

# Microchannel Porous Hydroxyapatite Scaffold Promotes Osteogenic Differentiation by Altering the Expression of miRNAs

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## Research Article

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# Abstract

Hydroxyapatite is a commonly used scaffold material for bone tissue engineering. However, the osteogenic mechanism of hydroxyapatite scaffolds remains unclear. Recently, we have prepared a hydroxyapatite scaffolds with microchannels and porous structures (HAG) which have good osteogenic effects in vitro and in vivo. In present study, we explained the mechanism of HAG scaffolds promoted the osteogenic differentiation from the perspective of miRNA differential expression. We used microarray assays to analyze the expression profiles of miRNAs from the osteogenic differentiation of hPMSCs with or without HAG; 16 miRNAs were upregulated and 29 miRNAs were downregulated between the two types of cells. And overexpression the differential miRNAs could promote the osteogenic differentiation of hPMSCs. Additionally, gene ontology analysis, pathway analysis, and miRNA-mRNA-network built were performed to reveal that the differentially expressed miRNAs participate in multiple biological processes, including cell metabolic, cell junction, cell development, differentiation, and signal transduction, among others. Furthermore, we found that these differentially expressed miRNAs connect osteogenic differentiation to processes such as axon guidance, MAPK, and TGF-beta signaling pathway. This is the first study to identify and characterize differentiability miRNAs derived from HAG-hPMSC cells.

## Introduction

Bone is a mineralized mesenchymal tissue that resists mechanical forces and regulates mineral homeostasis and energy metabolism<sup>1</sup>. However, bone defects due to trauma, disease and other causes seriously impair people's quality of life and cause a huge social and economic burden. Although bone has a certain ability of regeneration, serious bone injuries such as large bone defects still need to be solved by bone grafting<sup>2,3</sup>. Autografts is the most ideal way to avoid the occurrence of graft immune response, but it is limited by multiple surgeries and donor site injury. Therefore, bone tissue engineering, a scaffold material loaded seed cells and growth factors, has become a more promising method for clinical therapy. Importantly, biological scaffolds material is a key part of it. After the scaffold material is implanted in the affected area, the cells attach to the scaffold surface according to the properties of the material's surface including microstructure, charge, chemical composition, and so on<sup>4,5</sup>. Therefore, there is a growing demand for bone biomaterials with superior biological properties in recent years<sup>6</sup>.

MicroRNA (miRNA) is endogenous small noncoding ~ 22-nt RNA, which regulates a series of physiological and pathological processes including bone regeneration and metabolism by targeting mRNA sequences of related genes<sup>7,8</sup>. For example, miR-335-5p has been shown to be highly expressed in mouse embryo osteogenesis<sup>9</sup>. Similarly, two consecutive studies demonstrated that both miR-2861 and miR-3960 are high expression in original mouse osteoblasts<sup>10,11</sup>. Additionally, in different tissue sources, miR-140-5p and miR-140-3p are both confirmed to highly expressed, including human bone-marrow-derived stem cells (hBMSCs)<sup>12</sup>. Li et al. showed that miR-29b expression peaks at the mineral deposition stage of MC3T3 cell osteogenic differentiation<sup>13</sup>. Bhushan and his colleagues found that miR-181a expression was upregulated during BMP6-induced osteogenic differentiation of MC3T3 cells<sup>14</sup>. Also, miR-

210 was increased in ST2 cell line with BMP-4 dependent manner<sup>15</sup>. Furthermore, let-7, miR-20, miR-34a and miR-199 were confirmed to be increased during hBMSCs osteoblast differentiation<sup>16</sup>. As mentioned above, different miRNAs are involved in the complex process of osteogenic differentiation. However, the mechanism of scaffold material-induced specific osteogenic associated miRNAs has not been seen.

In this study, we first observed that the differential expression of miRNA in the process of cell osteogenic differentiation induced by HAG scaffolds in hPMSCs. Hydroxyapatite is the most commonly used scaffold material for bone regeneration, due to its mineral composition with natural bone and HAG is a porous hydroxyapatite scaffold with microchannel structure developed by our team, the osteogenic response of which had been verified both in vitro and vivo<sup>17,18</sup>. Here, we compared the expression profiles of miRNAs in hPMSCs with or without HAG by microarray assays. Moreover, we further explore the biological functions that these differential miRNAs might be associated with osteogenesis through bioinformatics analysis and revealed that they are closely related to osteogenic differentiation related signaling pathways, including axon guidance, MAPK, and TGF-beta signaling pathway. The work may lay the foundation for the further construction of bone scaffolds loaded with miRNAs.

## Materials And Methods

**Cell culture and transfection.** hPMSCs were cultured as before<sup>18</sup>. In details, cells were cultured in complete medium (DMEM with 10% FBS and 1% PS). When the cell density fusion was 80–90%, the digestive passage culture was conducted with 0.25% trypsin. The surface markers of hPMSCs were analyzed by flow cytometry, including CD29, CD90 and CD45 (Abcam, UK). The osteogenic differentiation of hPMSCs were cultured with conditional medium (DMEM with 10%FBS, 20 mmol/L  $\beta$ -glycerophosphate, 50 g/mL vitamin C and 10 mol/L dexamethasone). The  $8 \times 10^4$  cells were subcultured into 6-well plates into 6-well plates, the medium was changed every two to three days, and protein /RNA samples were collected at different time points. When the cell density approached 80 percent, 100pM miRNA mimics and NC were transfected to cells followed by the protocol of Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, USA). The mimics were synthesised from Genepharma. ALP staining was performed followed by the TRAP/ALP Stain kit (Wako, Japan). Alizarin Red staining was performed to detect calcium deposits after 14 days of induction culture in accordance with the instructions of the kit (Cyagen, China).

**Preparation of scaffolds and cell seeded.** The HAG scaffold was produced and cleaned by referring to previous description [18]. The scaffolds were incubated overnight in DMEM prior to the cell experiment at 37°C. The surface micromorphology and cell adhesion of HAG were confirmed by ZEISS Gemini 300 scanning electron microscopy (SEM). In details, 20 $\mu$ L cell suspension ( $4 \times 10^6$  /mL) were seeded onto the surface of HAG scaffolds and cultured for 3 day. Then, scaffolds were fixed with 15% glutaraldehyde overnight after washed three times with PBS. Dehydration was performed according to the following alcohol concentration gradient (25%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, 100% 15min each time). The sample after vacuum drying was used for SEM detection

**Osteogenic differentiation of hPMSCs with HAG and quantitative real-time PCR (qRT-PCR).** 20 $\mu$ L cell suspension ( $4 \times 10^6$  /mL) were seeded in a HAG. Then, the cells co-culture with the scaffold in incubator. After osteogenic induction for different time point, extracted the total RNAs (TRIzol™ Reagent, Invitrogen), and reversed to cDNAs (M-MLV, Invitrogen™). Quantitative fluorescence detection was performed using 2X ChamQ Universal SYBR Master Mix (Vazyme, China). ABI 7500 was applied for qRT-PCR. Primers specific for human miRNAs and internal control U6 were designed and synthesized from Tsingke, China. (5'-3'ALP: F-CTTGAAGTGTTGCATGGGC, R-CAAGTCTCAGGGTGAAGG; OCN: F-CAGCGAGGTAGTGAAGAGAC, R-TGAAAGCCGATGTGGTCAG; BMP2: F-CTATCAGGACATGGTTGTGGAG, R-GGAAATATTAAGTGTCAACTGGG)

**Western blotting.** The proteins were isolated by 10% SDS-PAGE gel, which transferred with NC membranes (BD). After incubation with GAPDH, ALP, OCN, Runx2 (Huabio, China) primary antibodies overnight, the membranes were washed for 5 times with PBST (2‰ Tween). Then, the HRP- conjugated secondary antibody was incubated for 1h. Finally, Super ECL kit (biosharp, China) was used to detect the protein bands. The Tanon-5200 was applied for exposure.

**Experimental animals and implantation.** All experimental protocols were approved by Medical Ethics Committee of Sichuan Provincial People's Hospital, Affiliated Hospital of UESTC. Male SD rats (8 weeks) were purchased from Chengdu Dossy Experimental Animals CO.,LTD. After a week of acclimatization, 6 rats were randomly allocated for HAG implantation, and the lateral femoral muscles of HAG-free were made the wound surface of the same size and depth used as the control. Then,each animal was given intramuscular injections of penicillin per day to combat infection for three days.

**Immunohistochemistry.** Paraffin sections were washed with xylene, repeated 3 times, then passed through alcohol gradient, and finally into distilled water. Nuclear were stained by Hematoxylin for 5min, and washed with distilled water, differentiated with 70% hydrochloric acid and alcohol, Distilled water wash to return blue. Sections were stained with Masson Lichun red acid solution for 5 min and soaked in 0.2% glacial acetic acid solution. 1% phosphotungstic acid aqueous solution after differentiation for 5 min, then they were directly dyed with toluidine blue for 5 min without washing and soaked in 0.2% glacial acetic acid solution for 5s. At last, 95% ethanol and anhydrous ethanol were used, slices were dehydrated, slices were transparent with xylene and sealed with neutral gum. The pictures were captured by BA200Digital and analysed by Image-Pro Plus 6.0 and SPSS17.0.

**Microarray Assays.** Microarray Assays were completed by Aksomics, China. Total RNA were extracted using Trizol™ reagent and used to build a miRNA library. The library quality was determined by Agilent 2100 Bioanalyzer. It was eventually sequenced on an Illumina NextSeq 500 sequencer.

**Bioinformatics Analysis.** Edge R was used to analyze the differential expression of miRNA between groups. Based on the database of TargetScan7.1 and mirdbV6, target genes were screened for the Top 10 miRNA up and down regulated in significant difference. Then the function enrichment analysis of target

genes was performed by Gene Ontology, including Molecular function, Cellular component and Biological process. The miRNA-mRNA networks were constructed using Cytoscape.

**Statistical Analysis.** Three independent repeated experiments were used to perform and analysis related results (mean  $\pm$  SD). The T test was used to analyze differences between groups. P values < 0.05 were considered significant.

We confirmed that all methods were carried out in accordance with relevant guidelines and regulations in the manuscript, and the study was carried out in compliance with the ARRIVE guidelines in the manuscript.

## Result

**HAG scaffolds promoted surface hPMSCs adhesion.** Microchannel porous hydroxyapatite scaffolds were used in this study<sup>[18]</sup>. As before, the surface morphology of the scaffold was detected by SEM and shown in Fig. 1A (750–900  $\mu$ m porous structure and 25–30  $\mu$ m groove structure). Then the properties of hPMSCs were detected by flow cytometry. The positive surface marker CD29 and CD90 were highly expressed (97.08%), while CD45 (0.10%) was hardly expressed in the cells (Fig. 1B). For cell adhesion, the phenotype of cells were detected by SEM after three days of co-culture of cells and scaffolds. As shown in Fig. 1C, the cells were attached to the surface of the scaffold and grew well.

**HAG scaffolds promoted the osteogenic differentiation in vitro and in vivo.** Consistent with previous studies, the HAG scaffolds could promote the osteogenic differentiation of hPMSCs. As shown in Fig. 2, after induction of osteogenesis for 7, 14 days, the expression of ALP, BMP2, OCN mRNA in cells co-cultured with scaffolds were increased, and the protein levels of ALP, OCN and Runx2 were increased significantly (Fig. 2A, B). The ALP activity of cells on HAG scaffolds for 7 days incubation was significantly higher than that of control cells (Fig. 2C). Moreover, in 4th week after implantation, paraffin sections were prepared for Masson staining. The results showed that compared with the control group, there were significant red staining of new bone around the scaffold (Fig. 3A). The statistical results of the area of new bone formation also showed that the amount of new bone formation in the HAG group was increased (Fig. 3B). Then, qRT-PCR was used to detect the expression of osteogenic markers in tissue samples surrounding the scaffolds, and showed that the expression of ALP, BMP2, OCN and Runx2 mRNA were increased (Fig. 3C).

**The miRNA expression in HAG-hPMSCs and control hPMSCs were differential.** In recent years, several studies revealed that miRNAs participated the process of osteogenic differentiation through targeting mRNA sequences of different genes related to osteogenesis<sup>19–22</sup>. However, roles of miRNAs in bone formation by HAG scaffolds mediated remain unclear. Thus, in order to uncover that how HAG scaffolds promoted osteogenic differentiation of hPMSCs, microarray assay was used to analyse the miRNA expression profiles between HAG-hPMSCs and hPMSCs. Indeed, we observed 45 mature miRNAs significantly differentially expressed in HAG-hPMSCs group (FC  $\geq$  2, Fig. 4A). Red represented highly

expressed genes, while green represents the down-regulated genes, and the darker the color, the more distinct the expression difference. Compared with differentiated hPMSCs, the expressions of 16 miRNAs in HAG-hPMSCs were up-regulated and 29 were down-regulated, as shown in the volcano plot ( $FC \geq 2$ , Fig. 4B). HAG scaffolds may specifically promote the expression of these miRNAs to achieve their bone-promoting effects.

**Pathway and functional analysis of differential miRNAs.** The differential expression of miRNAs in HAG-hPMSCs group may indicate that miRNA plays an important role in the process of osteogenic differentiation promoted by HAG scaffolds. Therefore, the gene ontology enrichment analyses, including BP (biological process), CC (cellular component), and MF (molecular function) by cluster Profiler in R software, were performed to explore the biological functions may be regulated by differential miRNAs. The statistically significant results ( $p$  values  $< 0.05$ ) showed that these miRNAs participated in the regulation of cell metabolic ( $p$  value =  $2.58E-12$ ), cell junction ( $p$  value =  $6.45E-08$ ), cell development ( $p$  value =  $1.82E-11$ ), cell differentiation ( $p$  value =  $4.04E-07$ ), and signal transduction ( $p$  value =  $8.12E-12$ ) (Fig. 5A Enrichment score; Fig. 5B Fold enrichment). Importantly, the process of osteogenic differentiation is closely related to these functions.

Furthermore, KEGG pathway analysis was used to understand the signaling pathways in which these differentially expressed miRNAs were involved in regulation. The results indicated that genes in multiple signaling pathways, including axon guidance ( $p$  value =  $5.02E-05$ ), MAPK signaling ( $p$  value =  $6.65E-07$ ), and the TGF- $\beta$  signaling pathway ( $p$  value =  $5.7E-04$ ), highly associated with osteogenic differentiation are potential target genes for these differentially expressed miRNAs (Fig. 5C). It suggested that HAG may achieve its osteogenic effect by promoting differential expression of these miRNAs.

**miRNAs validation and miRNA-mRNA network analysis.** According to the results of microarray analyses, the 6 miRNAs (miR-210-3p; miR-146a-5p; miR-483-5p; miR-3615; miR-125b-2-3p; miR-145-5p), with the most significant expression differences were verified by qPCR. Consistently, the expression of miR-210-3p, miR-146a-5p, miR-483-5p were obviously increased, while miR-3615, miR-125b-2-3p and miR-145-5p were significantly decreased ( $FC > 2$ ,  $p < 0.05$ ) (Fig. 6A). Furthermore, we observed that the expression of osteogenic genes were increased when miR146a-5p were overexpressed (Fig. 6B, C). Meanwhile, following osteogenic differentiation, the hPMSCs transfected with miR-146a-5p mimics showed a higher intensity of ALP staining and formed a larger number of calcified nodules (Fig. 6D, E).

As known, miRNAs achieve its regulatory effect by pairing with the complementary sequence of the target gene and degrading it or preventing its translation. More importantly, a miRNA can target multiple mRNA and the same mRNA can be regulated by multiple miRNAs<sup>23</sup>. Thus, TargetScan7.1 and mirdbV6 were used to predict the target genes of the above 6 miRNAs. Moreover, the miRNA-mRNA networks were constructed by Cytoscape (Fig. 6F). As shown in Fig. 6B, the miRNAs can target hundreds of mRNA, while the mRNA of BRCC3 can be recognized by different miRNA. More importantly, a great quantity of target mRNA were to be involved in osteogenic differentiation related pathways. For instance, the Brain-Derived Neurotrophic Factor (BDNF), a well-known growth factor of the neurotrophin family, is involved in

regulating the growth, survival of neurons and angiogenesis<sup>24</sup>. Interestingly, Yamashiro et al. found that the mRNA and protein of BDNF were expressed in osteoblasts<sup>25</sup>. More importantly, further studies showed that the osteogenic differentiation ability of hPMSCs were enhanced with the expression of BDNF<sup>26,27</sup>.

## Discussion

How to solve different conditions of bone defects is a major clinical challenge<sup>28</sup>. Autograft is the ideal way to repair bone defects<sup>29</sup>. However, due to the limited source, the high infection rate in donor sites, and increases the pain burden of patients made its clinical application rate low. With the rapid development of the field of biomaterials, and the advantages of good biocompatibility and no immunogenicity, bone biomaterials are the most widely material for bone repair. Among them, hydroxyapatite, which has the same chemical composition as natural bone, long-term biocompatibility and interacts well with soft tissue or bone in vivo, is widely used to prepare various bone materials<sup>30-32</sup>. Therefore, an in-depth study of the osteogenic mechanism of hydroxyapatite scaffolds is conducive to the preparation of more efficient and specific bone scaffolds. However, previous studies mainly focused on the structure of hydroxyapatite scaffold and ignored its possible influence on the expression of miRNA or mRNA and signal transduction in cells.

Nowadays, more and more studies have revealed that miRNAs expressed in the various stages of bone formation, including the differentiation of bone marrow stromal stem cells into osteogenic precursor and the further development of precursor cells into mature osteoblasts. During these processes, they may play either a positive or negative role, including miR-96, miR-124, and miR-199a and so on<sup>33-37</sup>. Thus, we tried to explain the mechanism of HAG scaffolds promoted osteogenic differentiation from the perspective of miRNA differential expression. Interestingly, we did detect differential expression of miRNAs in the HAG-hPMSCs group. Moreover, overexpression of miRNA, which was highly expressed in the HAG scaffolds group, promoted osteogenic differentiation of hPMSCs. Furthermore, the integration of pathway analysis, functional analysis and miRNA-mRNA cross network analysis showed that the miRNAs differentially expressed in HAG scaffolds group were highly correlated with axon guidance, MAPK, and TGF-beta signaling pathway. These signaling pathways are closely related to osteogenic differentiation. Therefore, it is of great significance to understand the biological-related mechanism of bone induced by hydroxyapatite scaffolds based on miRNA-mRNA or signaling pathway for the subsequent research and development of scaffolds targeting biological regulation.

## Conclusions

In present work, we revealed that the HAG scaffolds may promote the bone formation through regulating the differentially expressed of miRNAs. In details, we successfully uncovered the statistically significant miRNAs in HAG-hPMSCs and constructed the miRNA-mRNA network through different bioinformatics analysis. However, we did not elucidate the specific signaling pathway of action involved in osteogenic

differentiation promoted by HAG. That's what we're working on right now. We believe that figuring out the key target miRNA and pathway for HAG promoting osteogenic differentiation will lay the foundation for the preparation of bone scaffolds loaded with miRNA.

## Declarations

### Conflict of interest

There is no conflict of interest among the authors.

### Acknowledgement

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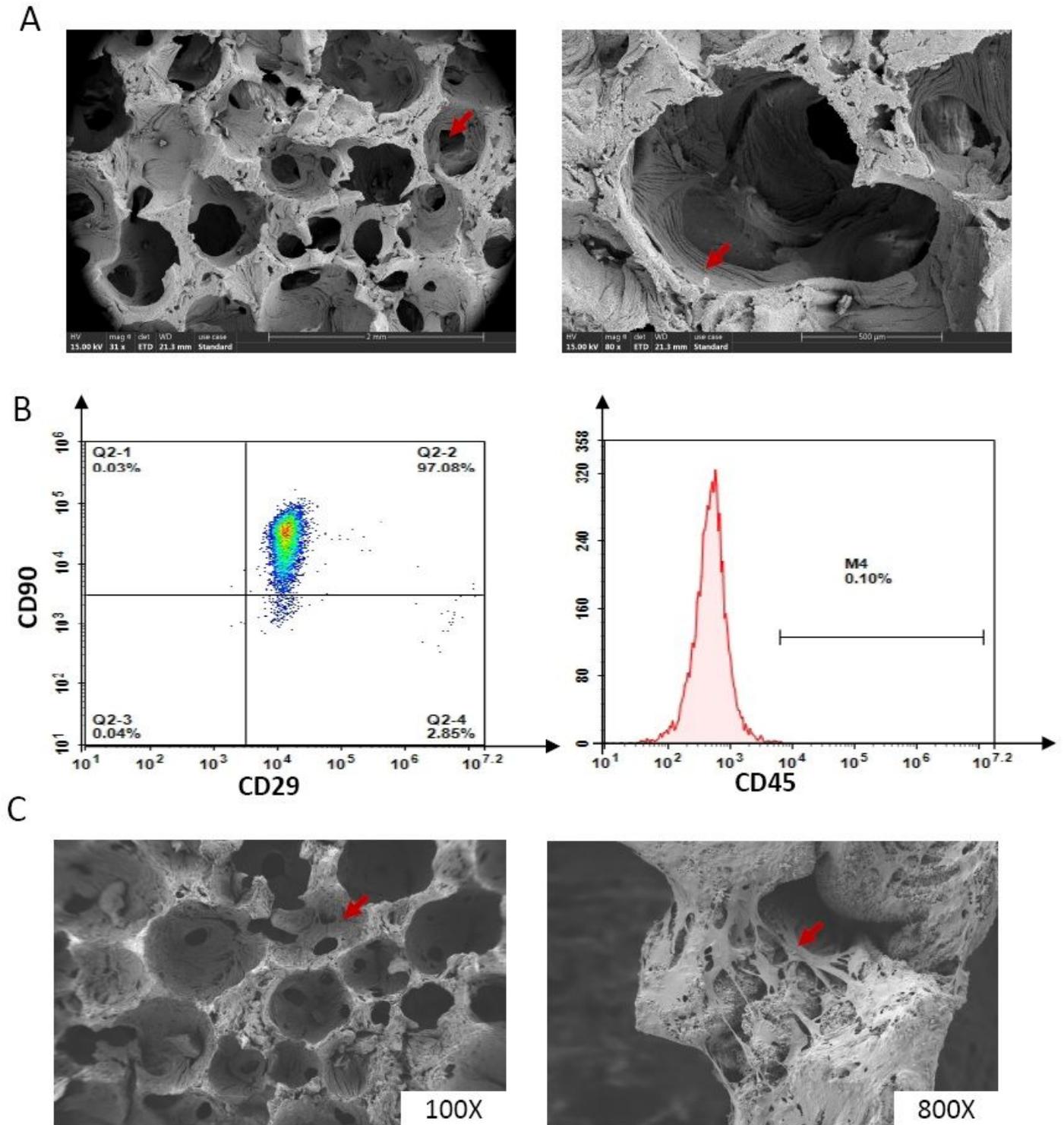
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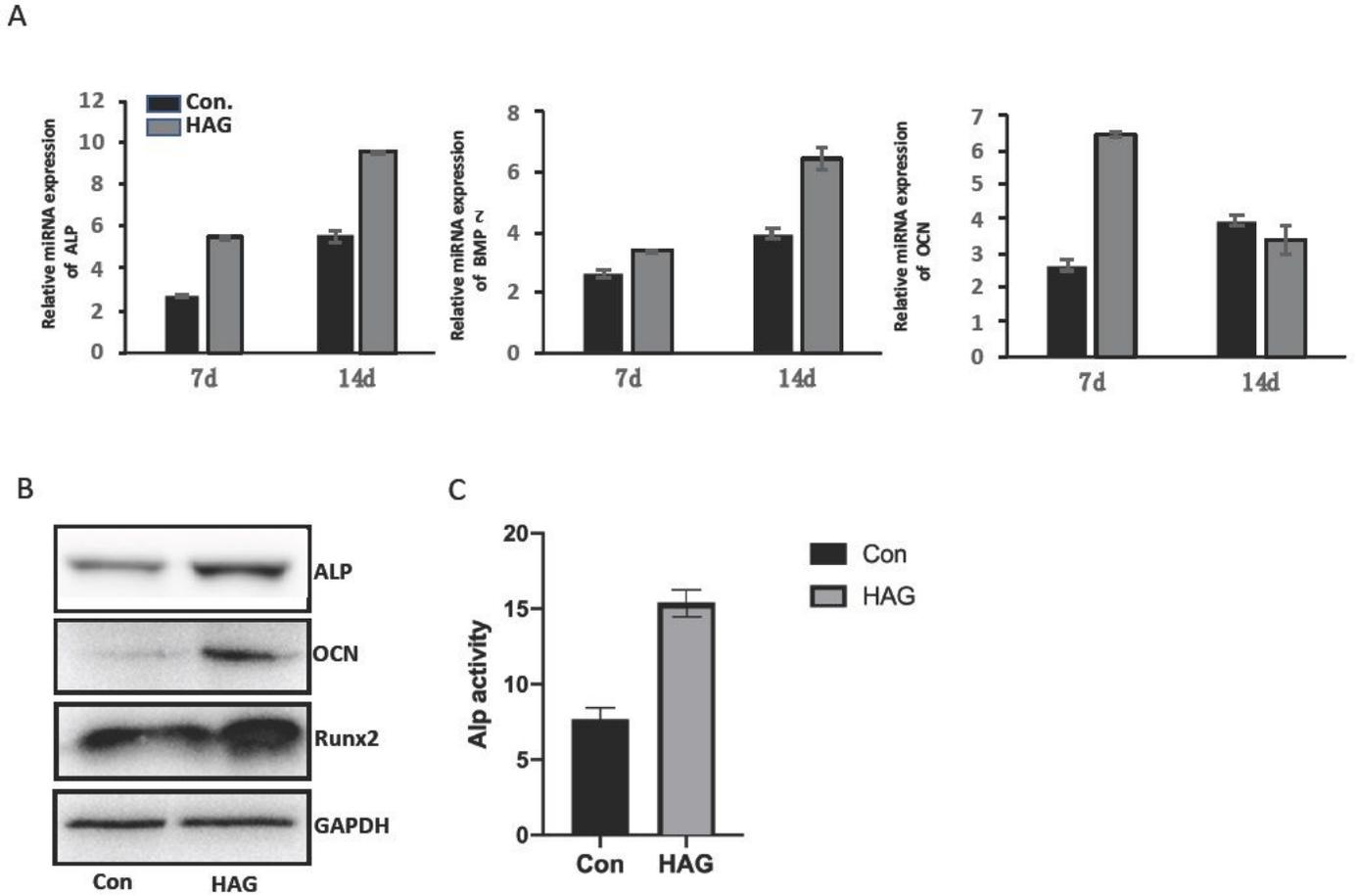
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## Figures



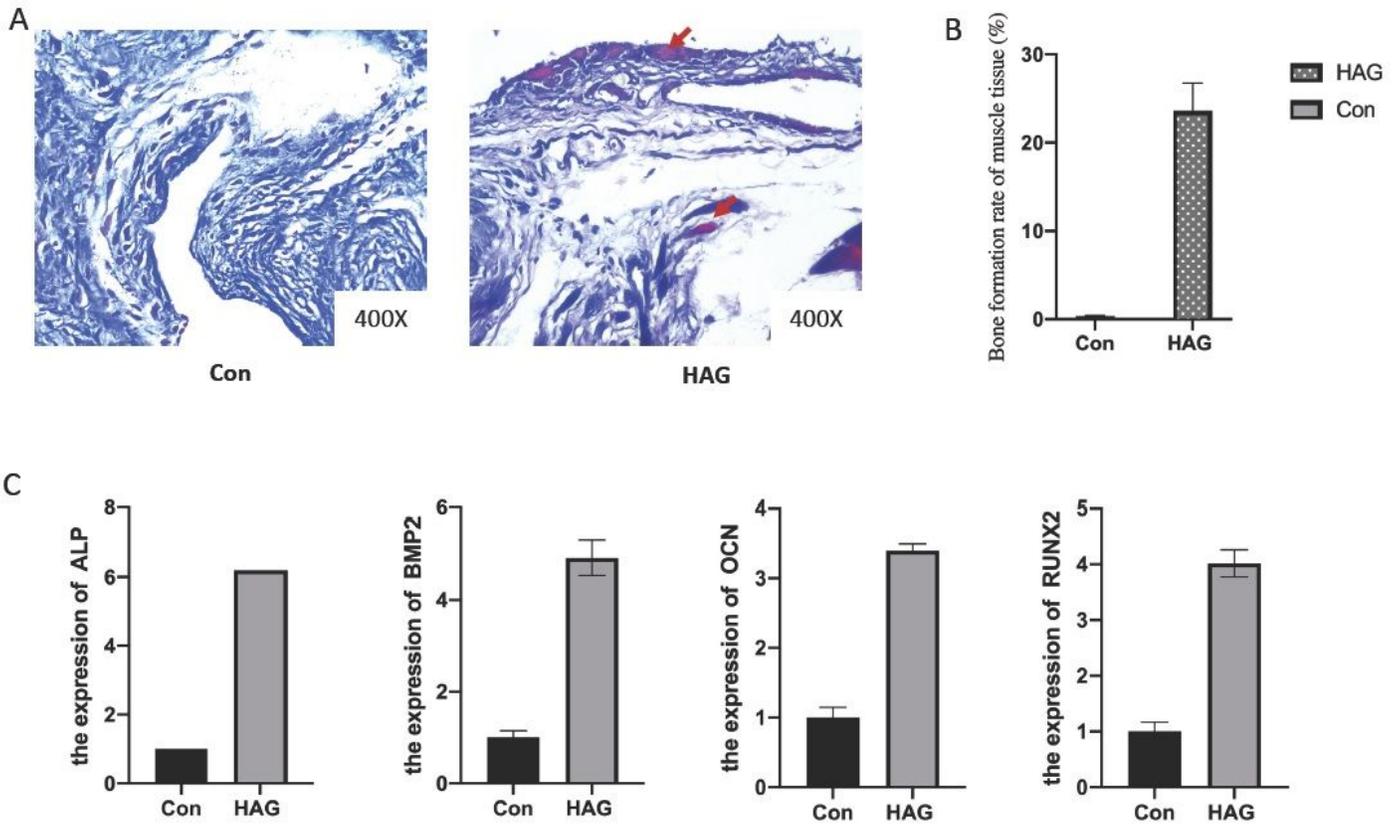
**Figure 1**

HAG scaffolds promoted surface hPMSCs adhesion A. Scanning electron microscopy (SEM) of HAG scaffolds surface morphology. B Flow cytometry was used to detect the expression of BMSCs surface markers (CD29, CD90, CD45). C. The phenotype of cells attached to the scaffold was observed by SEM.



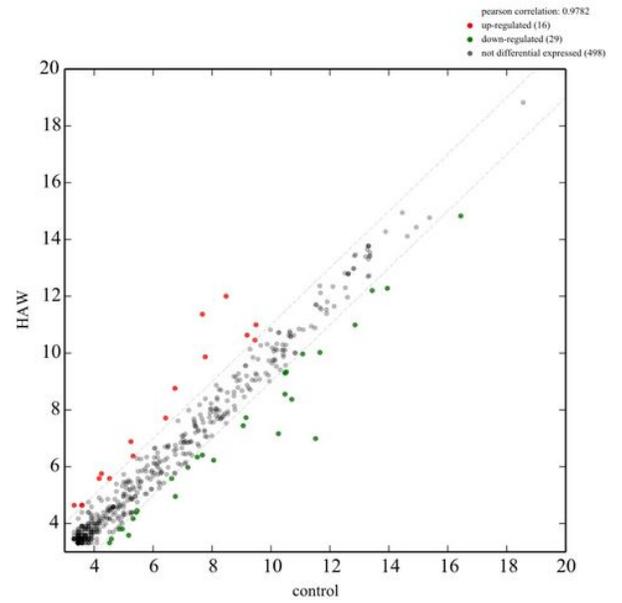
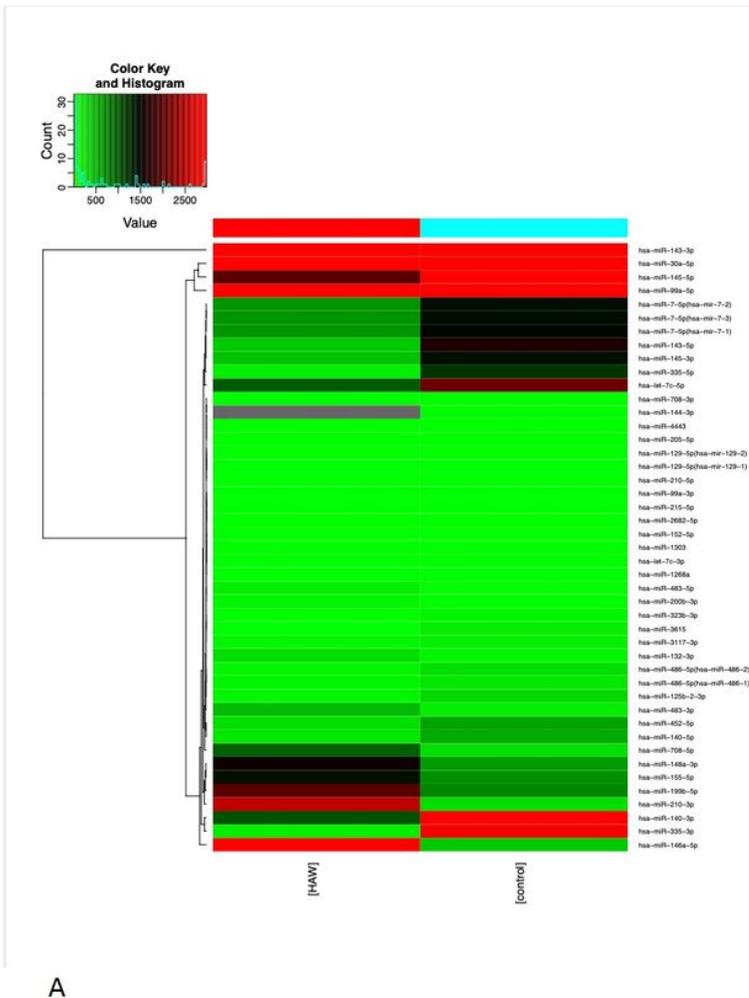
**Figure 2**

HAG scaffolds promoted the osteogenic differentiation of hPMSCs A. Expression of ALP, BMP2 and Ocn mRNA level in hPMSCs with or without HAG; GAPDH for internal reference. n = 3. Mean  $\pm$  SD was shown. B. Expression of ALP, BMP2 and Ocn protein level in hPMSCs with or without HAG, Gapdh for loading control. C. The ALP activities for 7 days incultation.



**Figure 3**

HAG scaffolds promoted the bone formation in vivo A. New bone formation was detected by Masson trichrome staining after HAG scaffolds implanted for 4 weeks (the red stain was new bone). B. Bone formation rate of muscle tissue (%) in 4th week after implanted. C. The relative mRNA expression level of Alp, Bmp2, Ocn and Runx2.

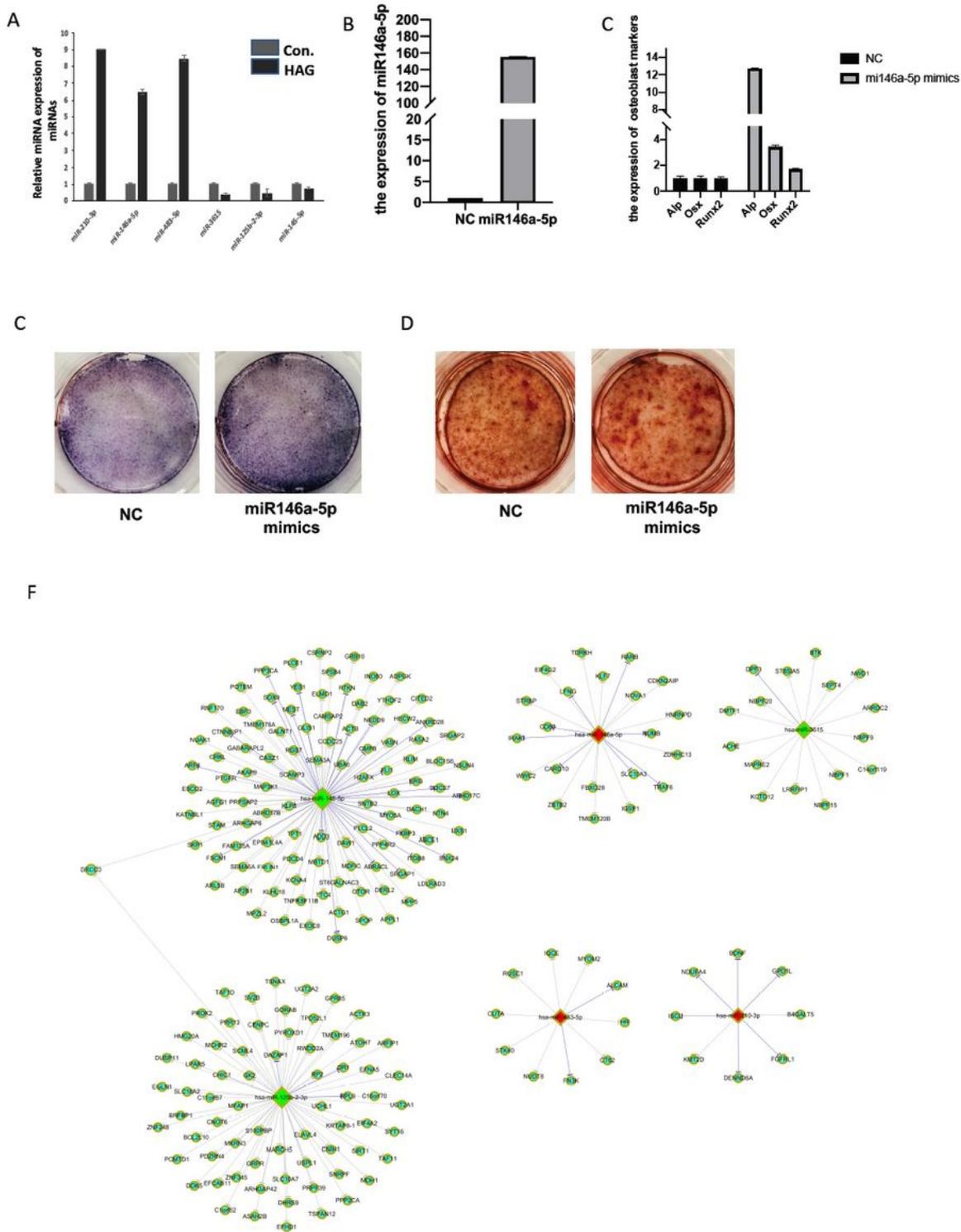


B

**Figure 4**

The miRNA differential expression profiles A. Different miRNAs heat map in hPMSC with or without HAG scaffolds. B. The volcano plot of miRNAs in HAG-hPMSC and hPMSC. The red means significant upregulation, while the green means significant downregulation (fold change  $\geq 2$ , p value  $< 0.05$ ). Notes: control: hPMSCs without HAG; HAW: hPMSC with HAG scaffolds.





**Figure 6**

The expressions of selected miRNAs were verified and the miRNA- mRNA interaction network was constructed A. The expression of chosen miRNAs were detected by qPCR in hPMSCs with or without HAG, normalized to U6 expression. B. Expression of miR146a-5p after mimics transfection. C. The expression of osteogenic markers mRNA after miR146a-5p mimics transfection and osteogenic differentiation induction for 7 days for 4d. D,E The ALP and Alizarin red staining were used to detected the ALP activity

and calcium deposits formation after osteogenic differentiation induction for 6 days and 14d. F. miRNA-mRNA cross network were made by Cytoscape. These mRNAs represent the potential downstream targets of chosen miRNAs.