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The effect and mechanism of ICA regulation of miR-122-5p which from osteoblasts-derived exosomes on osteogenesis and migration of BMSCs

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Abstract

Background: Avascular necrosis of femoral head (ANFH) is a common disease in orthopedics, which seriously affects the quality of life of patients. At present, there is no clear method to solve it in clinical practice. In order to cope with this worldwide problem, many scholars have devoted themselves to the research on the treatment of ANFH. Promoting the osteogenic differentiation and directed migration of stem cells is one of the main ideas for the treatment of ANFH. In recent years, more and more researches have begun to pay attention to Chinese medicine and Chinese medicine extracts. At present, in the field of bone regeneration, epimedium is the most in-depth study of Chinese herbal medicine, and Icariin is one of the main active ingredients of epimedium.

Methods: Different concentrations of Icariin were applied to bone marrow mesenchymal stem cells (BMSCs), and the optimal concentration of Icariin to promote osteogenic differentiation and migration of BMSCs was observed. Then the exosomes from osteoblasts were combined with the optimal concentration of Icariin to BMSCs, observe the effect of promoting osteogenic differentiation and migration of BMSCs. Using high-throughput sequencing, analyze the composition of osteoblast-derived exosomes, predict the target miRNA, and then use miRNA mimic and inhibitor to verify.

Results: The optimal concentration of Icariin to promote osteogenic differentiation and migration of BMSCs is 1×10^{-7} M. Icariin combined with osteoblast-derived exosomes can effectively promote the osteogenic differentiation and migration of BMSCs. The top four miRNAs which content in

osteoblast-derived exosomes are let-7a-5p, miR-100-5p, miR-21-5p, miR-122-5p, and analysis shows that miR-122-5p is related to osteogenic differentiation, The verification found that miR-122-5p can significantly promote the osteogenic differentiation and migration of BMSCs.

Conclusions: Icariin can clearly promote the osteogenic differentiation and migration of BMSCs, and osteoblast-derived exosomes can also effectively promote the osteogenic differentiation and migration of BMSCs, the combination of Icariin and osteoblast-derived exosomes can enhance this effect. In this process, one of its targets is miR-122-5p.

Keywords: Exosomes, Icariin, miRNA, osteogenic differentiation, migration

1 Background

Avascular necrosis of femoral head (ANFH) is a common and frequently-occurring disease in orthopedics[1], most of which are between 20 and 50 years old, which seriously affects the quality of life of patients [2]. At present, there is no clear method to solve it. In order to cope with this worldwide problem, many scholars have invested in the research of ANFH treatment [3, 4]. Among them, the idea of applying bone marrow mesenchymal stem cells (BMSCs) to the treatment of ANFH is popular. Femoral head necrosis, in the final analysis, is a decrease in bone mass, osteogenic differentiation in bone metabolism is reduced and osteoclasts are active, and bone metabolism balance is broken. Therefore, research to promote the osteogenic differentiation and directional migration of stem cells is the main idea for research and treatment of ANFH. The use of drugs, physical stimulation, weightlessness environment, etc. to intervene on stem cells to observe the effects of osteogenic differentiation and migration, and finding effective methods has become the focus of many scholars [5-10]. In recent years, more and more researches have begun to pay attention to traditional Chinese medicine because of its low toxicity. Traditional Chinese medicine and its extracts have aroused the interest of many scholars [11].

At present, in the field of bone regeneration, Epimedium is the most studied Chinese herbal medicine [12]. In traditional Chinese medicine research, Epimedium has the function of strengthening muscles and bones, nourishing the kidneys and producing marrow [13], and Icariin (ICA, C₃₃H₄₀O₁₅, molecular weight: 676.66) is one of the main active ingredients of Epimedium. At the same time, modern medical analysis believes that exosomes are a good medium for communication between cells in the body, and they can easily enter and exit cells for the delivery of biologically active ingredients. It is also a good drug carrier, participating in various regulation of the body. Exosomes directly or indirectly participate in the regulation of bone metabolism balance, and play a certain role in maintaining bone metabolism balance[14]. Exosomes can promote the osteogenic differentiation of BMSCs, which may be related to the miRNAs contained in them. Finding the target miRNAs that function in osteoblast-derived exosomes is the key to understanding the underlying mechanism of action.

This study expects that ICA in Epimedium, combined with targeted exosomes, will act on BMSCs with directional differentiation ability, and explore its ability to promote osteogenic differentiation of BMSCs, so as to find an effective method for the treatment of ANFH. We suspect that ICA can regulate a specific miRNA in osteoblast-derived exosomes to play the role of osteogenesis and migration, and it has a better effect than ICA and can play a synergistic effect.

2 Materials and methods

2.1 Materials and reagents

Bone Marrow Mesenchymal Stem Cell Complete Medium (Sciencell, USA), Rabbit Bone Marrow Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cyagen Biosciences, USA), Fetal Bovine Serum FBS (Gibco, USA), Icariin (Sigma Company, USA), EZNA Total RNA Extraction Kit (OMEGA Company, USA), RNA Reverse Transcription Kit (TOYOBO Company, Japan), SYBR Green qPCR Kit (TOYOBO Company, Japan), DMSO Solution (Sigma Company, United States), Rabbit Runx2 gene SiRNA (Guangzhou Ruibo Biotechnology Co., Ltd., China), AMD3100 (Invitrogen, United States), transfection reagent Lipofectamin2000 (Invitrogen, United States), CCK-8 kit (Dalian Meilunbio, China), GAPDH antibody (Abcam, USA), Runx2 antibody (Bioss, USA), BMP-2 antibody (Bioss, USA), Osterix antibody (Bioss, USA), Smad8 antibody (Santa, USA), Smad1 Antibody (Bioss, USA), Smad5 antibody (Bioss, USA), CXCR4 antibody (Santa, USA), SDF-1 α antibody (Bioss, USA), HRP-Goat anti Rabbit (Abcam, USA), HRP -Goat anti Mouse (Abcam, USA), Exosome extraction kit (System Biosciences, USA), CD63 antibody (Santa cruz, USA), Tsg101 antibody (Bioss, USA), HSP70 antibody (Proteintech, United States), miR-122-5p mimic NC (Guangzhou Ruibo Biotechnology Co., Ltd., China), miR-122-5p mimic (Guangzhou Ruibo Biotechnology Co., Ltd., China), miR-122-5p inhibitor NC (Guangzhou Ruibo Bo Biotechnology Co., Ltd., China), miR-122-5p inhibitor (Guangzhou Ruibo Biotechnology Co., Ltd., China).

2.2 Experimental method

2.2.1 Extraction, identification and culture of rabbit BMSCs

Refer to the previous experimental methods of our research team to extract, culture and identify BMSCs [15].

2.2.2 Extraction, culture and identification of rabbit osteoblasts (OB)

Refer to John's method [16], take the two ends and stems of the limbs of a 6-month-old New Zealand white rabbit, separate the periosteum on the bone surface, cut it, centrifuge (600 g/min, 5min), add 0.2% type I collagenase and put it in a 37°C thermostat for 3-4h. Take out the centrifuge tube and centrifuge (600g/min, 5min), discard the supernatant, add PBS containing double antibody and wash it again, centrifuge (600g/min, 5min) to remove the supernatant, repeat once. Finally, the precipitate obtained by removing the supernatant is mixed with 1640 medium by pipetting, and then aliquoted into T25 culture flasks, and cultured in a CO₂ incubator (37°C, 20% O₂ concentration, 5% CO₂ concentration), when the cell fusion reaches 80% ~90%, pass it down again. Observe the cell morphology under a microscope, and identify the cells by Giemsa staining and Alizarin red staining.

2.2.3 Extraction and identification of osteoblast-derived exosomes(OB-exo)

The P3 generation of osteoblasts are routinely cultured, and the status of the cells is observed with a microscope. If the cells are in good condition, subculture is carried out. The subcultured osteoblasts are then expanded and cultured. After the culture scale can meet the requirements for collecting the supernatant (the total volume of the supernatant is 240ml), stop expanding the culture and continue the culture for 24 hours. After the cell status is stable, discard the original medium and change to 1640 medium without FBS. After continuing the culture for 48 hours, place it in the ultra-clean workbench to collect the cell supernatant and use it in the next centrifugation. ① Kit extraction method: After the osteoblasts are expanded and cultured, the supernatant is taken,

centrifuged with a low-speed centrifuge (1200g/min, 15min), and the centrifuged supernatant is transferred to an ultrafiltration tube with a pore size of 100kD, under the condition of 4°C to ultrafiltration, concentration and centrifugation (4000g/min, 40-60min). Then discard the liquid at the bottom of the tube, transfer the concentrated liquid in the upper tube to the EP tube, add one-fifth volume of the exosome extraction reagent SBI, mix well, and incubate at 4°C overnight (not less than 16h). On the 2nd day, the above-mentioned mixed solution was centrifuged again (4000g/min, 3-5min), and the supernatant was discarded after centrifugation. A white flake precipitate appeared at the bottom of the EP tube, and this white substance was exosomes. Place the EP tube containing exosomes in the centrifuge again to continue centrifugation (4000g/min, 3-5min), and suck up the remaining liquid. Use 20μl PBS to resuspend the exosomes at the bottom of the EP tube, mix by pipetting, and store at -80°C for later use. ②Ultracentrifugation extraction method: After the osteoblasts are expanded and cultured, the supernatant is taken, centrifuged with a low-speed centrifuge (1200g/min, 15min) to remove cell debris, and the centrifuged supernatant is transferred to a high-speed centrifuge tube at 4°C high-speed centrifugation (4000g/min, 60min). Transfer the supernatant after centrifugation to an ultracentrifuge tube, ultracentrifuge at 4°C (4000g/min, 70min), discard the supernatant, repeat this ultracentrifugation operation once, when the second centrifugation is completed, the bottom of the centrifuge tube appears white flake precipitation, this white matter is exosomes. Gently discard the supernatant, pay attention to this step, be very careful, and move gently. Then use 200μl of PBS to resuspend the exosomes at the bottom of the centrifuge tube, pipette to mix, collect and transfer to a 1.6ml EP tube, store at -80°C for later use.

2.2.4 High-throughput sequencing and miRNAs analysis of OB-exo

When culturing osteoblasts, divide three independent culture flasks to extract OB-exo, and finally obtain three exosomes samples, perform high-throughput sequencing on the three samples, and finally take the average value , Get the sequencing result.

2.2.5 The effect of ICA on osteogenic differentiation and migration of BMSCs

2.2.5.1 The effects of different concentrations of ICA on the proliferation, osteogenesis and migration of BMSCs

P3 generation BMSCs were inoculated into six-well plates, and the following tests were performed respectively: ①Divided ICA into 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M, 1×10^{-5} M, 1×10^{-4} M each group. CCK-8 kit detects the activity and proliferation ability of BMSCs at different concentrations of ICA; ②Different concentrations of ICA (0, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M) and the mixture of culture medium was added to different wells after 2w culture and stained with Alizarin Red dye to observe the formation of mineralized nodules in each group; ③Different concentrations of ICA (0, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M) and medium mixtures were added to different wells, and RNA of each group of cells was extracted on 3d and 7d for osteogenic differentiation and migration related gene mRNA detection; ④Add the mixture of ICA (0, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M) and culture medium at different concentrations into different wells , Extract the protein of each group of cells on 3d and 7d, and perform Western blotting for osteogenic differentiation and migration related genes. Through these experiments we will determine the optimal concentration of ICA.

2.2.5.2 The effect of ICA at different times on the osteogenic differentiation and migration ability of BMSCs

P3 generation BMSCs were inoculated into six-well plates, and the following tests were performed: ①The mixture of $1 \times 10^{-7} \text{M}$ ICA and culture medium was added to different wells, and the cells of each group were extracted on 3d, 7d, and 14d. mRNA for osteogenic differentiation and migration related genes mRNA qPCR detection; ②Add $1 \times 10^{-7} \text{M}$ concentration of ICA and medium mixture to different wells, respectively on 1d, 2d, 3d, 4d, 5d, 6d, and 7d, then the mRNA of each group of cells was extracted, and the osteogenic differentiation and migration-related genes mRNA were detected by qPCR; ③The mixture of $1 \times 10^{-7} \text{M}$ ICA and culture medium was added to different wells, respectively, on 1d, 2d, 3d, 4d, 5d, 6d, and 7d, the cell proteins of each group were extracted, and Western blotting was performed to detect the proteins related to osteogenic differentiation and migration.

2.2.5.3 Observe the influence of ICA on the expression of Runx2 signal axis and CXCR4 signal axis

P3 generation BMSCs were evenly inoculated into 4 wells in a six-well plate. When the cell fusion reached 60%-80%, the culture medium was discarded, the four groups of different treatments were added to the corresponding culture medium, and the four groups were cultured. The four groups of culture medium are blank group, ICA $1 \times 10^{-7} \text{M}$ group, ICA $1 \times 10^{-7} \text{M}$ +si-Runx2 group, and ICA $1 \times 10^{-7} \text{M}$ +AMD3100 group. Among them, the preparation process of si-Runx2 solution is as follows. Dilute the pre-prepared si-Runx2 solution and DMEM at a ratio of 1:50. At the same time, the transfection reagent Lipofectamin 2000 and DMEM are also diluted at a ratio of 1:50. The two solutions are diluted at the same time and incubated at room temperature for 5 minutes. After 5 minutes, the two solutions are mixed in equal proportions and placed at 37°C and incubated for 20 minutes. This is to prepare si-Runx2 solution to be added; The solution concentration of AMD3100 is $50 \mu\text{mol/L}$ and added to the corresponding wells. After culturing for 3 days, the mRNA of each group of cells was extracted, and the mRNA of genes related to osteogenic differentiation and migration was detected.

2.2.6 The effect of ICA combined with OB-exo on the osteogenic differentiation and migration ability of BMSCs

Experimental grouping and culture conditions: The conclusion from the previous experiment is that the optimal concentration of ICA on BMSCs is $1 \times 10^{-7} \text{M}$, and the ICA at this concentration is used for follow-up research. P3 generation BMSCs were inoculated into 4 wells of two six-well plates. When the cell fusion reached 60%-80%, the culture medium was discarded, and the four groups of different treatments were added to the corresponding culture medium. The culture medium was blank group, ICA group, OB-exo group, and ICA+OB-exo group. Among them, the amount of exosomes in the OB-exo group and the ICA+OB-exo group were both $20 \mu\text{l}$. The PBS solution containing exosomes was thawed and mixed gently. On the 3rd day, one six-well plate was used to extract mRNA and one was used to extract protein. The mRNA and protein expression of genes related to bone differentiation and migration were detected by qPCR and Western blotting.

2.2.7 ICA regulates the effect of OB-exo miR-122-5p on the osteogenic differentiation and migration ability of BMSCs

In the previous experiment, the top twenty miRNAs in exosomes were screened through high-throughput sequencing, and miR-122-5p was selected for subsequent research. The mimic NC, mimic, inhibitor NC, and inhibitor of miR-122-5p synthesized by Ruibo were first divided into four groups (miR-122-5p mimic NC group, miR-122-5p mimic group, miR-122-5p mimic NC+ICA group, and

miR-122-5p mimic+ICA group), added to BMSCs for culture, cultured for 3 days, collect BMSCs, use qPCR and Western blotting to detect osteogenesis (Runx2, BMP-2, Osterix, Smad1 /5/8, OCN, OPN, ALP) and migration (SDF-1 α , CXCR4) mRNA and protein expression, observe the effect of each group. Among them, the final concentration of miR-122-5p mimic NC and miR-122-5p mimic solutions is 50nM. Secondly, divide into four groups (miR-122-5p inhibitor NC group, miR-122-5p inhibitor group, miR-122-5p inhibitor NC+ICA group, and miR-122-5p inhibitor+ICA group) , culture for 3 days, collect BMSCs, use qPCR and Western blotting to detect osteogenic (Runx2, BMP-2, Osterix, Smad1/5/8, OCN, OPN, ALP) and migration (SDF-1 α , CXCR4) mRNA and protein expression, observe the effect of each group. Among them, the final concentration of miR-122-5p inhibitor NC and miR-122-5p inhibitor solutions is 100nM.

First, observe the effect of miR-122-5p mimic and ICA on the osteogenic differentiation and migration ability of BMSCs. P3 generation BMSCs were inoculated into 4 wells of two six-well plates. When the cell fusion reached 60%-80%, the culture medium was discarded, and the four groups of different treatments were added to the corresponding culture medium. The culture media were miR-122-5p mimic NC group, miR-122-5p mimic group, miR-122-5p mimic NC+ICA group, and miR-122-5p mimic+ICA group. After culturing for 3 days, a six-well plate was used to extract mRNA and one was used to extract protein. The mRNA and protein expression of genes related to bone differentiation and migration were detected by qPCR and Western blotting.

Then, observe the effect of miR-122-5p inhibitor and ICA on the osteogenic differentiation and migration ability of BMSCs. P3 generation BMSCs were inoculated into 4 wells of two six-well plates. When the cell fusion reached 60%-80%, the culture medium was discarded, and the four groups of different treatments were added to the corresponding culture medium. The culture medium is the miR-122-5p inhibitor NC group, miR-122-5p inhibitor group, miR-122-5p inhibitor NC+ICA group, and miR-122-5p inhibitor+ICA group. After culturing for 3 days, a six-well plate was used to extract mRNA and one was used to extract protein. The mRNA and protein expression of genes related to bone differentiation and migration were detected by qPCR and Western blotting. The primer sequence of each gene is shown in Table 1.

Table 1 The primer sequence of each gene

GAPDH	Forward 5'-TGGAATCCACTGGCGTCTTC-3' Reverse 5'-GGTTCACGCCCATCACAAAC-3'
Runx2	Forward 5' -AGCGGTCCACTACGTTACCTG-3' ' Reverse 5' -TCCGACACGGTTCGACCGC-3'
BMP-2	Forward 5'-GCGAGTTGTATTTTCGTAACCA-3' Reverse 5'-GAACGTCCCGATCTCGGAC-3'
Osterix	Forward 5'-TCCCTGGATATGACTCA TCCCT-3' ' Reverse 5'-CCAAGGAGTAGGTGTGTTGCC-3'
Smad1	Forward 5'-CTAGCGTTAGCGACCGAGCGA-3' Reverse 5'-GCAGAGCGGGTTATGCGGA-3'
Smad5	Forward 5'-GTCGAGGTCAACCTACFCAG-3' Reverse 5'- GACCGGAATATGGCGAGCTC-3'
Smad8	Forward 5'-CCAGCAAGTGCGTCACCATCC-3' Reverse 5'-GCACTCCAGCGGCTTCAACTC-3'
CXCR4	Forward 5'-GCAGCAGCAGCTACTTTGACG-3' '

	Reverse 5'-GACTCGTTCAGTTTAAACGGGG-3'
SDF-1 α	Forward 5'-ATGCCCTGCCGATTCTTTG-3' Reverse 5'-GGGCACAGTTTGGAGTGTGA-3'
ALP	Forward 5'-TCTTGGGTCTCTTTGAGCCG-3' Reverse 5'-CTCGGGGGTTCTTCTTCAGG-3'
OPN	Forward 5'-TCCAAAGTCAGCCAGGAATCC-3' Reverse 5'-CGGAGTTGTCTGTGCTCTTCA-3'
OCN	Forward 5'-CTCCTTACCCGGATCCCCTG-3' Reverse 5'-GTAGAAGCGCTGGTAGGCGT-3'

2.3 Statistical analysis

For the data, use SPSS 21.0 statistical software to perform statistical analysis. Measurement data are expressed as mean \pm standard deviation ($\bar{X} \pm S$). Count data is subjected to chi-square test. One-way analysis of variance is used between multiple samples. The t test was used for comparison between groups. $p < 0.05$ means the difference is statistically significant. GraphPad Prism 6 software was used to process the data to generate histograms.

3 Results

3.1 Morphological observation and flow cytometry identification of BMSCs

Figure 1 shows that under different magnification microscopes, BMSCs are spindle-shaped and grow in a whirlpool shape. Flow cytometry identified the three cell phenotypes of CD29, CD44, and CD45. CD29 and CD44 were positive, and CD45 was negative.

3.2 Observation and identification of OB morphology

Figure 2 shows that the OB cells are flat and spread, with various shapes, such as fusiform, polygonal, triangular, etc., with protrusions of different lengths and nuclei. At the same time, the cytoplasm is abundant, with 2 nuclei occasionally, and a spiral growth trend. The OB cells stained by Giemsa are fusiform or triangular, with rich cytoplasm and stained purple blue, while the nucleus is stained dark blue. The nucleus is located in the center of the cell and is round, there are 2 nucleoli in some cells, which are more obvious. After OB cultured for 2 weeks, a large number of scattered mineralized nodules were seen, and the mineralized nodules were stained orange-red after staining with Alizarin Red.

3.3 Morphology and identification of OB-exo

Figure 3 shows that under different magnifications of the transmission electron microscope field of view, the OB-exo show a clear vesicle-like structure with a particle size between 50nm-200nm. Compared with the control group, the expression levels of Tsg101, Hsp70, and CD63 three marker proteins of the exosomes obtained by ultracentrifugation and the exosomes obtained by the kit were significantly higher, indicating that the two methods obtained were exosomes. At the same time, the amount of exosomes obtained by the kit is greater than that obtained by ultracentrifugation.

3.4 High-throughput sequencing of OB-exo

Figure 4 shows the components in the exosomes and the percentage of the top 20 miRNAs in the total exosomal miRNAs. Among them, you can see the number of the top 20 miRNAs target genes measured in the four software and the number of intersections displayed by the Venn diagram of the top 4 miRNAs target genes. There are 200 in let-7a-5p, and 38 in miR-100-5p, 197 in miR-21-5p, and 330 in miR-122-5p.

3.5 Observation of ICA on the proliferation, differentiation and osteoblast migration of BMSCs and its role in the signaling pathways

Figure 5 observes the activity and proliferation of BMSCs and the mineralization of BMSCs under the action of ICA. Compared with the control group, 24h culture found that ICA has a significant inhibitory effect on cell proliferation at two concentrations of $1 \times 10^{-4} \text{M}$ and $1 \times 10^{-5} \text{M}$, while ICA at a concentration of $1 \times 10^{-6} \text{M}$ does not inhibit or promote cell proliferation. ICA can promote cell proliferation at the three concentrations of $1 \times 10^{-7} \text{M}$, $1 \times 10^{-8} \text{M}$, $1 \times 10^{-9} \text{M}$; 48h culture found that ICA has a significant inhibitory effect on cell proliferation at three concentrations of $1 \times 10^{-4} \text{M}$, $1 \times 10^{-5} \text{M}$, $1 \times 10^{-6} \text{M}$, while ICA at a concentration of $1 \times 10^{-9} \text{M}$ has no inhibitory or promoting effect on cell proliferation. ICA at two concentrations of $1 \times 10^{-7} \text{M}$ and $1 \times 10^{-8} \text{M}$ has promote cell proliferation. After 2w culture, Alizarin Red staining found that the cells in the ICA concentration of $1 \times 10^{-7} \text{M}$ group had the most abundant mineralized nodules and the best mineralization effect.

Figure 6 is to observe the effect of different concentrations of ICA on the bone formation and migration of BMSCs. It can be seen in the figure that at 3d and 7d, ICA with a concentration of $1 \times 10^{-7} \text{M}$ co-cultured with BMSCs has the highest mRNA expression of osteogenic differentiation and migration related genes, and the difference is statistically significant ($P < 0.05$), indicating that the concentration of ICA on BMSCs to promote their osteogenic differentiation and migration is $1 \times 10^{-7} \text{M}$.

Figure 7 is to observe the change trend of osteogenic differentiation and migration ability of BMSCs by ICA on 1-7 days. Compared with the blank group, the mRNA expression of osteogenic differentiation-related genes (Runx2, BMP-2, Osterix, OPN, OCN) and migration-related genes (SDF-1 α , CXCR4) in the ICA group were higher at 3d, 7d, and 14d, and the difference was statistically significant ($P < 0.05$). The expression of osteogenic differentiation-related genes and migration-related genes mRNA reached the first peak on the 3rd day and reached the second peak on the 7th day. Among them, the expression was higher on the 7th day, the difference was statistically significant ($P < 0.05$). The expression of osteogenic differentiation-related and migration-related proteins reached the first peak on the 4th day, and reached the second peak on the 7th day, the difference between the two peaks was statistically significant ($P < 0.05$).

Figure 8 is to observe the signal pathway that ICA affects the osteogenic differentiation and migration ability of BMSCs. It can be seen in the figure that the difference between the ICA group and the ICA+si-Runx2 group, and the ICA+AMD3100 group is statistically significant ($P < 0.05$). It shows that both si-Runx2 and AMD3100 can effectively inhibit the promotion of ICA on the osteogenic differentiation and migration ability of BMSCs.

3.6 The effect of ICA combined with OB-exo on osteogenic differentiation and migration of BMSCs

Figure 9 shows that the ICA group, OB-exo group, and ICA+OB-exo group respectively compared with the blank group, the osteogenic differentiation and migration-related gene mRNA expression was significantly increased, while the ICA+OB-exo group was higher than the ICA group and OB-exo group, and the difference was statistically significant ($P < 0.05$). It shows that ICA combined with OB-exo can significantly promote the expression of mRNAs related to osteogenic differentiation and migration of BMSCs. Compared with the blank group, ICA group, OB-exo group, and ICA+OB-exo group had significantly higher expression of osteogenic differentiation and migration-related protein. At the same time, ICA+OB-exo was also higher than ICA and OB-exo protein expression, the difference was statistically significant ($P < 0.05$). It shows that ICA combined with OB-exo can significantly promote the expression of genes and proteins related to osteogenic differentiation and migration of BMSCs.

3.7 The effect of ICA combined with miR-122-5p on osteogenic differentiation and migration of BMSCs

Figure 10 and Figure 11 observe the effect of ICA combined with miR-122-5p on the osteogenic differentiation and migration ability of BMSCs. Figure 10 observes the effect of miR-122-5p mimic on the osteogenic differentiation and migration ability of BMSCs, and Figure 11 observes the effect of miR-122-5p inhibitor on the osteogenic differentiation and migration ability of BMSCs. It can be seen in the figure that the combined application of miR-122-5p mimic and ICA can significantly enhance the mRNA and protein expression of osteogenic differentiation and migration in BMSCs, comparison between groups, the difference was statistically significant ($P < 0.05$). While miR-122-5p inhibitor combined with ICA, the expression of osteogenic differentiation and migration in BMSCs decreased, comparison between groups, the difference was statistically significant ($P < 0.05$). Experimental results confirmed that the expression of miR-122-5p can significantly affect the osteogenic differentiation and migration ability of BMSCs, and the combination with ICA can synergistically enhance the osteogenic differentiation and migration ability of BMSCs.

4 Discussion

A large number of studies have found [17-30] that ICA can promote the proliferation and osteogenic differentiation of BMSCs. Cao et al. [31] showed that within 5 months of treatment, intragastric administration of ICA significantly accelerated the formation of callus and fracture healing in rats. Zhang et al. found that ICA inhibited the differentiation of BMSCs into adipocytes and promoted their differentiation into osteoblasts [32]. Fan et al. found that ICA not only promotes the proliferation of BMSCs in vitro in a dose-dependent manner, but also promotes their differentiation into osteoblasts at very low doses ($1 \times 10^{-9} \text{M}$ to $1 \times 10^{-6} \text{M}$). Of course, a higher concentration of $1 \times 10^{-5} \text{M}$ is toxic and inhibits its differentiation towards osteogenic [33]. Huang et al. [34] showed that ICA can effectively prevent femoral head necrosis, improve the proliferation of BMSCs induced by prednisolone, enhance osteogenic differentiation and inhibit adipogenic differentiation. In addition, low concentrations of ICA ($1 \times 10^{-9} \text{M}$ to $1 \times 10^{-5} \text{M}$) significantly enhanced the proliferation ability of BMSCs, especially at the concentration of $1 \times 10^{-6} \text{M}$ [33]. The above studies show that ICA can significantly promote the osteogenic differentiation of BMSCs. Some studies have shown that the optimal concentration of ICA on the osteogenic differentiation of BMSCs is $1 \times 10^{-6} \text{M}$ [33,34], and most researchers believe that the optimal concentration of ICA on the osteogenic differentiation of BMSCs is $1 \times 10^{-7} \text{M}$ [17,18,35], this concentration has also been further confirmed in our study.

In the experiment, we also observed that high-concentration ICA ($> 1 \times 10^{-5} \text{M}$) has a toxic effect on cells. In the CCK-8 test, most of the cells in the $1 \times 10^{-4} \text{M}$ group have died, which is basically consistent with the results reported in the literature. ICA acts on BMSCs, not only can promote their differentiation toward osteogenic direction, but also enhance their migration ability, which is not clearly proposed in the previous literature. Our research team has verified in the previous study that the Runx2 signal axis and the CXCR4 signal axis can cross-talk, suggesting that there may be a connection between osteogenic differentiation and migration [36]. But it is the first time to clarify that ICA can clearly promote the osteogenic differentiation and migration of BMSCs at the same time. At the same time, this study found that the optimal concentration of ICA to promote osteogenic differentiation and migration of BMSCs is $1 \times 10^{-7} \text{M}$. In the research, we also found that over time, ICA with a concentration of $1 \times 10^{-7} \text{M}$ promotes the osteogenic differentiation and migration of BMSCs, which has certain regularity. In 1-7d, the 3rd and 7th days are the two peak periods of osteogenic differentiation and migration-related gene mRNA expression. Among the two, the 7th

day's expression is higher; and Western blotting test found that in 1-7 days, the 4th and 7th days are the two peak periods of osteogenic differentiation and migration protein expression, of which the 7th day's expression is higher. The results of this study showed that ICA has the best time to act on BMSCs and has a certain periodicity, reaching another peak at an interval of about 3 days, which may be related to the metabolic cycle of cells. At the same time, it was found that the first peak of mRNA for detecting bone formation and migration occurred on the 3rd day and the first peak of protein occurred on the 4th day, the results of mRNA and protein expression are inconsistent, but the second peak of mRNA and protein both occurred on the 7th day. According to our analysis, in the early stage of cell culture, the expression of mRNA occurs before the protein, and the protein immediately follows. However, when the 7th day is reached, the role of ICA in promoting the osteogenic differentiation and migration of BMSCs becomes stronger and stronger, so that the peaks of mRNA and protein appeared in the same period. This research result provides a very important reference for the future of ICA to clinical trials and even clinical application of medication. In addition, it was also found in this study that ICA is positively related to the occurrence of bone formation and migration of BMSCs. Our previous study[36] have revealed that there will be a cross-talk between osteogenic differentiation and migration, and there is a crossover point between the two, namely CXCR4, but it has not been demonstrated that they are a co-occurring relationship. Then, whether osteogenic differentiation initiates migration, or migration promotes osteogenic differentiation, remains to be further studied.

In addition, in experiments to explore whether ICA plays a role through the Runx2 signal axis and CXCR4 signal axis, we found that both si-Runx2 and AMD3100 can effectively inhibit ICA's role in promoting osteogenic differentiation and migration. This result, from the opposite side, also verified that osteogenic differentiation and migration occur concurrently, inhibiting one of them, and the other effect is also inhibited. There is a cross-talk between these two signal axes, which can promote and inhibit each other.

Exosomes, due to their unique structural advantages, have the potential to become a good tool for transporting drugs to target cells to play a biological role. The outer layer of its phospholipid bilayer structure can effectively protect the contents from various biological enzymes in the environment, and at the same time, it can maintain the activity of various biological molecules. After reaching the target cell, its content communicates with the cell's content, thereby exerting a biological effect. Studies have found [37-41] that the use of electroporation, ultrafiltration, centrifugation, co-incubation and other methods can load specific drugs into the exosomes through the double-layer lipid structure of the exosomes, allowing the exosomes to carry the specific drug reaches the target cell and takes effect. Based on this, the pharmaceutical carrier has become an important direction of application of exosomes, which has broad application prospects.

The experimental results suggest that there is a concomitant relationship between osteogenic differentiation and migration. At the same time as osteogenic differentiation, they can reach a distant place through migration, and then continue to demonstrate the role of osteogenic. In this process, both ICA and OB-exo can promote the osteogenic differentiation and migration of BMSCs, and putting them together can enhance this effect. The results of detection of mRNA and protein related to osteogenic differentiation and migration by qPCR and Western blotting found that compared with the ICA group or the OB-exo group, the combined effect of ICA and OB-exo on BMSCs is better than the other two groups. It shows that ICA and OB-exo have a synergistic enhancement effect in effectively promoting the osteogenic differentiation and migration of BMSCs. In addition, the

experimental results also show that, compared with the control group, the ICA group and the OB-exo group had significant differences in the expression of osteogenesis and migration mRNA and protein, indicating that both ICA and OB-exo can promote the osteogenic differentiation and migration of BMSCs. So, when ICA and OB-exo are used at the same time, in what way do they play a synergistic effect? Is it a simple superposition or a more specific synergy mechanism? We think that the mechanism by which ICA and OB-exo can play a synergistic effect is roughly based on three factors: ① Both ICA and OB-exo have their own effects on BMSCs, promoting their osteogenic differentiation and migration, and finally their effects are superimposed, but from the experimental results, the result of the ICA+OB-exo group is not a simple addition of the results of the ICA group and the OB-exo group; ② ICA can enter the BMSCs more efficiently through the transport function of OB-exo, and then play a biological role. At the same time, OB-exo also exerts a biological effect on BMSCs; ③ ICA not only uses the transport function of OB-exo, but also regulates certain components in OB-exo such as miRNAs, promotes its expression, and then released into BMSCs to promote the expression of osteogenic differentiation and migration. The above are just our conjectures and still need to be verified.

The chemical composition of ICA is very clear, but the composition of OB-exo is more complicated, we hope to find the target miRNA that plays a role through a comprehensive analysis of the composition of OB-exo, so as to complete further verification work. High-throughput sequencing detection is an important method of modern scientific research, which allows people to obtain more accurate sequencing information. In the experiment, we sequenced OB-exo and analyzed the composition of it and found that miRNAs accounted for 17.47% of the total exosomes, indicating that miRNAs are abundant in OB-exo. Among them, the content of each miRNA is also very different. The existence of this difference implies that each miRNA has a different degree of activity in osteoblast metabolism. The high content of miRNA indicates that it is highly related to osteogenic differentiation, however, there may also be some miRNAs in various exosomes with high content, so further analysis is needed. Based on this, we select the top 20 miRNAs with content to enter the next step of analysis.

In order to screen out miRNAs related to osteogenic differentiation, we first searched for target genes related to the regulation of bone metabolism in the predicted target gene library, through software prediction, we found that miR-let-7a-5p, miR-100-5p, and miR-21-5p, none of the top 3 candidate gene banks have the above-mentioned “bone” related genes, but a qualified gene was found in the candidate gene library of miR-122-5p, and miR-122-5p was ranked 4th. In addition, our previous research found that miR-122-5p can clearly promote the proliferation of osteoblasts [42]. Combined with the results of this previous research, we predict that miR-122-5p is one of the target miRNAs for osteogenic differentiation.

MiR-122-5p has been used as a target for the study of many diseases, including acute kidney injury [43], cardiomyocyte injury [44], gastric cancer [45-48], kidney cancer [49], non-alcoholic Steatohepatitis [50], breast cancer [51], cervical cancer [52], etc. However, there are few studies related to the regulation of osteogenic differentiation and migration of BMSCs. A study by our team suggests that [42] miR-122-5p is involved in the regulation of osteoblast proliferation, but it does not involve the promotion of osteogenic differentiation and migration of stem cells.

In the experiment, we found that miR-122-5p mimic and miR-122-5p mimic+ICA can effectively promote the expression of osteogenesis and migration-related mRNAs and proteins, while miR-122-5p inhibitor and miR-122-5p inhibitor+ICA can effectively inhibit the expression of mRNAs and

proteins related to osteogenic differentiation and migration. This shows that miR-122-5p can target and activate related genes on the Runx2 signal axis and CXCR4 signal axis, and promote the expression of mRNAs and proteins related to osteogenic differentiation and migration. At the same time, in the presence of ICA, miR-122-5p target activation of Runx2 signal axis and CXCR4 signal axis related genes expression is more obvious, the expression of osteogenic and migration-related proteins is also more obvious, which in turn also shows that ICA promotes the osteogenic differentiation and migration of BMSCs, and miR-122-5p is playing a promoting role.

Based on the experimental results, we further analyzed that miR-122-5p in OB-exo is one of the target miRNAs that regulates the differentiation and migration of BMSCs. We can infer that:①ICA regulation of miR-122-5p promotes the osteogenic differentiation and migration of BMSCs;②miR-122-5p is the target of OB-exo to promote the osteogenic differentiation and migration of BMSCs;③miR-122-5p is also a target for ICA and OB-exo to promote osteogenic differentiation and migration of BMSCs. Based on this, we believe that after ICA-carrying exosomes (or ICA and exosomes are in a coexisting state) enter BMSCs, both ICA and miR-122-5p to activate the Runx2 signal axis and the CXCR4 signal axis related genes (such as Runx2, BMP-2, Osterix, SDF-1 α , CXCR4) to promote their expression, and then promote the expression of related proteins. Regarding the status of ICA and miR-122-5p in exosomes, and how they target and activate related genes on the Runx2 signal axis and CXCR4 signal axis, further studies are needed.

5 Conclusions

Icariin can clearly promote the osteogenic differentiation and migration of BMSCs, and osteoblast-derived exosomes can also effectively promote the osteogenic differentiation and migration of BMSCs, the combination of Icariin and osteoblast-derived exosomes can enhance this effect. In this process, one of its targets is miR-122-5p.

Abbreviations

ANFH: Avascular necrosis of femoral head; BMSCs: bone marrow mesenchymal stem cells; ICA: Icariin; OB: Osteoblast; OB-exo: osteoblast-derived exosomes; Runx2: Runt-related transcription factor 2; BMP-2: bone morphogenetic protein-2; CXCR4: C-X-C chemokine receptor type 4; SDF-1 α : Stromal-derived factor 1 α ; Smad: Drosophila mothers against decapentaplegic protein; ALP: Alkaline phosphatase.

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Authors' contributions

Zhanghua Li designed the study. Aofei Yang, Xiangzhong Liu, Lu Yang, Yu Ning, Hantao Cai and Jing Hu collated the data and carried out the data analyses. Aofei Yang, Xiangzhong Liu and Lu Yang wrote the main manuscript text. Zhanghua Li revised the manuscript. All authors have read and approved the final submitted manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Wuhan Third Hospital, Tongren Hospital of Wuhan University and followed the Guide for the Care and Use of Laboratory Animals. All animal experiments were conducted in the Animal Management Center of Wuhan Third Hospital, Tongren Hospital of Wuhan University. All efforts were made to minimize the number and suffering of the included animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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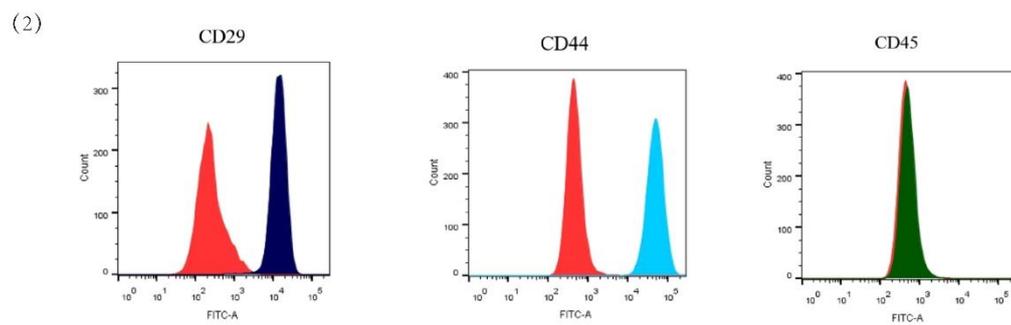
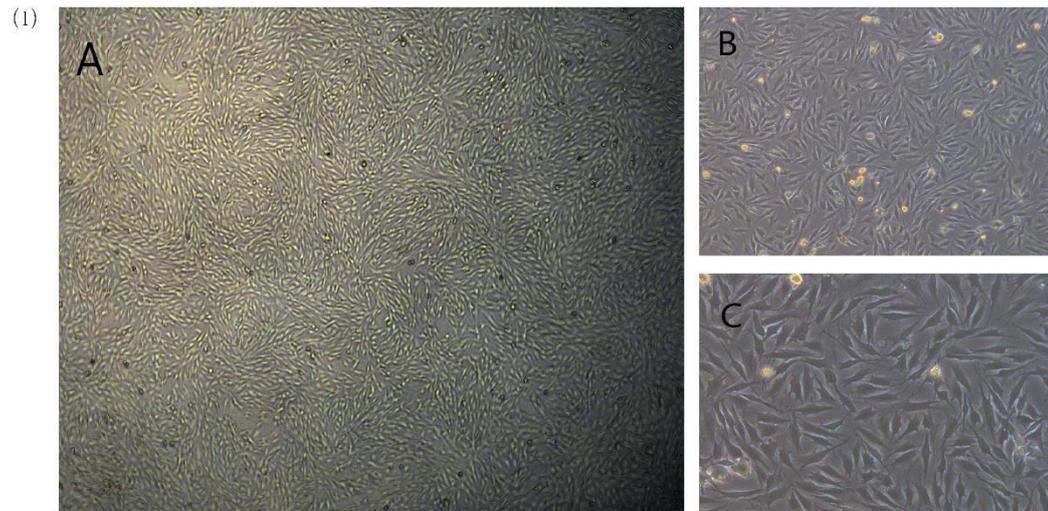


Figure 1 Cultivation and identification of BMSCs

Note: (1) AC showed that the P3 generation of BMSCs cultured for 24 hours, under the microscope, the morphological performance of different magnifications, where A is 4 \times , B is 10 \times , and C is 20 \times ; (2) Flow cytometry is used to measure the cell surface Markers (CD29, CD44, CD45) for identification.

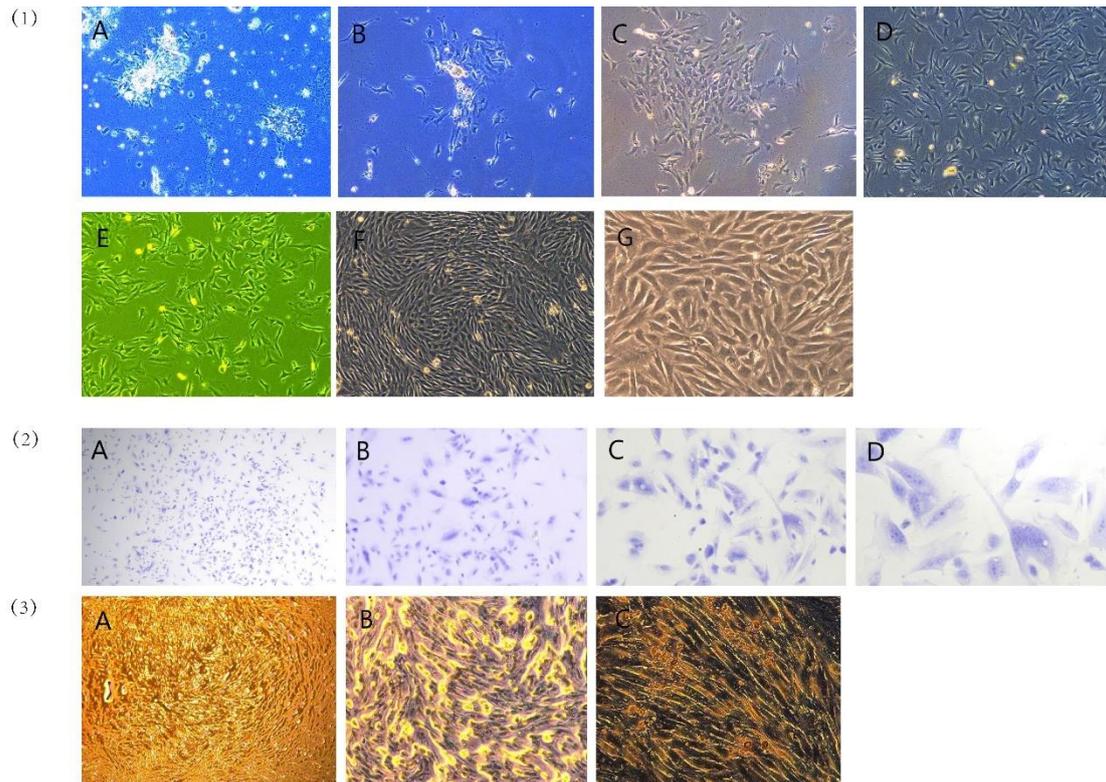


Figure 2 Cultivation and identification of OB

Note: (1) A-G shows the state of OB cells under the microscope. After OB cells were extracted and cultured for 3 days, until the 10th day, the cells slowly crawled out and continued to proliferate in large numbers. Among them, A is 3d, 10 \times , B is 4d, 10 \times , C is 5d, 10 \times , D is 7d, 10 \times , E is 8d, 10 \times , the first resuspended cell, F is 10d, 10 \times , G is 10d, 20 \times ; (2) A-D showed that Giemsa staining results of OB cells were observed under different magnification microscopes. Among them, A is 4 \times , B is 10 \times , C is 20 \times , D is 40 \times ; (3) A-C shows the mineralized nodules after 2w magnified by different magnifications of the microscope. Among them, A is 4 \times , B is 10 \times , C is 20 \times .

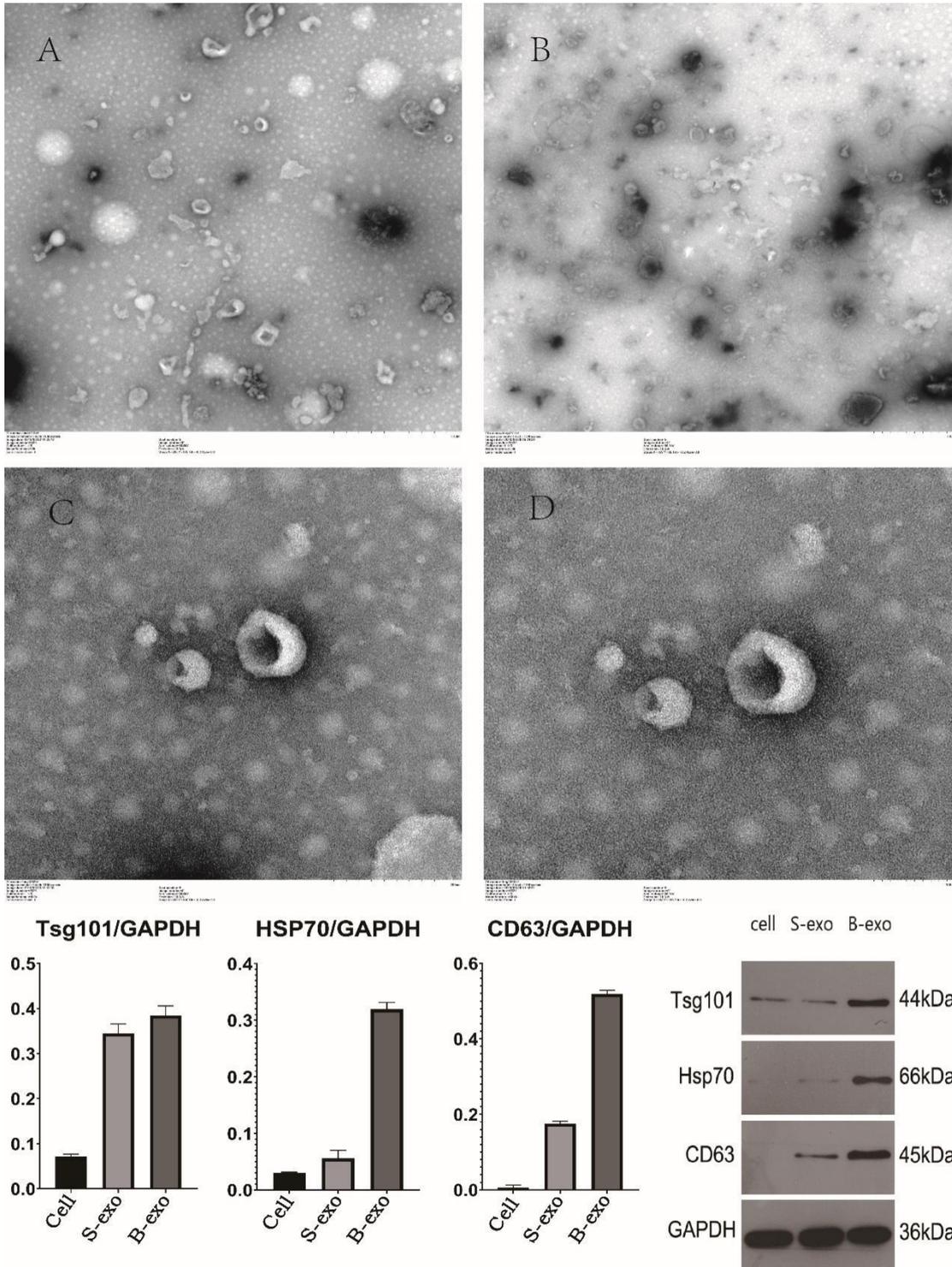
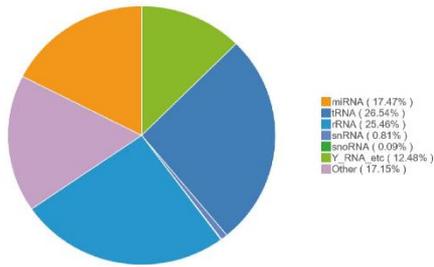


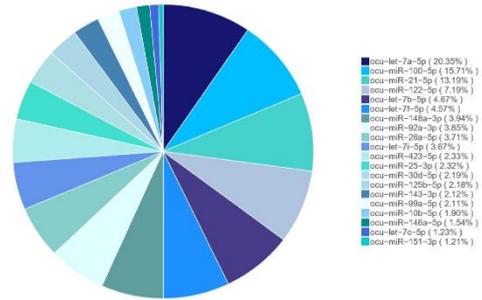
Figure 3 Extraction and identification of OB-derived exosomes

Note: (1) A-D shows the morphology and size of OB cell-derived exosomes under different transmission electron microscope fields. Among them, A and B are in a 1.0 μm field of view, C is in a field of 200 nm, and D is in a field of 100 nm. (2) Western blotting for the identification of exosomal marker proteins: detection of Tsg101, Hsp70, and CD63 (cell group, superisolated exosomes group, box exosomes group).

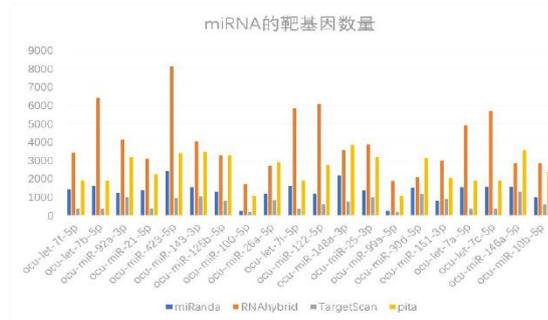
(1) Total Reads Annotation



(2)



(3)



(4)

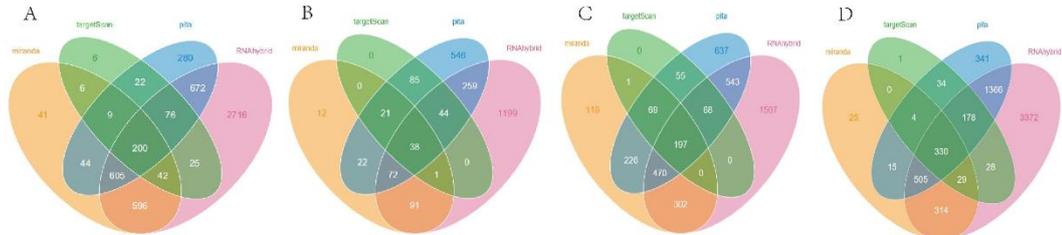


Figure 4 High-throughput sequencing and analysis of OB-derived exosomes

Note: (1) Distribution diagram of the internal components of exosomes. (2) The percentage of the top 20 miRNAs in the total miRNAs of exosomes. (3) The number of target genes of the top 20 miRNAs measured in the four software. (4) The intersection of the target genes of the top 4 miRNAs in the four software displayed by the Venn diagram (A is let-7a-5p, B is miR-100-5p, C is miR-21-5p, D is miR-122-5p).

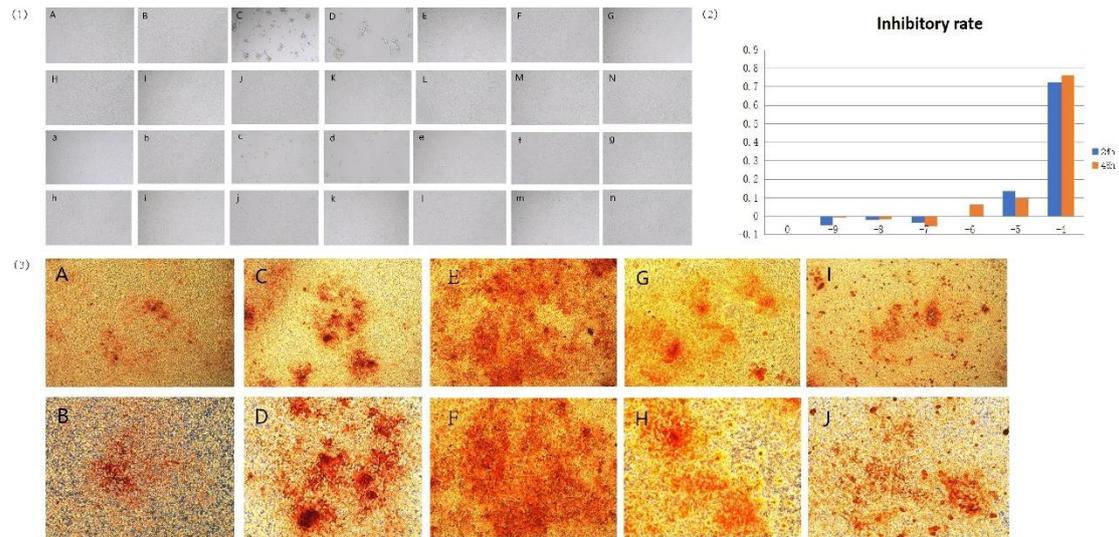


Figure 5 BMSCs activity and proliferation detection and BMSCs mineralization under the action of ICA

Note: (1) A-N is 24h. Under different magnification microscopes, the status of BMSCs under different concentrations of ICA, where A is ICA 0M, 10 \times , B is ICA 0M, 20 \times ; C is ICA 1×10^{-4} M, 10 \times , D is ICA 1×10^{-4} M, 20 \times ; E is ICA 1×10^{-5} M, 10 \times , F is ICA 1×10^{-5} M, 20 \times ; G is ICA 1×10^{-6} M, 10 \times , H is ICA 1×10^{-6} M, 20 \times ; I is ICA 1×10^{-7} M, 10 \times , J is ICA 1×10^{-7} M, 20 \times ; K is ICA 1×10^{-8} M, 10 \times , L is ICA 1×10^{-8} M, 20 \times ; M is ICA 1×10^{-9} M, 10 \times , N is ICA 1×10^{-9} M, 20 \times . a-n is 48h, under different magnification microscopes, the state of BMSCs under different concentrations of ICA, where a is ICA 0M, 10 \times , b is ICA 0M, 20 \times ; c is ICA 1×10^{-4} M, 10 \times , d is ICA 1×10^{-4} M, 20 \times ; e is ICA 1×10^{-5} M, 10 \times , f is ICA 1×10^{-5} M, 20 \times ; g is ICA 1×10^{-6} M, 10 \times , h is ICA 1×10^{-6} M, 20 \times ; i is ICA 1×10^{-7} M, 10 \times , j is ICA 1×10^{-7} M, 20 \times ; k is ICA 1×10^{-8} M, 10 \times , l is ICA 1×10^{-8} M, 20 \times ; m is ICA 1×10^{-9} M, 10 \times , n is ICA 1×10^{-9} M, 20 \times ; (2) CCK-8 kit detects the effects of ICA on BMSCs at various concentrations at 24h and 48h The inhibitory effect of activity and proliferation; (3) After 2 weeks of culture, stained with Alizarin Red, the mineralized nodules of BMSCs cultured with different concentrations of ICA under different magnification fields were observed with a microscope. Among them, A is ICA 0M, 4 \times , B is ICA 0M, 10 \times ; C is ICA 1×10^{-6} M, 4 \times , D is ICA 1×10^{-6} M, 10 \times ; E is ICA 1×10^{-7} M, 4 \times , F is ICA 1×10^{-7} M, 10 \times ; G is ICA 1×10^{-8} M, 4 \times , H is ICA 1×10^{-8} M, 10 \times ; I is ICA 1×10^{-9} M, 4 \times , J is ICA 1×10^{-9} M.

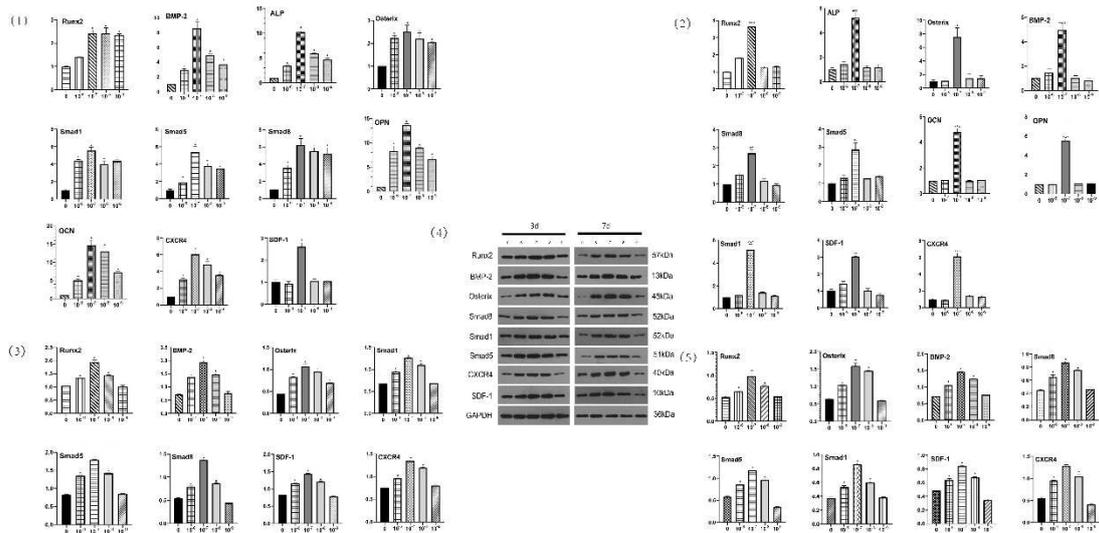


Figure 6 The effects of different concentrations of ICA on osteogenic differentiation and migration of BMSCs

Note: (1) and (2) mean that different concentrations of ICA (0 , $1 \times 10^{-6}M$, $1 \times 10^{-7}M$, $1 \times 10^{-8}M$, $1 \times 10^{-9}M$) act on the formation of BMSCs at 3d and 7d respectively. MRNA expression of genes related to bone differentiation and migration; (3) and (4) 3d and 7d with different concentrations of ICA (0 , $1 \times 10^{-6}M$, $1 \times 10^{-7}M$, $1 \times 10^{-8}M$, $1 \times 10^{-9}M$) Protein expression of osteogenic differentiation and migration related genes acting on BMSCs.

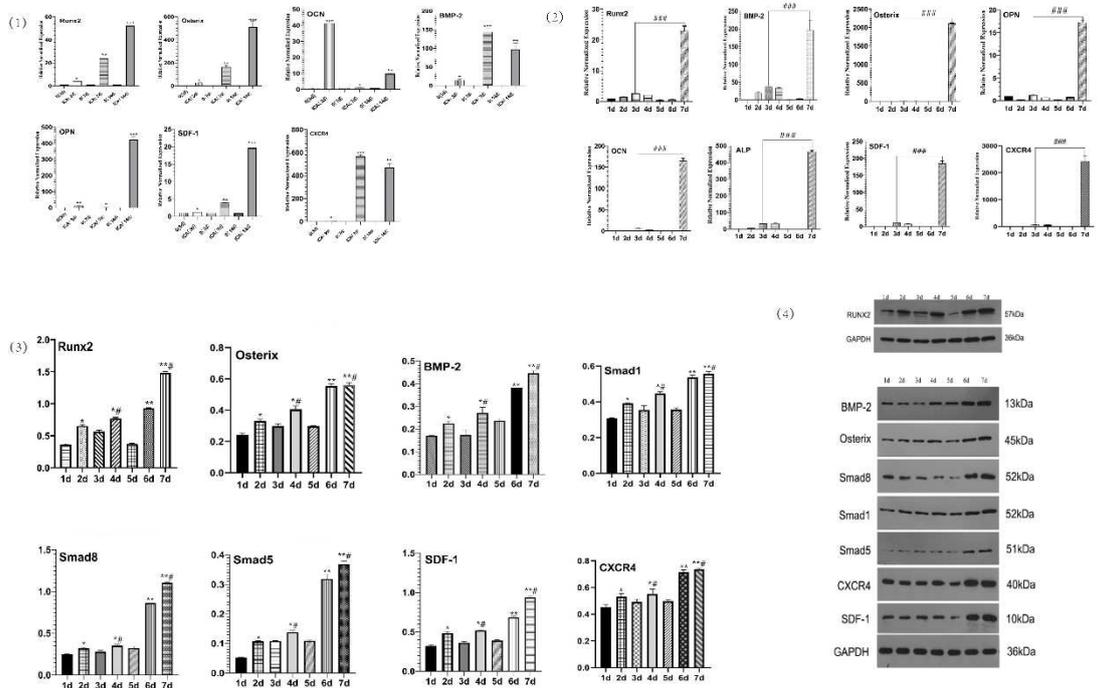


Figure 7 The osteogenic differentiation and migration trend of 1-7d ICA on BMSCs

Note: (1) BMSCs osteogenic differentiation-related genes (Runx2, BMP-2, Osterix, OPN, OCN) and migration-related genes (SDF-) in the blank control group and $1 \times 10^{-7}M$ ICA group at 3d, 7d, and 14d, respectively 1α , CXCR4) mRNA expression; (2) 1-7d concentration of $1 \times 10^{-7}M$ ICA acts

on osteogenic differentiation-related genes (Runx2, BMP-2, Osterix, OPN, OCN, ALP) and migration-related genes (SDF-1 α , CXCR4) mRNA expression. *, # stands for P<0.05, **, ## stands for P<0.01, ***, ### stands for P<0.001; (3) 1-7d at a concentration of 1 \times 10⁻⁷M ICA acts on the osteogenesis of BMSCs (Runx2, BMP-2, Osterix, Smad1/5/8), migration (SDF-1 α , CXCR4) expression of each target protein.

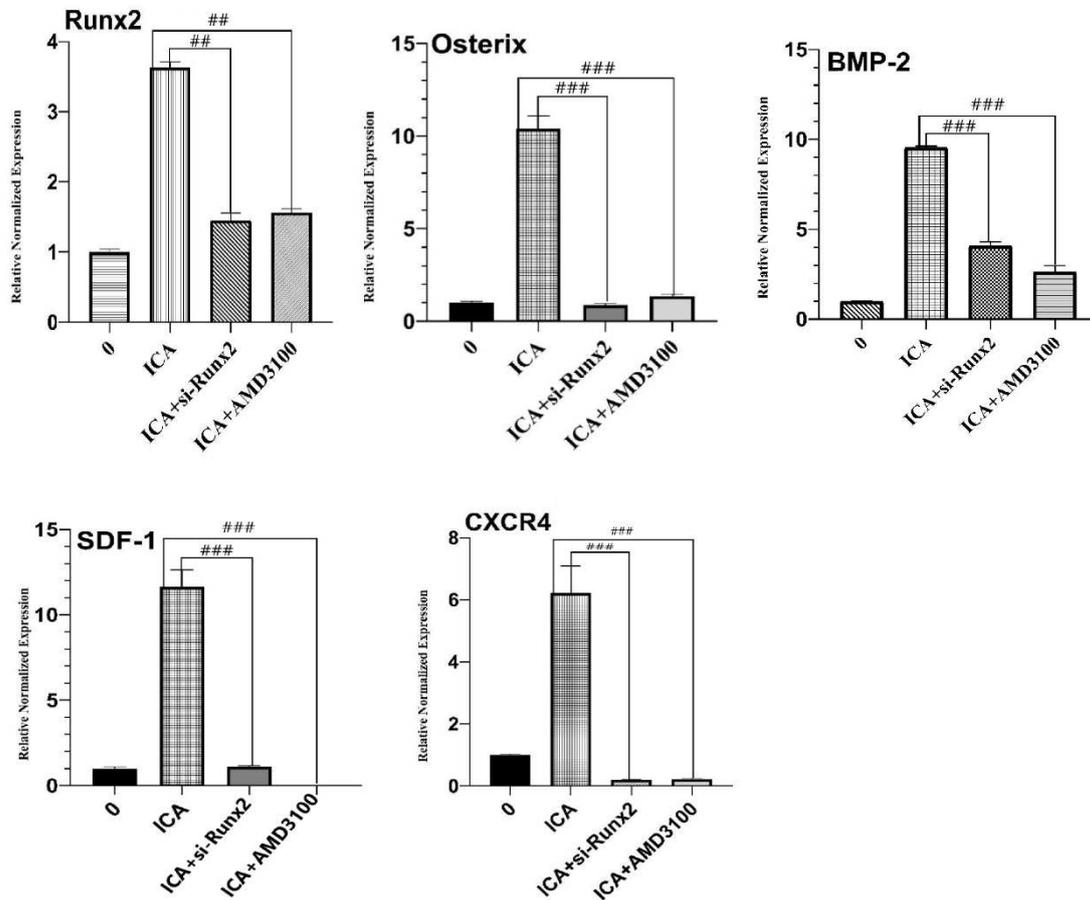


Figure 8 Signal pathway detection of ICA's role in osteogenic differentiation and migration
 Figure 8 Signal pathway detection of ICA's role in osteogenic differentiation and migration
 Note: The four groups are the blank group, ICA 1 \times 10⁻⁷M group, ICA 1 \times 10⁻⁷M +si-Runx2 group, ICA 1 \times 10⁻⁷M +AMD3100 group, ## represents P<0.01, ### represents P<0.001.

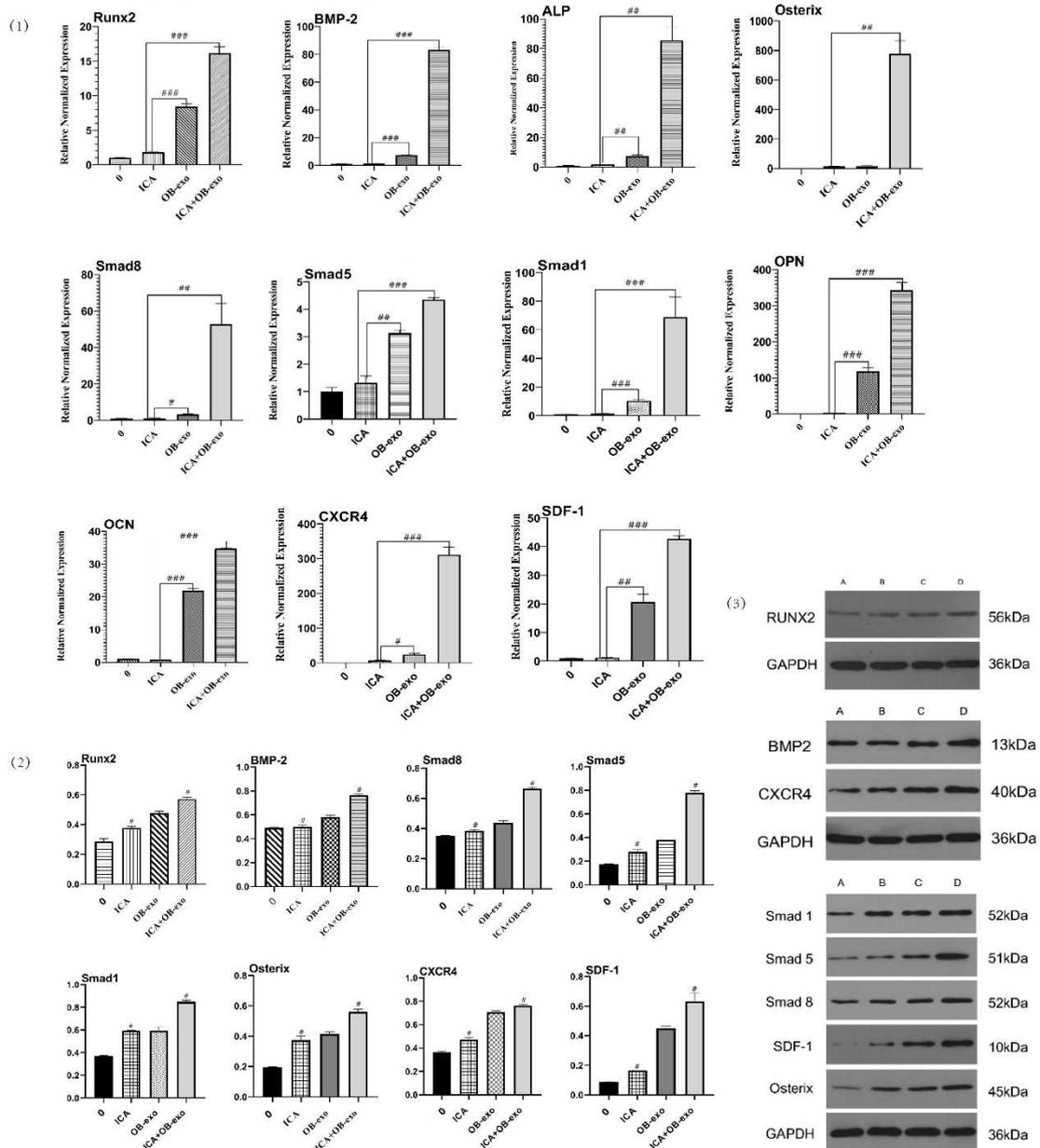


Figure 9 ICA combined with OB-exo exerts osteogenic differentiation and migration effects on BMSCs

Note: (1) Osteogenesis (Runx2, BMP-2, Osterix, Smad1/5/8, ALP, OCN, OPN) and migration (SDF-1) in blank group, ICA group, OB-exo group, ICA+OB-exo group -1 α , CXCR4) mRNA expression. # Represents $P < 0.05$, ## represents $P < 0.01$, ### represents $P < 0.001$; (2) Osteogenesis in blank group, ICA group, OB-exo group, ICA+OB-exo group (Runx2, BMP-2, Osterix, Smad1/5/8) and migration (SDF-1 α , CXCR4) protein expression (where A, B, C, D each represents the blank group, ICA group, OB-exo group and ICA+OB-exo group).

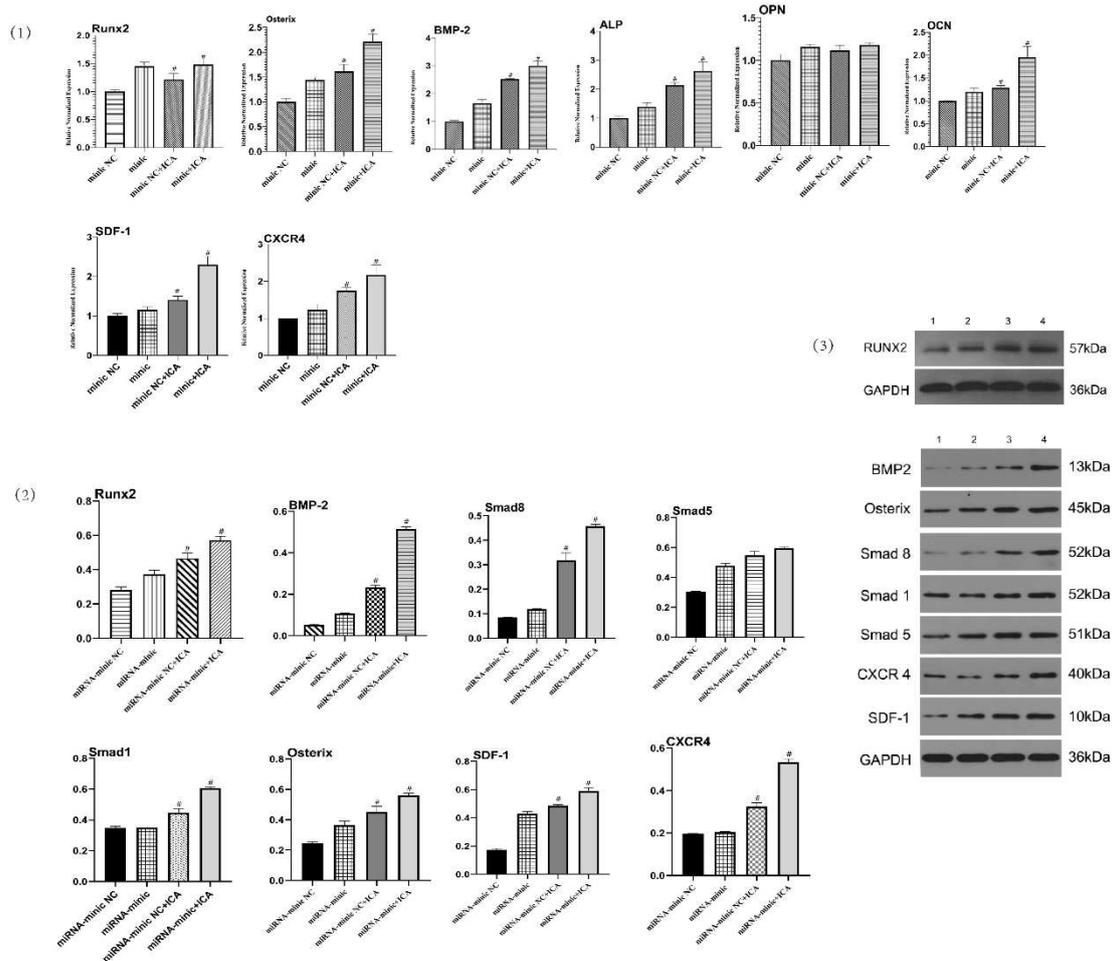


Figure 10 Detection of the osteogenic differentiation and migration effect of ICA combined with miR-122-5p mimic on BMSCs

Note: (1) Osteogenic differentiation and migration related genes in miR-122-5p mimic NC group, miR-122-5p mimic group, miR-122-5p mimic NC+ICA group, miR-122-5p mimic+ICA group mRNA expression. In the mRNA related to osteogenic differentiation (Runx2, Osterix, BMP-2, ALP, OPN, OCN) and migration (SDF-1 α , CXCR4), # represents P<0.01, ## represents P<0.05; (2) miR-122-5p mimic NC group, miR-122-5p mimic group, miR-122-5p mimic NC+ICA group, miR-122-5p mimic+ICA group, the expression of osteogenic differentiation and migration related gene protein (1, 2, 3, and 4 respectively represent the miR-122-5p mimic NC group, miR-122-5p mimic group, miR-122-5p mimic NC+ICA group, miR-122-5p mimic+ICA group).

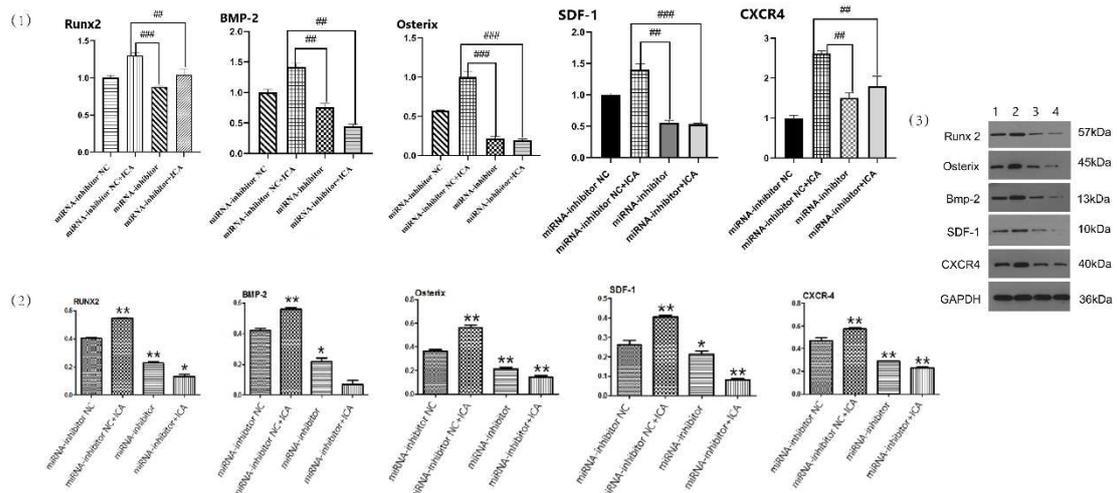


Figure 11 Testing the osteogenic differentiation and migration effects of ICA combined with miR-122-5p inhibitor on BMSCs

Note: (1) miR-122-5p inhibitor NC group, miR-122-5p inhibitor NC+ICA group, miR-122-5p inhibitor group, miR-122-5p inhibitor+ICA constitute mRNA expression of genes related to bone differentiation and migration . ##Represents P<0.01, ###represents P<0.001; (2) miR-122-5p inhibitor NC group, miR-122-5p inhibitor NC+ICA group, miR-122-5p inhibitor group, miR-122-5p inhibitor+ICA constitutes the expression of bone differentiation and migration-related proteins. Among the proteins related to osteogenic differentiation (Runx2, Osterix, BMP-2) and migration (SDF-1 α , CXCR4), * represents P<0.05, ** represents P<0.01.

Figures

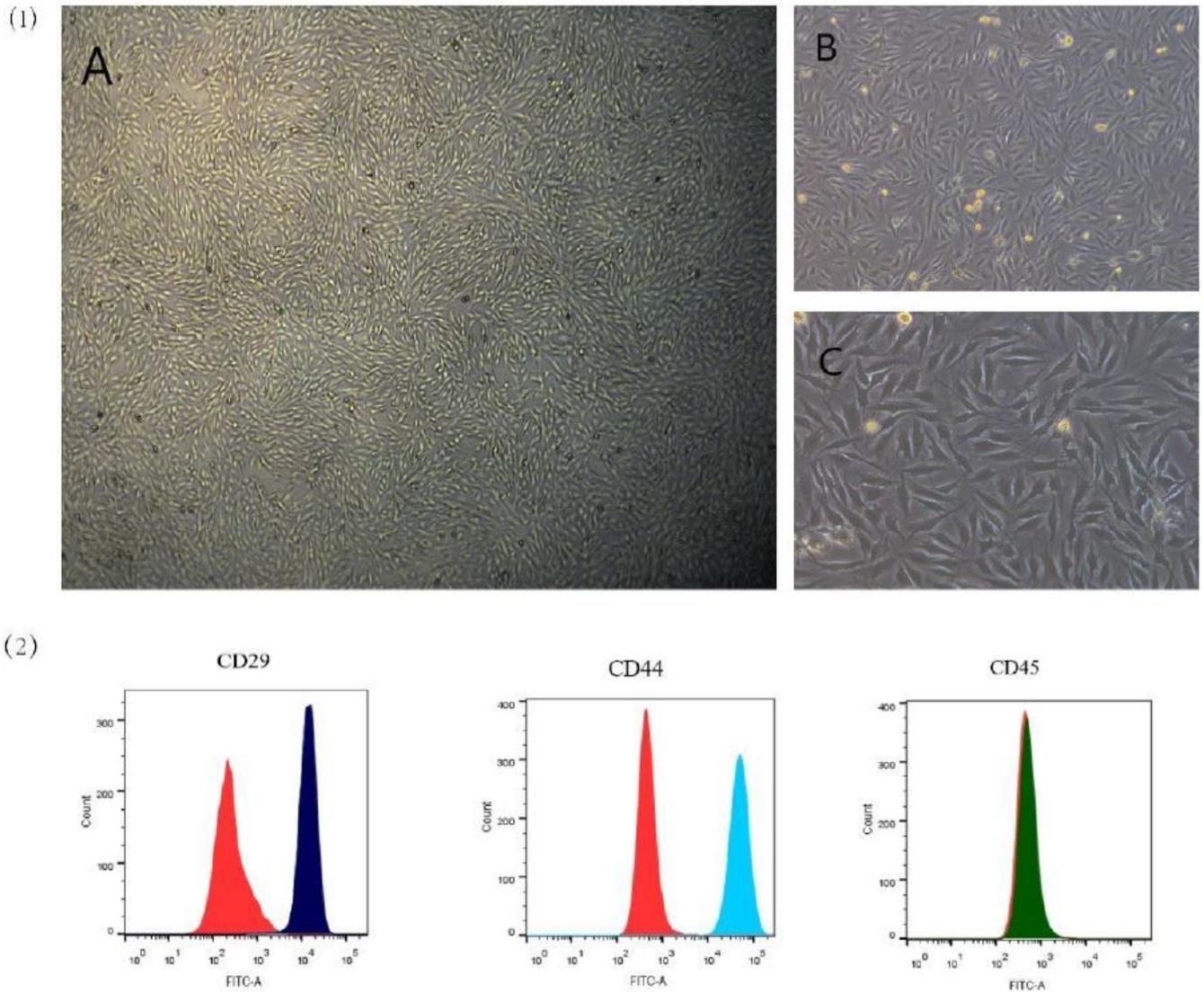


Figure 1

Cultivation and identification of BMSCs Note: (1) AC showed that the P3 generation of BMSCs cultured for 24 hours, under the microscope, the morphological performance of different magnifications, where A is 4x, B is 10x, and C is 20x; (2) Flow cytometry is used to measure the cell surface Markers (CD29, CD44, CD45) for identification.

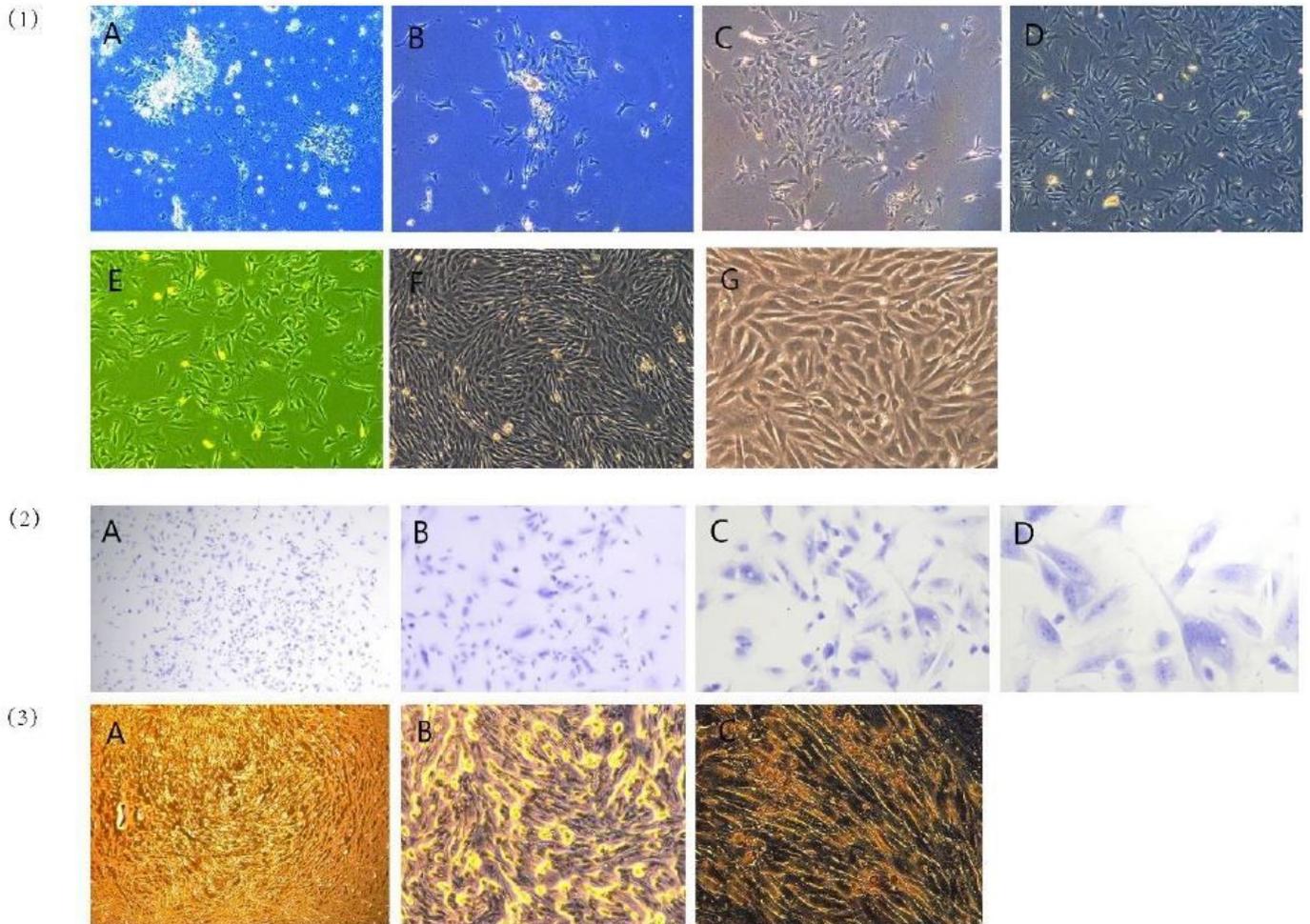


Figure 2

Cultivation and identification of OB Note: (1) A-G shows the state of OB cells under the microscope. After OB cells were extracted and cultured for 3 days, until the 10th day, the cells slowly crawled out and continued to proliferate in large numbers. Among them, A is 3d, 10 \times , B is 4d, 10 \times , C is 5d, 10 \times , D is 7d, 10 \times , E is 8d, 10 \times , the first resuspended cell, F is 10d, 10 \times , G is 10d, 20 \times ; (2) A-D showed that Giemsa staining results of OB cells were observed under different magnification microscopes. Among them, A is 4 \times , B is 10 \times , C is 20 \times , D is 40 \times ; (3) A-C shows the mineralized nodules after 2w magnified by different magnifications of the microscope. Among them, A is 4 \times , B is 10 \times , C is 20 \times .

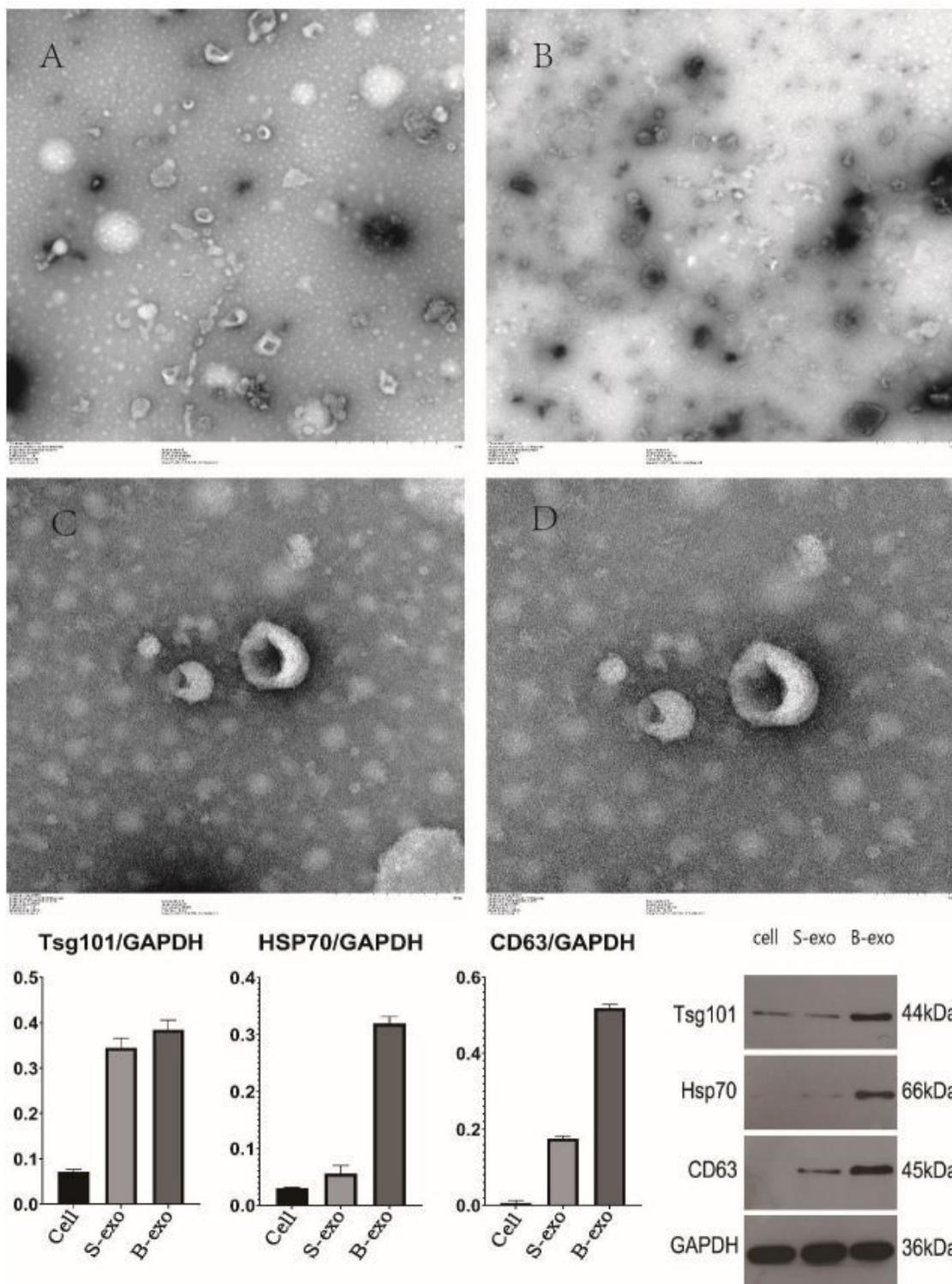
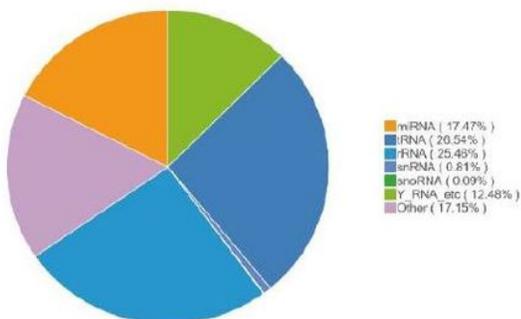


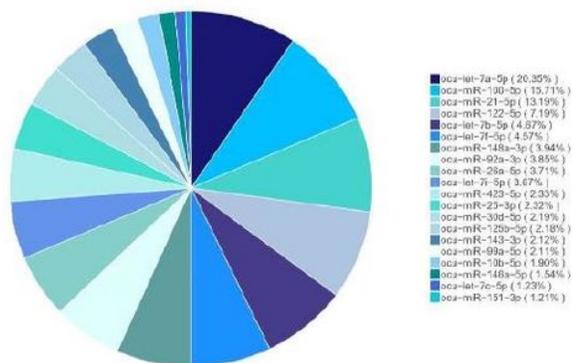
Figure 3

Extraction and identification of OB-derived exosomes Note: (1) A-D shows the morphology and size of OB cell-derived exosomes under different transmission electron microscope fields. Among them, A and B are in a 1.0 μm field of view, C is in a field of 200 nm, and D is in a field of 100 nm. (2) Western blotting for the identification of exosomal marker proteins: detection of Tsg101, Hsp70, and CD63 (cell group, superisolated exosomes group, box exosomes group).

(1) Total Reads Annotation



(2)



(3)



(4)

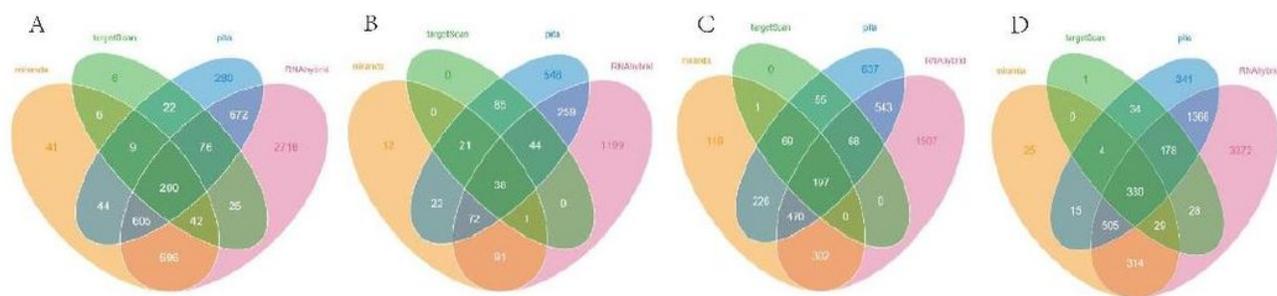


Figure 4

High-throughput sequencing and analysis of OB-derived exosomes Note: (1) Distribution diagram of the internal components of exosomes. (2) The percentage of the top 20 miRNAs in the total miRNAs of exosomes. (3) The number of target genes of the top 20 miRNAs measured in the four software. (4) The intersection of the target genes of the top 4 miRNAs in the four software displayed by the Venn diagram (A is let-7a-5p, B is miR-100-5p, C is miR-21-5p, D is miR-122-5p).

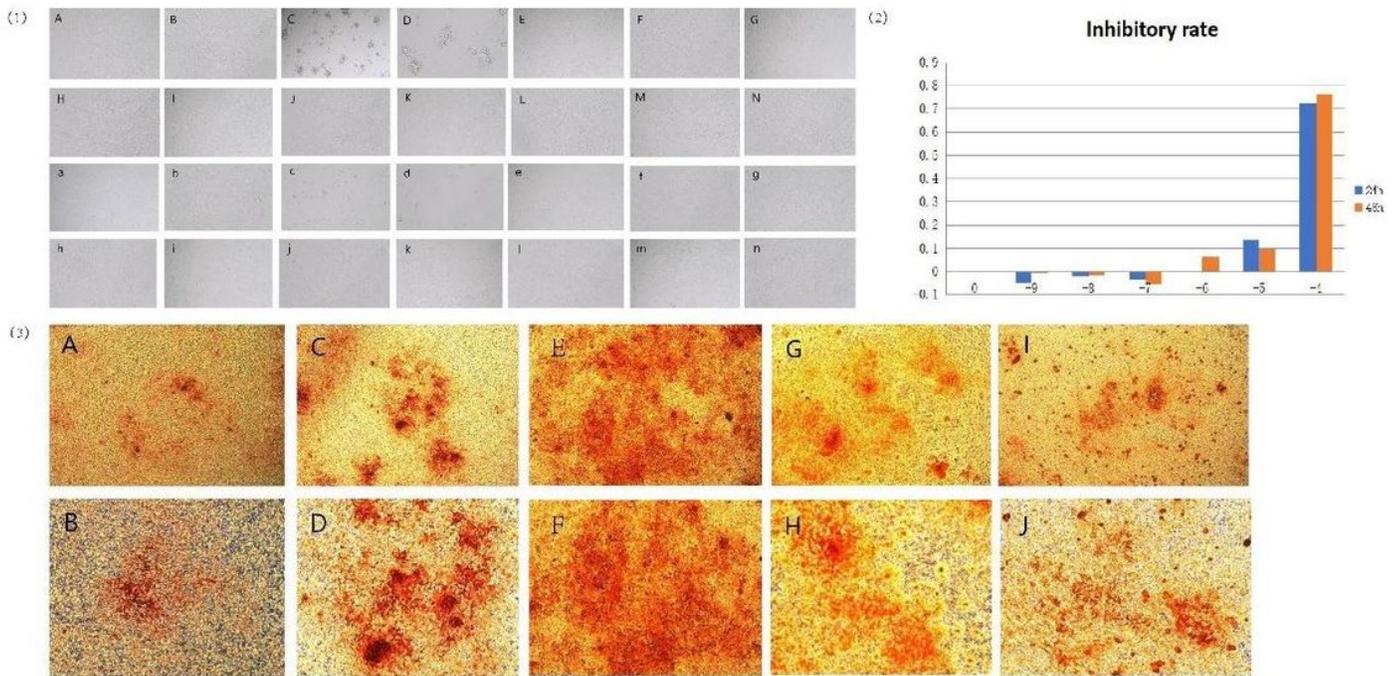


Figure 5

BMSCs activity and proliferation detection and BMSCs mineralization under the action of ICA Note: (1) A-N is 24h. Under different magnification microscopes, the status of BMSCs under different concentrations of ICA, where A is ICA 0M, 10 \times , B is ICA 0M, 20 \times ; C is ICA 1 \times 10⁻⁴M, 10 \times , D is ICA 1 \times 10⁻⁴M, 20 \times ; E is ICA 1 \times 10⁻⁵M, 10 \times , F is ICA 1 \times 10⁻⁵M, 20 \times ; G is ICA 1 \times 10⁻⁶M, 10 \times , H is ICA 1 \times 10⁻⁶M, 20 \times ; I is ICA 1 \times 10⁻⁷M, 10 \times , J is ICA 1 \times 10⁻⁷M, 20 \times ; K is ICA 1 \times 10⁻⁸M, 10 \times , L is ICA 1 \times 10⁻⁸M, 20 \times ; M is ICA 1 \times 10⁻⁹M, 10 \times , N is ICA 1 \times 10⁻⁹M, 20 \times . a-n is 48h, under different magnification microscopes, the state of BMSCs under different concentrations of ICA, where a is ICA 0M, 10 \times , b is ICA 0M, 20 \times ; c is ICA 1 \times 10⁻⁴M, 10 \times , d is ICA 1 \times 10⁻⁴M, 20 \times ; e is ICA 1 \times 10⁻⁵M, 10 \times , f is ICA 1 \times 10⁻⁵M, 20 \times ; g is ICA 1 \times 10⁻⁶M, 10 \times , h is ICA 1 \times 10⁻⁶M, 20 \times ; i is ICA 1 \times 10⁻⁷M, 10 \times , j is ICA 1 \times 10⁻⁷M, 20 \times ; k is ICA 1 \times 10⁻⁸M, 10 \times , l is ICA 1 \times 10⁻⁸M, 20 \times ; m is ICA 1 \times 10⁻⁹M, 10 \times , n is ICA 1 \times 10⁻⁹M, 20 \times ; (2) CCK-8 kit detects the effects of ICA on BMSCs at various concentrations at 24h and 48h The inhibitory effect of activity and proliferation; (3) After 2 weeks of culture, stained with Alizarin Red, the mineralized nodules of BMSCs cultured with different concentrations of ICA under different magnification fields were observed with a microscope. Among them, A is ICA 0M, 4 \times , B is ICA 0M, 10 \times ; C is ICA 1 \times 10⁻⁶M, 4 \times , D is ICA 1 \times 10⁻⁶M, 10 \times ; E is ICA 1 \times 10⁻⁷M, 4 \times , F is ICA 1 \times 10⁻⁷M, 10 \times ; G is ICA 1 \times 10⁻⁸M, 4 \times , H is ICA 1 \times 10⁻⁸M, 10 \times ; I is ICA 1 \times 10⁻⁹M, 4 \times , J is ICA 1 \times 10⁻⁹M, 10 \times .

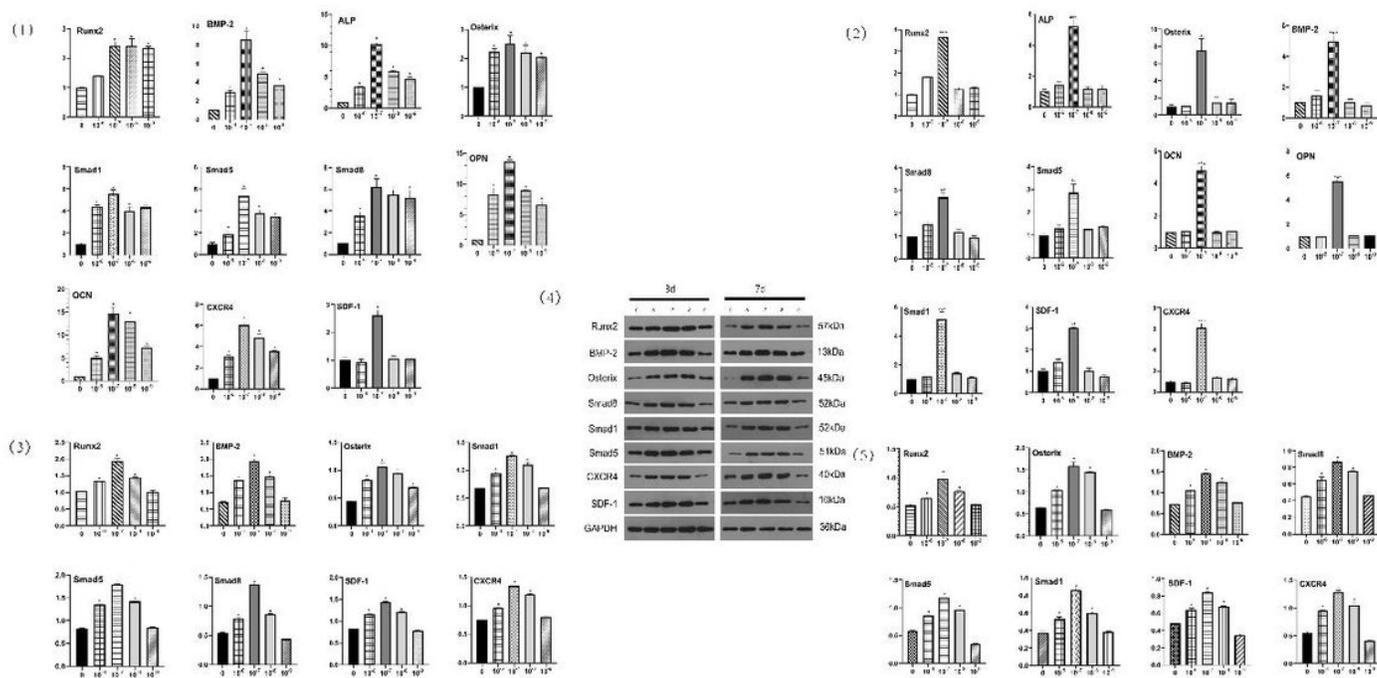


Figure 6

The effects of different concentrations of ICA on osteogenic differentiation and migration of BMSCs
 Note: (1) and (2) mean that different concentrations of ICA (0, $1 \times 10^{-6}M$, $1 \times 10^{-7}M$, $1 \times 10^{-8}M$, $1 \times 10^{-9}M$) act on the formation of BMSCs at 3d and 7d respectively. MRNA expression of genes related to bone differentiation and migration; (3) and (4) 3d and 7d with different concentrations of ICA (0, $1 \times 10^{-6}M$, $1 \times 10^{-7}M$, $1 \times 10^{-8}M$, $1 \times 10^{-9}M$) Protein expression of osteogenic differentiation and migration related genes acting on BMSCs.

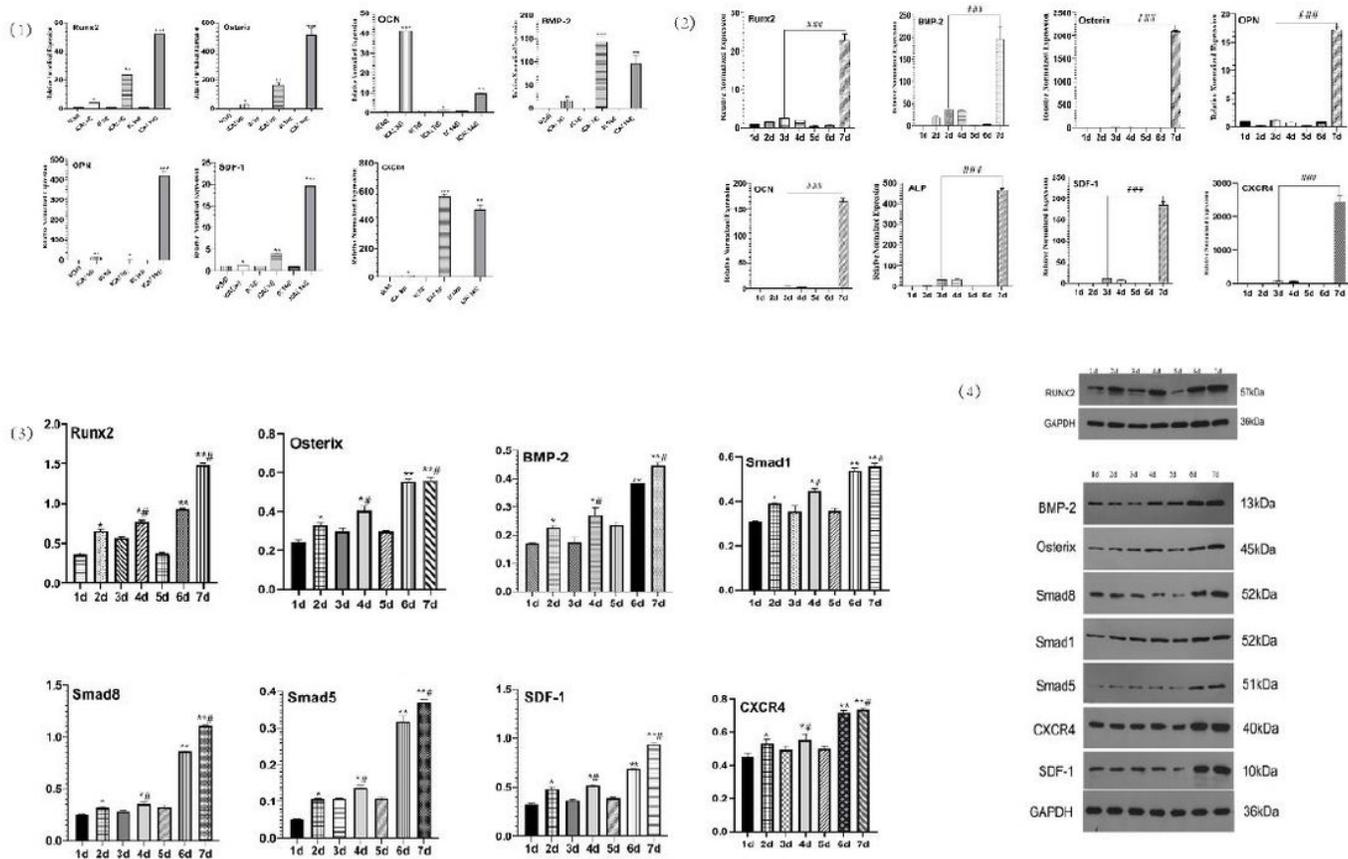


Figure 7

The osteogenic differentiation and migration trend of 1-7d ICA on BMSCs Note: (1) BMSCs osteogenic differentiation-related genes (Runx2, BMP-2, Osterix, OPN, OCN) and migration-related genes (SDF-) in the blank control group and 1×10^{-7} M ICA group at 3d, 7d, and 14d, respectively (1 α , CXCR4) mRNA expression; (2) 1-7d concentration of 1×10^{-7} M ICA acts on osteogenic differentiation-related genes (Runx2, BMP-2, Osterix, OPN, OCN, ALP) and migration-related genes (SDF-1 α , CXCR4) mRNA expression. *, # stands for $P < 0.05$, **, ## stands for $P < 0.01$, ***, ### stands for $P < 0.001$; (3) 1-7d at a concentration of 1×10^{-7} M ICA acts on the osteogenesis of BMSCs (Runx2, BMP-2, Osterix, Smad1/5/8), migration (SDF-1 α , CXCR4) expression of each target protein.

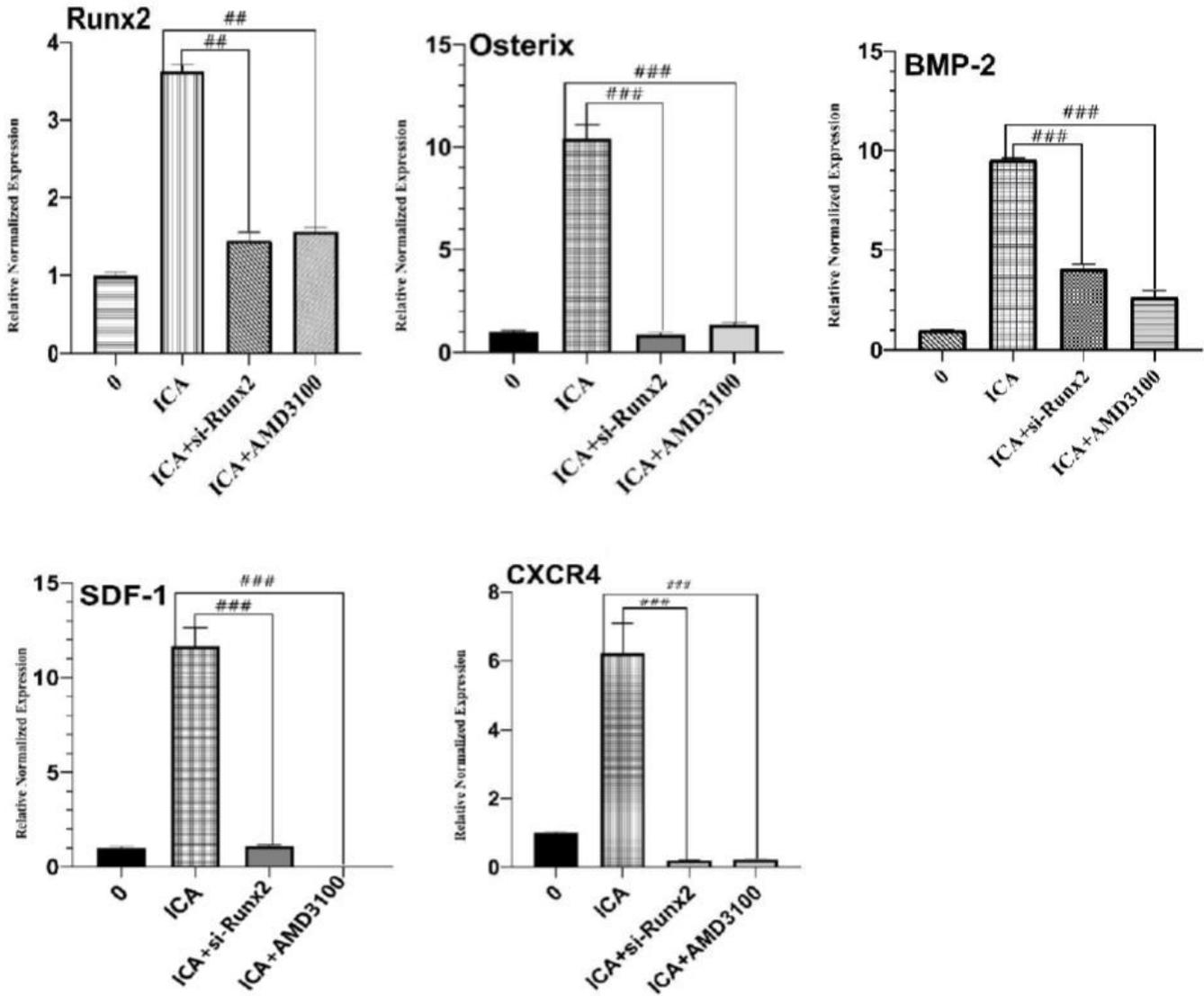


Figure 8

Signal pathway detection of ICA's role in osteogenic differentiation and migration Figure 8 Signal pathway detection of ICA's role in osteogenic differentiation and migration Note: The four groups are the blank group, ICA 1×10⁻⁷M group, ICA 1×10⁻⁷M +si-Runx2 group, ICA 1×10⁻⁷M +AMD3100 group, ## represents P<0.01, ### represents P<0.001.

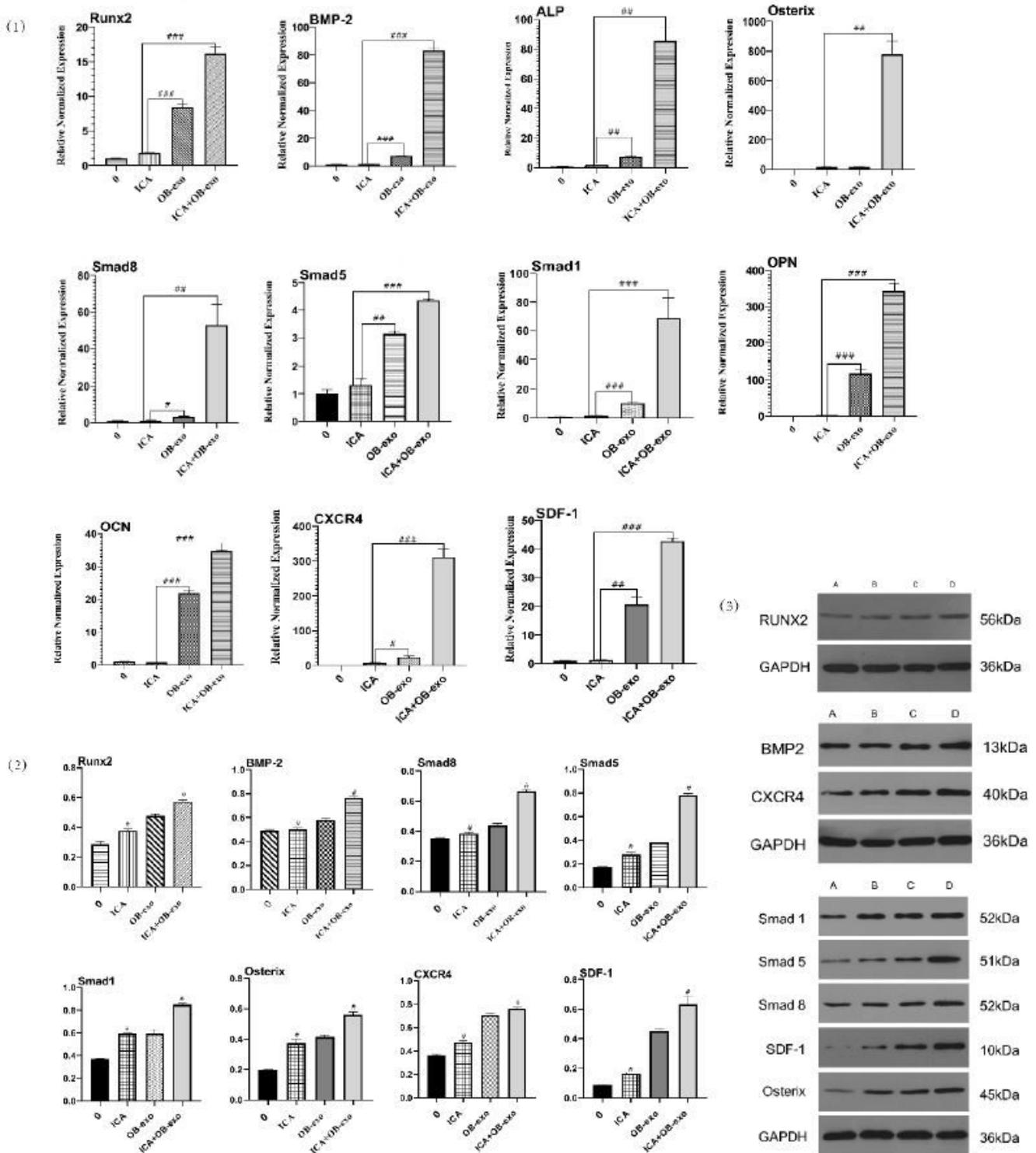


Figure 9

ICA combined with OB-exo exerts osteogenic differentiation and migration effects on BMSCs Note: (1) Osteogenesis (Runx2, BMP-2, Osterix, Smad1/5/8, ALP, OCN, OPN) and migration (SDF) in blank group, ICA group, OB-exo group, ICA+OB-exo group (α , CXCR4) mRNA expression. # Represents $P < 0.05$, ## represents $P < 0.01$, ### represents $P < 0.001$; (2) Osteogenesis in blank group, ICA group, OB-exo group, ICA+OB-exo group (Runx2, BMP-2, Osterix, Smad1/5/8) and migration (SDF-1 α , CXCR4) protein

expression (where A, B, C, D each represents the blank group, ICA group, OB-exo group and ICA+OB-exo group).

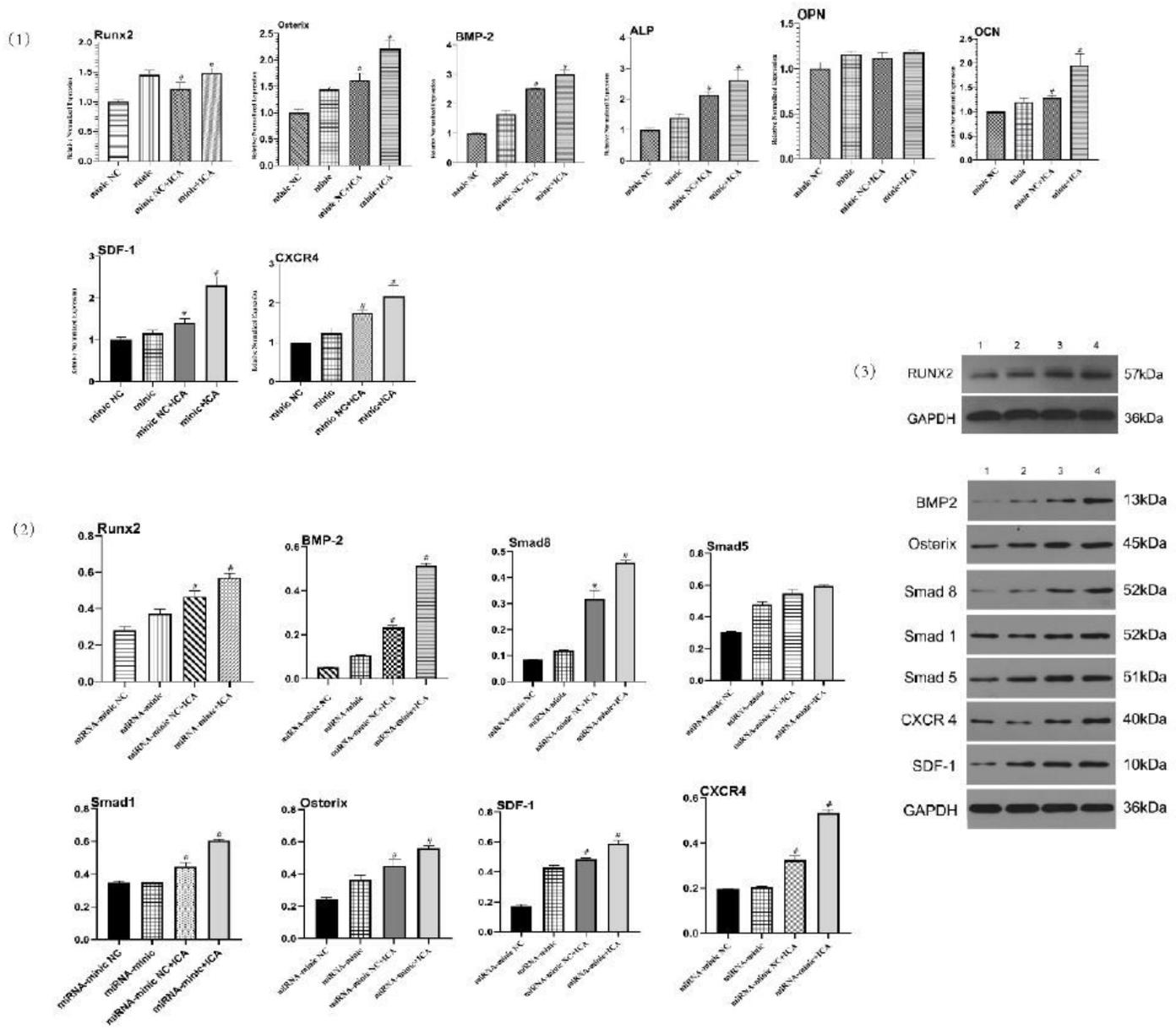


Figure 10

Detection of the osteogenic differentiation and migration effect of ICA combined with miR-122-5p minic on BMSCs Note: (1) Osteogenic differentiation and migration related genes in miR-122-5p minic NC group, miR-122-5p minic group, miR-122-5p minic NC+ICA group, miR-122-5p minic+ICA group mRNA expression. In the mRNA related to osteogenic differentiation (Runx2, Osterix, BMP-2, ALP, OPN, OCN) and migration (SDF-1 α , CXCR4), # represents $P < 0.01$, ## represents $P < 0.05$; (2) miR-122-5p minic NC group, miR-122-5p minic group, miR-122-5p minic NC+ICA group, miR-122-5p minic+ICA group, the expression of osteogenic differentiation and migration related gene protein (1, 2, 3, and 4 respectively represent the miR-122-5p minic NC group, miR-122-5p minic group, miR-122-5p minic NC+ICA group, miR-122-5p minic+ICA group).

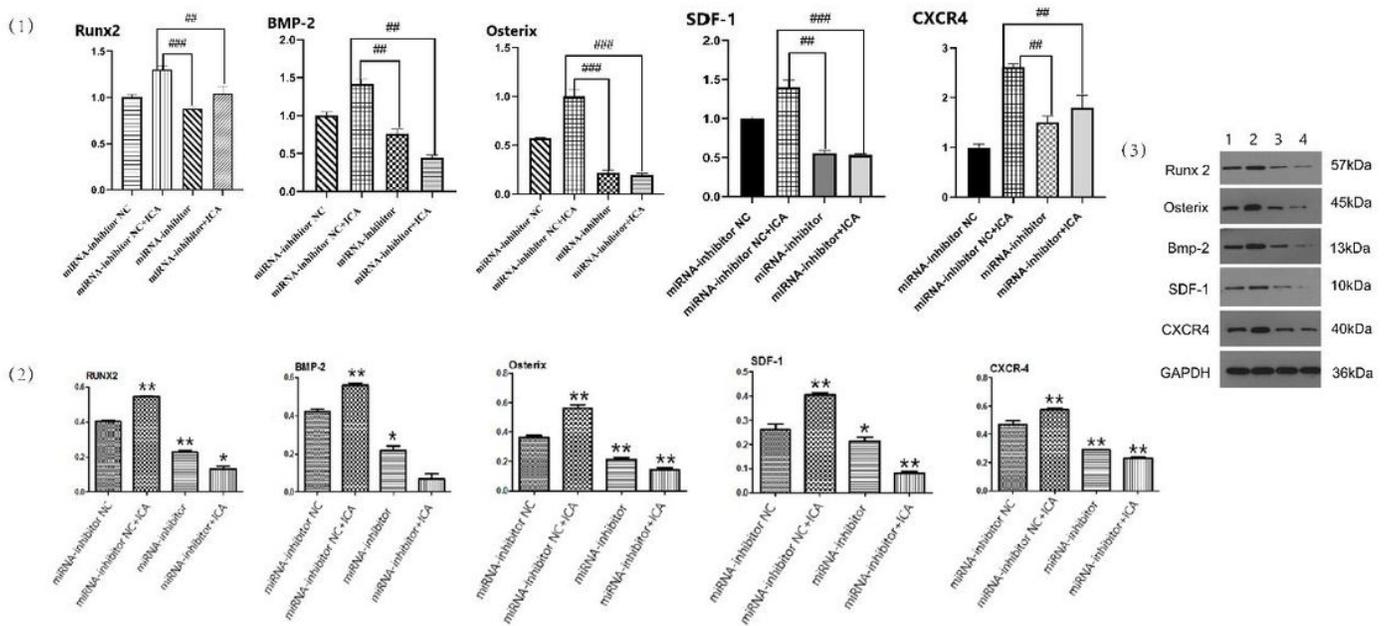


Figure 11

Testing the osteogenic differentiation and migration effects of ICA combined with miR-122-5p inhibitor on BMSCs Note: (1) miR-122-5p inhibitor NC group, miR-122-5p inhibitor NC+ICA group, miR-122-5p inhibitor group, miR-122-5p inhibitor+ICA constitute mRNA expression of genes related to bone differentiation and migration. ##Represents $P < 0.01$, ###represents $P < 0.001$; (2) miR-122-5p inhibitor NC group, miR-122-5p inhibitor NC+ICA group, miR-122-5p inhibitor group, miR-122-5p inhibitor+ICA constitutes the expression of bone differentiation and migration-related proteins. Among the proteins related to osteogenic differentiation (Runx2, Osterix, BMP-2) and migration (SDF-1 α , CXCR4), * represents $P < 0.05$, ** represents $P < 0.01$.