2 could partly impair the angiogenic capabilities of ECs. In conclusion, we concluded that exosomal let-7f-5p derived from MOBs could promote the angiogenesis of ECs via activating the DUSP1/Erk1/2 signaling pathway, which might be a promising target for promoting the angiogenesis of tissue-engineered bone.

KEYWORDS
angiogenesis, DUSP1, endothelial cells, Erk1/2 signaling pathway, exosomes, let-7f-5p, osteoblasts
INTRODUCTION

Angiogenesis plays a key role in the survival of tissue-engineered bone (Litvinova et al., 2020). Seeded cells outside of 200 μm from capillaries could not obtain sufficient nutrition through diffusion of blood and interstitial fluid (Shang et al., 2021). Our previous study found that tissue-engineered bone formation rate was related to that of the blood vessel, and the central part of the tissue-engineered bone exhibited cell death and scaffold non-degradation because of the lack of sufficient blood vessel in-growth (He et al., 2014, 2015). The utilization of "angiogenesis-osteogenesis" coupling mechanism was beneficial for promoting the formation of tissue-engineered bone (Rather et al., 2019). Thus, investigating the regulatory effects of osteogenies (Tang et al., 2019). And the MSC derived exosomes (MOB) in inferred of pro-angiogenesis (Yu et al., 2016). Exosomal miR-935 promoted the osteogenic differentiation of OBs (Zhang et al., 2020), while miR-23a-5p inhibited their osteogenic differentiation (Yang et al., 2020). Exosomal miR-141 promoted the angiogenesis of lung cancer (Wang et al., 2021), while miR-6785-5p played the opposite role in gastric cancer (Chen et al., 2021). BMSCs-derived exosomal miR-29a could both promote the osteogenic differentiation of BMSCs and angiogenic capabilities of ECs (Lu, Cheng, et al., 2020). Therefore, exosomes and containing miRNAs could regulate osteogenesis and angiogenesis, which indicated that some specific exosomal miRNAs could be the potential factors for promoting the formation of tissue-engineered bone.

Our previous study demonstrated that mineralized osteoblast-derived exosomes (MOB-Exos) could promote the angiogenesis of ECs (Tang et al., 2019). And the MSC-derived exosomal let-7f was inferred of pro-angiogenic effect (Gong et al., 2017). However, whether the MOB-derived exosomal let-7f-5p can promote the angiogenesis of ECs has not been studied or systematically verified. The present study was designed to investigate the regulatory effects of exosomal let-7f-5p derived from MOBs on angiogenic capabilities of ECs, expecting to provide a potential target for promoting tissue-engineered bone formation.

MATERIALS AND METHODS

2.1 Cell culture, osteogenic induction, and identification

OB cell line MC3T3-E1 and EC line bEnd.3 were purchased from the cell bank of the Chinese Academy of Sciences, and respectively cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 100 U/ml penicillin-streptomycin (Amersco) in the incubator under the condition of 37°C, 5% CO2 and 95% saturated humidity. The medium was changed every 3 days. OBs were cultured in the osteogenic inducing medium composed of DMEM complete medium, 1 x 10^{-8} M dexamethasone, 50 mg/L L-ascorbic acid, and 10 mM β-Sodium glycerophosphate (Sigma) for 21 days. Alkaline phosphatase (ALP) staining and Alizarin Red S (ARS) staining were respectively performed to identify the osteogenic induction efficiency.

2.2 Extraction and identification of exosomes

Before the extraction of exosomes, MOBs were cultured in DMEM with 10% exosome removed FBS (exosome-free medium) for 1 week. Exoquick reagent (Invitrogen) was applied to extract MOB-Exos according to the manufacturer’s protocol with some adjustments. Briefly, the culture supernatant was sequentially centrifuged in a centrifuge tube (Corning) at 2000g for 30 min and in a 100kDa ultrafiltration tube (Millipore) at 4000g for 30 min. And then, a total exosome isolation reagent with 0.5 vol of concentrated supernatant was added and well mixed. After incubated at 4°C overnight, the mixture was centrifuged at 10,000g for 1 h at 4°C. Exosomes were obtained in the deposition at the bottom of the tube, and then resuspended with 1×PBS with 0.01 vol of the initial culture supernatant, and identified by Nanosight light scattering technology, transmission electron microscope scanning, and Western blot. BCA Protein Assay Kits (Thermo) were applied to determine the concentration of exosomes. And the exosome concentration of 100 μg/ml was applied in the intervention of ECs.

2.3 Immunofluorescence staining

Two-hundred μg MOB-Exos were prepared, subjected to PKH-67 green fluorescent dye (Sigma), and incubated for 10 min. The dyeing procedure was terminated by an equal volume of 1% BSA. The labeled exosomes were extracted by Exoquick reagent, washed and resuspended with 1×PBS, and then added into the culture medium of bEnd.3. After co-incubated with exosomes for 4 h, the adherent ECs were fixed with 4% neutral paraformaldehyde for 20 min and
subsequently dyed with DAPI for 5 min. Finally, the labeled ECs were washed and photographed with the inverted phase-contrast fluorescence microscope. As a parallel experiment, micrON™ miRNA mimic and micrOFF™ miRNA inhibitor (RiboBio) were applied to detect the transfection efficiency of let-7f-5p mimics and inhibitors according to the manufacturer’s protocol.

2.4 | CCK-8 assay

CCK-8 kits (Yeasen) were applied to examine cell proliferation. Briefly, ECs were respectively resuspended with an exosome-free medium to a density of $5 \times 10^5$ cells/ml. One-hundred μl of the cell suspension was added into wells of 96-well plates. Three duplicates were set for each group at each time point. At 0, 24, 48, and 72 h after cell adhesion, 10 μl CCK-8 solution was added into each well respectively and incubated at 37°C for 1 h. The light absorption values at 450 nm ($A_{450}$) of each well were measured, according to which the cell growth curves were drawn.

2.5 | Wound healing assay

ECs were resuspended with exosome-free medium and inoculated into wells of the six-well plates at a density of $5 \times 10^5$ cells/well. Three duplicates were set for each group at each time point. Two-hundred μl pipette heads were used to draw lines vertically at the well bottom. After washing out the exfoliated cells, ECs were cultured in the exosome-free medium. The scratches were observed and photographed with the inverted phase-contrast microscope at 0, 24, 48, and 72 h after scribing. The scratch areas and scratch heights were measured in Image J software, and then the average migration widths were calculated by using the formula: average migration width = (0 h scratch area – scratch area at measurement time)/scratch area height.

2.6 | Transwell migration assay

ECs were resuspended with the pure DMEM medium to a density of $5 \times 10^5$ cells/ml. One-hundred μl cell suspension was added into the upper chamber of the Transwell chamber (Corning), and 600 μl exosome-free medium was added into the lower chamber. After being cultured for 24 h, cells in the upper chambers were wiped up. ECs migrating to the lower chambers were washed, fixed with methanol for 30 min, and stained with 0.1% crystal violet solution for 20 min after air drying. Five fields were randomly selected to count the number of migrated cells in Image J software.

2.7 | Tube formation assay

ECs were resuspended with the exosome-free medium to a density of $5 \times 10^5$ cells/ml. Matrigel (Corning), 96-well plates, and 200 μl pipette tips were placed in a 4°C refrigerator for precooling overnight. Fifty μl Matrigel was added to each well and incubated at 37°C for 30 min. Then 50 μl cell suspension was added to each well and cultured for 6 h. The tubule-like structures were observed and photographed. The total segment lengths of tubule-like structures were measured in Image J software.

2.8 | Real-time quantitative PCR

Total RNA was isolated by RNA extraction reagents (Servicebio) and then subjected to an ultramicro spectrophotometer (Thermo) to detect the RNA concentration and purity according to the manufacturer’s instructions. After the RNA concentration was adjusted to 200 ng/μl, RevertAid First Strand cDNA Synthesis Kits (Thermo) were applied to the RNA reverse transcription according to the manufacturer’s instructions. The quantitative PCR was performed with ABI 7300 real-time PCR system (ABI) according to the recommendations. Data were standardized to the threshold cycle values according to the internal control of U6. The primer sequences were listed in Table 1.

| RNA name | Primer sequence |
|----------|----------------|
| U6       | Forward 5'-CTCGGTTCCCGGACACGAC-3’ | Reverse 5'-AACGCTTTCAAGAATTCGCT-3’ |
| mmu-let-7f-5p | Forward 5'-CTCAACTGTGGTCGGAGGTGCAAT | Reverse 5'-ACACTCCGCTGCTGGTAGTTATGT-3’ |
| mmu-let-7f-5p mimics | Forward 5'-UGAGGUAGUAGAUGUAGUAGU-3’ | Reverse 5'-AATATACAAATCTACTACCTCA-3’ |
| mmu-let-7f-5p inhibitor | Single-stranded 5'-AATATACAAATCTACTACCTCA-3’ | |
| DUSP1    | Forward 5'-AGGACACCAACCAAGGCCAGAC-3’ | Reverse 5'-ATACTCCGCTCTGCTTCAC-3’ |
| DUSP4    | Forward 5'-GTACATCGACGCACTAAAGGAC-3’ | Reverse 5'-GCTTGACGAACTCAAAAGCCTC-3’ |
| β-actin  | Forward 5'-GGCTGATTTCCCCCTCATCG-3’ | Reverse 5'-CCAGTTGGAATCGGACATCT-3’ |

Abbreviation: DUSP, dual specificity phosphatase.
2.9 | Western blotting analysis

Western blotting was performed as previously described (Tang et al., 2019). Briefly, cells and exosomes were lysed by RIPA buffer containing protease and phosphatase inhibitors (Sevenbio-tech). The protein concentration was quantified with a BCA Protein Assay kit (Thermo), and adjusted to the same with RIPA buffer. Twenty μg total protein was loaded, subjected to electrophoresis, and then transferred to PVDF membranes (Millipore). Subsequently, the membranes were blocked with 5% non-fat milk in TBST for 2 h and then incubated with related primary antibodies for CD63 (Invitrogen), CD81 (Invitrogen), Erk1/2 (CST), pErk1/2 (Santa Cruz), GAPDH (CST), β-actin (CST) at 4°C overnight. Afterward, the membranes were washed and incubated with secondary antibodies (CST) for 1 h and visualized with an ECL detection kit (Bio‐tech). The protein concentration was quantified with a BCA kit (Bio‐tech). The protein concentration was quantified with a BCA kit (Bio‐tech). The protein concentration was quantified with a BCA kit (Bio‐tech). The protein concentration was quantified with a BCA kit (Bio‐tech). Finally, the photographs were taken with Fluor Chem E system (Proteinsimple), and analyzed in Image J software.

2.10 | Dual luciferase reporter assays

The dual luciferase reporter assays were performed with some modifications. Briefly, wild-type and mutant 3'-UTRs of dual specificity phosphatase-1 (DUSP1) and dual specificity phosphatase-4 (DUSP4) were respectively amplified and cloned downstream of the luciferase gene within the pmirGLO vectors (Promega), with the Renilla luciferase plasmid (Promega) co-transfected as a control. After transfected with 1 μg vectors and subjected to 60 μM let-7f-5p mimics, the luciferase activities of the transformed cells were measured with Dual-Luciferase Reporter assay kits (Promega) according to the manufacturer’s instructions.

2.11 | Subcutaneous xenograft model

The protocol was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (No. m20210910). Matrigel plugs were produced by respectively mixing 500 μl matrigel (BD Biosciences) with 4 × 10^6 ECs pretreated with MOB-Exos, let-7f-5p mimicked and inhibited MOB-Exos. Five repetitions were set for each group. Then the plugs were subcutaneously injected into the dorsal region of nude mice (female, 7–8 weeks, BALB/c) after the mice were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium (200 mg/kg). After 2 weeks, the mice were sacrificed with narcotic overdose. And then the plugs were harvested, embedded in O.C.T., and sectioned. The sections were subjected to immunofluorescence staining, immunohistochemistry staining, and western blotting to detect the infiltration of ECs and the expression of pErk1/2 and DUSP1.

2.12 | Statistical analysis

The experimental data were analyzed with Graphpad prism 8 software. Unpaired t-test was used for two groups of independent measurement samples, and chi-square test was used for counting samples. p < 0.05 and p < 0.01 were both regarded as statistical difference.

3 | RESULTS

3.1 | Identification of MOBs and MOB-Exos

The intracellular expressed ALP and calcium nodules deposited on the surface of OBs were respectively detected by ALP and ARS staining and exhibited a gradual increase with time (Figure 1a,b), which demonstrated that pre-OBs were successfully induced to MOBs. The diameters of extracted microvesicles detected by Nano‐sight light scattering technology ranged from 30.25 to 149.25 nm with a mean of 73.80 ± 18.70 nm (Figure 1c). And the microvesicles exhibited "saucer"-like shapes and diameters <200 nm in transmission electron microscope scanning (Figure 1d). Besides, high expression of biomarkers CD63 and CD81 was determined by Western blot and NanoFCM (Figure 1e–g). Those results demonstrated that the microvesicles extracted from MOBs were exosomes.

3.2 | MOB-Exos enhanced the angiogenic capabilities of ECs

PKH-67 immunofluorescence staining showed that the green fluorescent labeled MOB-Exos were absorbed by ECs, and wrapped the blue fluorescent labeled nucleus of ECs (Figure 2a). The regulatory effects of MOB-Exos were investigated by evaluating the angiogenic capabilities of ECs after intervention with an equal volume of PBS (control group) and MOB-Exos (MOB-Exos group). CCK-8 showed that the proliferative abilities of ECs treated with MOB-Exos were significantly higher than those of the control group at 48 and 72 h (Figure 2e). The migration abilities of ECs detected by Wound healing and Transwell assays exhibited a remarkable increase after treatment with MOB-Exos (Figure 2b,c,f,g). Furthermore, the tubule forming abilities of ECs in the MOB-Exos group were also significantly higher than those of the control group (Figure 2d,h). Those results demonstrated that MOB-Exos could be absorbed by ECs and then significantly promoted the angiogenic abilities of ECs.

3.3 | let-7f-5p in MOB-Exos promoted the angiogenesis of ECs

High expression levels of let-7f-5p were detected in ECs after the co-culture of ECs and MOBs, while its expression levels in
ECs significantly decreased with the addition of extracellular vesicle secretion inhibitor GW4896 (Figure 3f), revealing that MOBs could transport extracellular let-7f-5p into ECs via an exosome-dependent manner. To systematically verify the effects of let-7f-5p, a mimic-inhibiting was established by extracting exosomes from MOBs pretreated with let-7f-5p mimics and inhibitors (miR mimic and inhibitor groups). Cy3 immunofluorescence staining showed that Cy3 labeled let-7f-5p mimics and inhibitors evenly distributed in MOBs (Figure 3a), and RT-PCR analysis showed that let-7f-5p expressed in MOB-Exos remarkably increased and decreased by let-7f-5p mimics and inhibitors respectively (Figure 3g), confirming the successful establishment of the mimic-inhibiting system.

The regulatory effects of let-7f-5p were determined by contrastively investigating the angiogenic capabilities of ECs treated with MOB-Exos (MOB-Exos group), let-7f-5p mimicked MOB-Exos (miR mimic group), and let-7f-5p inhibited MOB-Exos (miR inhibitor group). CCK-8 experiments showed that the proliferative abilities of ECs significantly increased in the miR mimic group and decreased in the miR inhibitor group (Figure 3e). Wound healing assays and Transwell assays revealed that the migrating abilities of ECs were significantly enhanced by mimicking let-7f-5p and weakened by inhibiting let-7f-5p (Figure 3b,c,h,i). Furthermore, the total segment lengths of tubule-like structure formed in the miR mimic group were significantly higher than those of the other two groups (Figure 3d,j).

These assays confirmed that let-7f-5p played a positive regulatory role in MOB-Exos promoting the angiogenesis of ECs.

### 3.4 MOB-Exo let-7f-5p promoted the angiogenesis of ECs via activating the Erk1/2 signaling pathway

Western blotting assays showed that pErk1/2 expression levels remarkably increased by mimicking let-7f-5p and decreased by inhibiting let-7f-5p (Figure 4d,e), revealing the important role of Erk1/2 signaling pathway in MOB-Exo let-7f-5p promoting angiogenesis of ECs. To verify the effects, the Erk1/2 signaling pathway in ECs in miR mimic group was inhibited by PD98059 (Erk inhibitor group), whose effectiveness was confirmed by the significantly decreased expression of pErk1/2 compared to the miR mimic group (Figure 4d,e). The proliferative abilities of ECs in the Erk inhibitor group were significantly lower than those of the miR mimic group but greater than the MOB-Exos group (Figure 4f). The migration abilities of ECs also decreased by the inhibition of Erk1/2 signaling pathway in ECs in miR mimic group (Figure 4a,b,g,h). Moreover, the tubule-like structures formed by ECs remarkably decreased in the Erk inhibitor group (Figure 4c,i). These assays confirmed that inhibition of the Erk1/2 signaling pathway could partially inhibit the MOB-Exo let-7f-5p-induced angiogenesis of ECs, which demonstrated that the...
Erk1/2 signaling pathway was one target for the positive regulatory effects of MOB-Exo let-7f-5p.

3.5 | MOB-Exo let-7f-5p activated the Erk1/2 signaling pathway via suppressing DUSP1

After retrieving TargetScan, PicTar, miRanda, and microT databases, 198 shared target genes were obtained via Venn analysis (Figure 5a). GO database analysis found that 12 target genes were involved in the regulation of mitogen-activated protein kinase (MAPK) signaling pathway, of which 10 target genes except for DUSP1 and DUSP4 positively regulated MAPK signaling pathway (Figure 5b). And DUSP1 and DUSP4 enriched in MAPK phosphatase site of Erk1/2 signaling pathway (Figure 5c). Based on above analysis, DUSP1 and DUSP4 were likely to be the target genes of let-7f-5p.

Then, RT-PCR and dual luciferase reporter assays were performed. The results showed that mimicking exosomal let-7f-5p significantly suppressed the expression of DUSP1 in ECs (Figure 5d). let-7f-5p mimics suppressed the luciferase activity of cells transfected with vectors of wide-type DUSP1 by 39%, while mutation of let-7f-5p seeding region within DUSP1 abrogated the repressive ability of let-7f-5p (Figure 5e), confirming the specificity of the target sequence for DUSP1. Besides, DUSP4 expression levels was confirmed to be unregulated by let-7f-5p (Figure 5f,g).

3.6 | MOB-Exo let-7f-5p promoted the angiogenesis via the DUSP1/Erk1/2 signaling pathway in vivo

Based on the in vitro results that MOB-Exo let-7f-5p promoted the angiogenesis of ECs via the DUSP1/Erk1/2 signaling pathway, matrigel plug assays were performed to investigate the pro-angiogenic effects of MOB-Exo let-7f-5p in vivo. The gross view of xenografts showed that more blood vessels formed in matrigel plugs in the miR mimic group and the less in miR inhibitor group (Figure 6a). And also, the higher expression of CD31 and VEGF in the miR mimic group and the lower in the miR inhibitor group were respectively detected by immunofluorescence staining and western blotting (Figure 6b,e,h,i), which revealed the pro-angiogenic effects of MOB-Exo let-7f-5p on the matrigel plugs. Corresponding to the angiogenesis, the expression...
of pErk1/2 was much higher in the miR mimic group and lower in the miR inhibitor group (Figure 6c,f,j,k), revealing the important role of the Erk1/2 signaling pathway in MOB-Exo let-7f-5p promoting angiogenesis in vivo. Meanwhile, the expression of DUSP1 was conversely lower in the miR mimic group and higher in the miR inhibitor group (Figure 6d,g,l,m), which exhibited a reverse trend to that of pErk1/2 expression and indicated that DUSP1 was most probably the target gene that MOB-Exo let-7f-5p activated the Erk1/2 signaling pathway.

4 | DISCUSSION

As important structures for supplying nutrition and removing metabolic wastes, blood vessels are crucial for maintaining the normal physiological function of tissues. A close synergy existed between angiogenesis and osteogenesis. Angiogenesis promoted the continuous differentiation of BMSCs into OBs and then participated in bone formation and homeostasis maintenance (Cheng et al., 2018). OBs could secrete various angiogenic factors to enhance the angiogenic abilities of ECs (Yang, Jiang, et al., 2019). OB-derived VEGF promoted the angiogenic response in the inflammation phase of bone repair and strengthened the coupling of angiogenesis and osteogenesis in repairing areas by intramembranous ossification (Hu & Olsen, 2016), which proved that OBs could directly influence endothelium angiogenesis and trigger the synergy of angiogenesis and osteogenesis. Because of the key role of angiogenesis in the formation of tissue-engineered bone (Litvinova et al., 2020), this “angiogenesis-osteogenesis” coupling mechanism might provide a new potential way to promote the formation of tissue-engineered bone.
Exosomes, as important intercellular messengers, were proved of great importance in promoting fracture healing by accelerating angiogenesis and osteogenesis (Zhang et al., 2020). However, the functions of exosomes were closely related to the source cell types and pathophysiological states. Exosomes derived from OBs exhibited relatively weaker inhibition on T cell proliferation activity and did not affect on promoting T cell regulatory phenotype compared to exosomes derived from osteosarcoma cells (Troyer et al., 2017). Exosomes derived from OBs in patients with hip osteoarthritis significantly inhibited the activity and osteogenic differentiation of BMSCs compared to those in patients with osteoporosis (Niedermair et al., 2020). Therefore, although exosomes derived from BMSCs and MSCs were proved of proangiogenic effects (Gong et al., 2017; Zhang et al., 2020), whether these effects existed in exosomes derived from MOBs was undefined. To this end, our study utilized MC3T3-E1, a frequently-used cell line for tissue-engineered bone which was of the same genetic materials as the relevant living cells and extremely similar pathophysiological functions, to carry out experiments, and applied exosome-removed FBS to avoid the interference of FBS-derived exosomes, and ultimately demonstrated that MOB-Exos could significantly promote the angiogenesis of ECs, which revealed that MOB-Exos might be the potential factors for accelerating tissue engineering bone formation.

let-7f-5p, as a member of the let-7 family, was closely related to immune response, inflammatory response, and cell differentiation, for which it had become a hot target for early diagnosis and treatment of a variety of diseases (Li et al., 2019; Shen et al., 2019). Studies had shown that let-7f-5p could effectively reverse the inhibition of angiogenesis induced by smoking (Dhahri et al., 2017), and the plasma-derived exosomal let-7f-5p had significant sensitivity and specificity in this process (Singh et al., 2020). The current study demonstrated the exosome-dependent manner of MOBs transporting let-7f-5p into ECs and confirmed the positive regulatory effects of MOB-Exo let-7f-5p on the angiogenic capabilities of ECs.
Erk1/2 signaling pathway, as the most important pathway in the MAPK signaling pathway, was closely related to cell proliferation, migration, apoptosis, skeleton formation, and maintenance (Koga et al., 2019), and had been considered as an important target for the treatment of malignant tumors (Ghali & Ghali, 2020) for its close relationship with angiogenesis (Medfai et al., 2019; Zhang et al., 2019). Exosomal miRNAs regulating angiogenesis was also closely associated with the Erk1/2 signaling pathway. Exosomal miRNA-21 from adipose-derived stem cells promoted angiogenesis by significantly up-regulating the expression of pErk1/2 (An et al., 2019). BMSC-derived exosomal miRNA-21-5p promoted angiogenesis by inhibiting spry2 gene expression and then activating the Erk1/2 signaling pathway (Wu et al., 2020). The current study found that mimicking let-7f-5p of MOB-Exos could promote the expression of pErk1/2, while inhibiting let-7f-5p obtained opposite results, indicating the important role of the Erk1/2 signaling pathway in MOB exosomal let-7f-5p promoting angiogenesis of ECs, which was further confirmed by comparative studies via inhibiting the Erk1/2 signaling pathway in the let-7f-5p mimic group. These results were also supported by the important effects of the Erk1/2 signaling pathway on exosomal miRNAs regulating angiogenesis.

miRNAs could not directly activate the Erk1/2 signaling pathway, but by reducing the target mRNA stability or promoting its degradation, thus inhibiting the expression of target genes at the protein translation level (Yang, Chen, et al., 2019). DUSPs could inactivate MAPK by dephosphorylating threonine and tyrosine at the T-X-Y site of the MAPK kinase domain, thus antagonizing cell signaling cascades (Chuang & Tan, 2019). DUSP1 and DUSP4, as members of the DUSPs family, negatively regulated the Erk1/2 signaling pathway (Haddock et al., 2019; Yang, Sun, et al., 2019), and were closely associated with some miRNAs’ functions. miRNA-101 in the ventrolateral orbital cortex improved the depression-like behavior of rats by inhibiting the expression of DUSP1 to activate the downstream Erk1/2 signaling pathway (Zhao et al., 2017). miRNA-122-5p promoted the expression of DUSP4 and inhibited the Erk1/2 signaling pathway in pulmonary microvascular ECs (Lu, Feng, et al., 2020). Our study found that let-7f-5p could suppress DUSP1, but not DUSP4 expression, and confirmed that DUSP1 was the target gene of let-7f-5p. Our study didn’t investigate the effects of DUSP1 on pErk1/2 expression, but studies confirmed that suppressing DUSP1 expression could upregulate the expression of pErk1/2 (Ge et al., 2019; Shao et al., 2021), which strongly supported our conclusion.

5 | CONCLUSION

Our findings demonstrated that exosomal let-7f-5p derived from MOBs could significantly promote the angiogenesis of ECs, and the underlying mechanism might be that let-7f-5p activated the Erk1/2 signaling pathway by suppressing the expression of DUSP1. MOB-derived exosomal let-7f-5p might be a promising target for tissue engineering bone formation.
Yiqun He carried out the cytological studies and the statistical analysis, participated in the qPCR and western blotting assays, and drafted the manuscript. Hailong Li mainly carried out the qPCR and western blotting assays. Zuochong Yu mainly carried out the Dual luciferase reporter assays. Linli Li participated in the qPCR and western blotting assays. Xujun Chen participated in the Dual luciferase reporter assays. Aolei Yang participated in the statistical analysis. Feizhou Lyu conceived of the study, participated in its design and coordination, and helped to draft the manuscript. Youhai Dong designed the study, participated in its coordination, and revised the manuscript. All authors read and approved the final manuscript.

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**CONFLICT OF INTEREST**

The authors confirm that there are no conflicts of interest.

**DATA AVAILABILITY STATEMENT**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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