

miR-196a-5p Promoted the Osteogenic Differentiation and Calvarial Bone Defect Repair of Wharton's Jelly Umbilical Cord Stem Cells

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Research

Keywords: miR-196a-5p, Mesenchymal Stem Cells, Wharton's jelly umbilical cord Stem Cells, Osteogenic Differentiation, Bone Regeneration

Posted Date: October 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-962654/v1>

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Abstract

Background: To investigate the effect of miR-196a-5p on the osteogenic differentiation and defected bone repair of Wharton's jelly umbilical cord stem cells (WJCMSCs).

Methods: miR-196a-5p mimic or inhibitor was applied to overexpress or silence miR-196a-5p expression in WJCMSCs. The alkaline phosphatase (ALP) activity, mineralization ability, and osteogenic markers expression were used to test WJCMSCs osteogenic potential *in vitro*. Calvarial bone defect model of rat was used to evaluate WJCMSCs bone regeneration ability *in vivo*. mRNA microarray was used to reveal the underlying mechanisms that miR-196a-5p regulated bone repair.

Results: miR-196a-5p inhibition reduced the ALP activity, mineralization ability, and level of osteogenic markers OCN, DSPP, DMP1 and BSP, while miR-196a-5p overexpression enhanced the ALP activity, mineralization ability, and level of OCN, DSPP, DMP1 and BSP of WJCMSCs *in vitro*. Next, the micro-CT and histopathology results showed miR-196a-5p-overexpressed-WJCMSCs obviously promoted the new bone tissue regeneration and calvarial bone defect repair after MSCs transplanted for 12 weeks. Further, mRNA microarray of miR-196a-5p-overexpressed-WJCMSCs revealed totally 959 significantly differentially expressed genes (DEGs), among which 34 upregulated and 925 downregulated. Also, 241 miR-196a-5p targeted genes were predicted by using miRNA targeted websites and only 19 predicted genes were consistent with microarray results. On this basis, one significantly downregulated gene SERPINB2 was selected and revealed that SERPINB2 deletion obviously enhanced the ALP activity and mineralization ability of WJCMSCs *in vitro*.

Conclusions: miR-196a-5p promoted the osteogenic differentiation potential and calvarial bone defect repair ability of WJCMSCs. And SERPINB2 acted as one key downstream gene to participate in the miR-196a-5p promoted MSCs osteogenic differentiation.

Background

The maxillofacial bone plays the major supporting and protecting role of tooth. Such as periodontitis, tumor, or trauma may cause the supporting destruction of bone, thus leading to functional and aesthetic problems [1, 2]. Nowadays, the clinical therapies for bone defects mainly rely on the grafts of bone granule filling materials or allogeneic and autologous tissue transplantation [3]. However, considering that there is a certain degree of alveolar bone loss caused by periroot lesions treatments, the volume of bone loss is not enough to provide sufficient space for material and bone tissue to be replanted, and these bone defects are of great interest for the support and protection of tooth periroot [4]. More suitable restoration methods still need to be considered for the repair of alveolar bone shape and further function. Because of self-renewal ability and multiple differentiation potentials, Mesenchymal stem cells (MSCs) based therapy is thought as a nascent method to damaged bone repair/regeneration [5, 6]. Yet, the mainly limitation is the low MSCs osteogenesis and bone defect repair ability. Other part, simple stem cell clumps or replantation with simple support materials still need to be improved for early tissue bonding of

such bone defects. Therefore, to find key regulator promoted MSCs osteogenic and extracellular matrix formation is helpful for the clinical usage of MSCs on traumatic alveolar bone loss repair.

Recent study revealed that MSCs osteogenic initiation was directly contributed by some small molecule regulators, especially MicroRNA (miRNA) [7]. MiRNA is a significantly conserved and non-protein-encoding RNA, which usually contains 19-25 nucleotides and acts as a key translational silencing regulator by interacting with the targeted mRNAs at 3' untranslated region [8, 9]. To find key miRNAs promoted MSCs osteogenic and extracellular matrix formation is helpful to elucidate the key regulatory mechanisms that block osteogenic differentiation of MSCs. Research revealed miR-196a-5p activated the Hedgehog signaling pathway and down-regulated GNAS expression, thereby promoted Bone marrow mesenchymal stem cells (BMSCs) osteogenic differentiation [10, 11]. Another study indicated that miR-196a-5p promoted the osteogenic differentiation of human Adipose derived stem cells (ADSCs) by down-regulating the level of HOXC8, which is a predicted target of miR-196a-5p [12]. All above revealed that miR-196a-5p might act as one regulator during osteogenic differentiation of MSCs. Yet, the role and mechanism of miR-196a-5p regulates MSCs osteogenic differentiation under bone defect conditions is still unclear.

Numerous studies have revealed MSCs repair potential for calvarial bone defect including BMSCs, Dental pulp stem cells (DPSCs) [13], and Wharton's jelly umbilical cord Stem Cells (WJCMSCs) [14]. WJCMSCs are extracted from neonatal umbilical cord tissue, which have a wide range of sources, low immunogenicity and non-invasive to the human body [15]. High biological safety, strong proliferation and differentiation potential and no ethical controversy make WJCMSCs a research hotspot in stem cell therapy [16–18]. Randomized controlled clinical trials have shown that WJCMSCs were effective when using for a variety of autoimmune diseases treatment, like Crohn's disease and Diabetes Mellitus [19, 20]. Researches have also shown that WJCMSCs as a valuable alternative to mesenchymal stem cells for osteogenic differentiation and craniofacial bone defect [21–24]. In our research, the function and mechanism of miR-196a-5p in the osteogenic differentiation and calvarial bone defect repair of WJCMSCs were investigated. The results revealed that miR-196a-5p promoted the potential of osteogenic differentiation and calvarial bone defect repair in WJCMSCs, and Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2) acted as one key targeted agent of the miR-196a-5p promoted MSCs osteogenic differentiation.

Methods

Animals

A total of twelve eight-week-old male Sprague Dawley (SD) rats were used in the present study. The animal study was approved by the animal care and use committee of Beijing Stomatological Hospital Animal laboratory (Reference number: 81,670,948). The animal care and experimental procedure were performed follow the guidelines of the Beijing Experimental Animal Management Ordinance.

Cell culture

Human stem cells study complied with the ISSCR “guidelines for the Conduct of Human Embryonic Stem Cell Research”. Human WJCMSCs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured as described before [24].

Synthesis of miRNA and viral infection

Lentivirus miR-196a-5p mimics and inhibitor were obtained from Genepharma Company (Suzhou, China). Virus transfection and puromycin selection for MSCs were performed as described before [25]. The sequence of miR-196a-5p mimics used in our study was 5'-TAGGTAGTTTCATGTTGTTGGG-3', The targeted sequence of miR-196a-5p inhibitor was 5'-CCCAACAACATGAAACTACCTA-3'.

Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

The extraction of WJCMSCs total RNA, synthesis of cDNA, and reactions of realtime RT-PCR were established as previously described [26]. The special target genes' primers were collected in Additional files 5.

Western blot analysis

As our previously described, 25 µg total protein extracted from WJCMSCs was separated by SDS gel electrophoresis [27]. The special target primary antibodies were including: Anti-Bone sialoprotein (BSP) (cat no. sc-73630, Santa Cruz Biotechnology, USA), Anti-Dentin matrix protein 1 (DMP1) (cat no. sc-81249, Santa), Anti-Osteocalcin (OCN) (cat no. sc-73464, Santa), and Anti-Dentin sialophosphoprotein (DSPP) (cat no. sc-73632, Santa). The endogenous control was detected using monoclonal antibodies against Histone H3 (cat no. sc-56616, Santa).

Alkaline Phosphatase (ALP) activity and mineralization analysis

As previously manufacturer's protocol, the WJCMSCs ALP activity in osteogenesis differentiation 5 days was analyzed by using ALP activity kit (Sigma) and WJCMSCs mineralization ability in 2 weeks were normalized by using Alizarin red staining and 10% cetylpyridinium chloride (CPC; Sigma) [28].

MSCs sheets preparation

As previous described for the MSCs sheets induction, 4×10^5 WJCMSCs were plated in each six-well culture plate and 20 mg/ml Vitamin C (Sigma) was used for 10 days induction [29, 30].

***In vivo* calvarial bone defect model and MSCs sheets transplantation**

Twelve of 8 weeks male Sprague Dawley (SD) rat were employed for calvarial bone defect model. Experimental procedure was performed under general anesthesia with 3% sodium pentobarbital solution intraperitoneal injection (1.5 mL / kg weight). The surgical area skin was prepared, and a 3 cm sagittal incision from frontal to occipital bone was made. The skin and subcutaneous tissue were cut in order, and the periosteum was carefully separated. A 5 mm diameter planer ring drill was used to carefully drill and generated calvarial bone defects at both sides of the center of the parietal area. Removing the bone piece completely and avoid damaging the dura mater and the surface blood vessels. Then, the sizeable WJCMSCs sheets were transplanted and skin defect was layered sutured. Rats were sacrificed after 12 weeks.

Micro-computed tomography analysis

Rat calvarial bones were harvested and scanned for Micro-CT analysis. The scanning parameters were 70 kV, 114 mA, and thickness was 150 μm . On this scanning basis, three-dimensional structure of each calvarial bones was established and quantitative analysis of newly formed bone was took by CTan software.

Histological evaluation of regenerated bone

All Rat calvarial bones samples were harvested and decalcified, dehydrated, and embedded in paraffin, then made into 5 μm -thick sections. The conventional H&E staining and microscope (OLYMPUS BX53) were used for histomorphological analysis.

Transcriptome microarray analysis

WJCMSCs total RNA was extracted, then RNA labeling, hybridization, and scanning were performed as described in the Affymetrix technical manual (Affymetrix, CA, USA). The selected threshold for differentially mRNAs was that fold change > 2 (up genes) or < 0.5 (down genes) and p value < 0.05 . The microarray raw data were normalized with Expression Console software (Affymetrix) and then bioinformatic analysis was performed.

Statistical analysis

Each experiment was done at least in triplicate. All statistical results were analyzed by SPSS10 statistical software. Statistical significance was assessed by one-way analysis of variance (ANOVA) or Student's t test; $p \leq 0.05$ was defined as significant.

Results

miR-196a-5p inhibition decreased the osteogenic differentiation ability of WJCMSCs in vitro

To confirm the role of miR-196a-5p, we used the miR-196a-5p inhibitor to knock down miR-196a-5p expression in WJCMSCs. After 2 $\mu\text{g}/\text{mL}$ puromycin selection for 3 days, the inhibition efficiency of miR-196a-5p was detected by realtime-RT-PCR (Figure 1A). Then WJCMSCs were induced by using of osteogenic-inducing medium to decide miR-196a-5p effect on osteogenic. After 5 days induction, the WJCMSCs ALP activity was dramatically decreased in the miR-196a-5p inhibition group compared with the Consh group (Figure 1B). After 2 weeks induction, Alizarin red staining (Figure 1C) and quantitative calcium results (Figure 1D) revealed that the mineralization ability of WJCMSCs was dramatically reduced in the miR-196a-5p inhibition group compared with the Consh group. Also, the osteogenic markers' level were tested, western blot results showed that BSP, DSPP, DMP1, and OCN were significantly down-expressed at osteogenic induced 7 day and 14 day (Figure 1E).

miR-196a-5p overexpression enhanced the osteogenic differentiation ability of WJCMSCs in vitro

To further investigate its effect for WJCMSCs osteogenic ability, we overexpress miR-196a-5p in WJCMSCs by miR-196a-5p mimics transfection. After 3 days of selection with 2 $\mu\text{g}/\text{mL}$ puromycin, realtime RT-PCR was applied to test the overexpression efficiency of miR-196a-5p (Figure 2A). After osteogenic induced for 5 days, the ALP activity of WJCMSCs showed considerably increased in the miR-196a-5p mimics group compared to the Consh group (Figure 2B). After 2 weeks induction in vitro, Alizarin red staining (Figure 2C) and quantitative calcium analysis (Figure 2D) showed the mineralization ability of WJCMSCs was considerably increased in the miR-196a-5p mimics group compared to the Consh group. Next, the results of western blot indicated the osteogenic markers' level of BSP, DSPP, DMP1, and OCN were significantly up-regulated in the miR-196a-5p mimics group on day-7 and day-14 during osteogenic induction (Figure 2E).

miR-196a-5p promoted the calvarial bone defect repair ability of WJCMSCs in SD rat

Since miR-196a-5p overexpression enhanced the osteogenic differentiation ability of WJCMSCs *in vitro*, we hypothesized whether miR-196a-5p regulated bone regeneration *in vivo*. To test this hypothesis, we

established a calvarial bone defect models for a 5mm diameter round which difficultly to heal in SD rats. To promote the healing potential of MSCs, WJCMSCs sheets were formed by using 20 µg/mL vitamin C induction 7 days (Figure 3A). The H&E staining revealed that compared to the Consh group, the miR-196a-5p mimics group formed a better two- or three-layered uniformly spread structure in whole cell sheets (Figure 3B).

Then, WJCMSCs sheets was transplanted in calvarial bone defect of SD rat. After 12 weeks, the gross morphological manifestation showed that calvarial bone defect significantly healed in the miR-196a-5p mimics group (Figure 3C). And micro-CT reconstructed image (Figure 3D) and quantitative analysis results (Figure 3E) showed that the miR-196a-5p mimics group formed more new bone tissue compared to the Consh group. The 20× objective view of H&E staining showed more mature new bone tissue was formed in the miR-196a-5p mimics group compared to the Consh group (Figure 3F; Black line showed).

The profiling of differentially expressed genes in miR-196a-5p overexpressing WJCMSCs and negative control group

To explore the mechanism that miR-196a-5p regulated osteogenic differentiation of WJCMSCs, microarray and bioinformatics analyses were performed. In general, totally 959 differentially expressed genes (DEGs) regulated by miR-196a-5p were revealed. Among which 34 genes were significantly upregulated (fold change ≥ 2.0 , P value ≤ 0.05), and 925 genes were significantly downregulated (fold change ≤ 0.5 , P value ≤ 0.05) (Additional files 6). Here, we used a heatmap to show the mainly DEGs between the miR-196a-5p mimics group and the control group in WJCMSCs (Figure 4A). Among these DEGs, we chosen CLDN11, IL6, ENPP1, SERPINB2, and VCAM1 to verify that the accuracy of microarray results was credibly (Figure 4B).

Also, GO terms analysis was performed to detected the significantly changed functions caused by the differentially expressed genes in WJCMSCs. The significantly top 3 GO terms of miR-196a-5p up-regulated DEGs in WJCMSCs mainly included in proteinaceous extracellular matrix (biological process; GO:0005578), extracellular matrix component (biological process; GO:0044420), fibrillar collagen trimer (biological process; GO:0005583); extracellular matrix structural constituent (cellular component; GO:0005201), growth factor binding (cellular component; GO:0019838), insulin-like growth factor binding (cellular component;GO:0005520); extracellular matrix organization (molecular function; GO:0030198), extracellular structure organization (molecular function; GO:0043062), negative regulation of cell motility (molecular function; GO:2000146) (Additional files 1). The significantly top 3 GO terms of miR-196a-5p down-regulated DEGs in WJCMSCs mainly included in chromosomal region (biological process; GO:0098687), nuclear envelope (biological process; GO:0005635), spindle (biological process; GO:0005819); ATPase activity (cellular component; GO:0016887), helicase activity (cellular component; GO:0004386), cell adhesion molecule binding (cellular component; GO:0050839); chromosome segregation (molecular function; GO:0007059), cell cycle checkpoint (molecular function; GO:0000075), sister chromatid segregation (molecular function; GO:0000819) (Additional files 2).

Pathway enrichment analysis was then performed to detect the significantly associated major pathways with differentially expressed genes regulated by miR-196a-5p. The KEGG analysis results showed that the ECM-receptor interaction pathway (KEGG:hsa04512), the amoebiasis pathway (KEGG:hsa05146), and the leishmaniasis pathway (KEGG:hsa05140) were more correlative with the miR-196a-5p up-regulated DEGs in WJCMSCs (Additional files 3), and the RNA degradation pathway (KEGG:hsa03018), the steroid biosynthesis pathway (KEGG:hsa00100), and the endocytosis pathway (KEGG:hsa04144) were more correlative with the miR-196a-5p down-regulated DEGs in WJCMSCs (Additional files 4). And a network was analysed to reveal the relationship between the significantly miR-196a-5p regulated DEGs associated pathways in WJCMSCs (Figure 5).

In addition to transcriptome microarray analysis, we also use websites miRDB (<http://www.mirdb.org>), miRwalk (<http://mirwalk.umm.uni-heidelberg.de>), and TargetScan (<http://www.targetscan.org>) to predict the targeted genes of miR-196-5p. The predicted targeted genes by at least two sites were included in the analysis. Interestingly, there are 241 predicted genes were got (Additional files 7). And only 19 predicted targeted genes were consistent with our microarray results (Additional files 8).

Deletion of SERPINB2 enhanced the osteogenic differentiation potential of WJCMSCs in vitro

Here, a significantly downregulated gene by miR-196a-5p, SERPINB2 drawn our interest. We tried to predict the possible binding sites of miR-196a-5p and SERPINB2 mRNA, but unfortunately, SERPINB2 mRNA does not contain the possible binding sites of miR-196a-5p, which suggested the miR-196a-5p regulation to SERPINB2 seem not by binding to the 3' untranslated region of SERPINB2 mRNA. Therefore, we further hypothesized that the key role of miR-196a-5p in regulating osteogenesis does not simply depend on its classical regulatory role. So, Therefore, we further investigated the effect of SERPINB2 on the osteogenesis function in WJCMSCs.

Firstly, lentiviral shRNA infection was performed to knock down the SERPINB2 expression in WJCMSCs. Real-time RT-PCR determined that about 80% knockdown efficiency was achieved (Figure. 5A). Then, the ALP activity was tested at day 5 by osteogenic induction, and the result showed that the ALP activity of the SERPINB2sh group was significantly increased compared to the Scramsh group in WJCMSCs (Figure 5B). The two weeks Alizarin red staining (Figure 5C) and the quantitatively calcium results (Figure 5D) showed that the mineralization ability of the SERPINB2sh group significantly increased compared to the Scramsh group in WJCMSCs.

Discussion

According to the pre-clinical trials, MSCs-based biological therapy is a potent approach in the clinical treatment of bone defects [31]. miR-196a-5p has been reported its level was significantly lower in peripheral blood from postmenopausal osteoporosis patients [19, 32]. Research revealed miR-196a-5p

promoted BMSCs osteogenic differentiation by activating the Hedgehog signaling pathway [10, 11]. And miR-196a-5p promoted human ADSCs osteogenic differentiation by down-regulating HOXC8 [12]. Our study clarified miR-196a-5p acted as a key regulator to promote the osteogenic differentiation and bone defect repair ability of WJCMSCs. The choice for appropriate MSCs source and the regulation for its osteogenic differentiation potential is crucial for gaining insight into MSCs-based bone regeneration [33]. Compared with BMSCs and ADSCs, WJCMSCs have a better ability to secrete external matrix, suggesting WJCMSCs also as a valuable alternative for osteogenic differentiation and craniofacial bone defect [21–24]. cell-cell communication supports the early MSCs alive and further repair niches creation of bone defects. Numerous bioactive exogenous matrix protein and extracellular vesicles (EVs), play important roles in cell-cell communication, osteogenic genes regulation, and osteoblastic differentiation. miR-196a-5p plays an essential role in EVs exogenesis during BMSCs' osteoblastic differentiation [18]. Our study found that miR-196a-5p promoted the formation of a better two- or three-layered uniformly spread structure in whole WJCMSCs cell sheets. Together, these suggested miR-196a-5p acts as one key promoted regulator of MSCs osteogenic differentiation and was conducive to support the bone defects repair structure creation of MSCs.

The main obstacle of MSCs-based tissue regeneration is to form high-quality tissue structures that match the body's functional needs [34]. We used subcutaneous implantation and a pair of 5mm critical-size calvarial bone defect model to evaluate miR-196a-5p bone formation capacity of rats *in vivo* [20]. Besides, the structure of the calvarial bone is similar with alveolar bone, both of which are surface cortical bone and internal cancellous bone. In this study, we combined with "cell sheet" technology to further enhance the osteogenesis of WJCMSC. The cell sheet, which contains intact extracellular matrix (ECM) and cell surface proteins, simulate the physical structure and biological function of natural ECM [35]. Due to easily fabricated and biodegradable, MSCs sheet has been efficaciously utilized in the regeneration of bone tissues [36, 37]. We found miR-196a-5p enhanced WJCMSCs-mediated new bone formation and the high quality repair/regeneration of damaged bone tissue.

The mechanism of miR-196a-5p to stimulate bone regeneration has not yet been elucidated. Therefore, the differential expression genes profiles of miR-196a-5p in WJCMSCS were determined by mRNA microarray analysis. Among this, 34 genes were significantly upregulated and 925 genes were significantly downregulated. In addition, we also predicted miR-196-5p might targeted genes of by using websites miRDB (<http://www.mirdb.org>), miRwalk (<http://mirwalk.umm.uni-heidelberg.de>), and TargetScan (<http://www.targetscan.org>). There are 241 predicted genes that all three sites overlap. Integrated the two different methods of transcriptome gene chip and website prediction, there are only 19 downstream target genes that meet the two screening conditions. Therefore, we added details of miR-196-5p possible downstream targeted genes. Also, significantly GO terms of miR-196a-5p up-regulated DEGs in WJCMSCs mainly included in extracellular matrix component and structural constituent, fibrillar collagen trimer, and growth factor binding while miR-196a-5p down-regulated DEGs mainly included in chromosomal structure change and ATPase activity. The KEGG analysis results showed that the miR-196a-5p mainly activated ECM-receptor interaction pathway (KEGG:hsa04512) and down-regulated the RNA degradation pathway (KEGG:hsa03018), which was consistent with our observation that miR-196a-

5p promoted extracellular matrix formation in WJCMSCs. Here, we reveal specific whole-mRNA regulatory target genes and detailed pathway mechanisms of miR-196a-5p.

SERPINB2 was a significantly downregulated gene (2.6 fold) of miR-196a-5p in WJCMSCs. Previous study revealed SERPINB2 played a negative regulatory role in human BMSCs osteogenic differentiation through TGF- β signaling pathway [38]. Our result confirmed SERPINB2 played a negative role in the mineralization of WJCMSCs. The classical pathway of miRNA regulation is combination with 3'UTR of downstream target genes, thus degrade or induce translational silence of target genes [8]. Unfortunately, although SERPINB2 is negatively correlated with miR-196a-5p, websites of miRDB, miRwalk and TargetScan could not predict the directly interaction site between miR-196a-5p and SERPINB2 mRNA. The result demonstrated miR-196a-5p regulation of SERPINB2 might not through the classical way, and the specific mechanism needs to be further explored. Given the large differences between the microarray and the predicted results, the study of miR-196a-5p underlying mechanisms should not focus solely on classical 3'UTR binding way, especially the revelation of these key negative suppressed genes and involved pathways.

In contrast with our study, other researchers revealed SERPINB2 involvement in physiological mineralization. SERPINB2 was detected significantly high expression in mineralized ECM producing cells and SERPINB2-deficient severely impair cell mineralization function [39]. These contradictory results for bone formation may be related with the environment which SERPINB2 involved in. Previous study shown that intracellular(47kDa)and extracellular (60kDa) SERPINB2 isoforms execute different functions [40]. The intracellular isoform plays the cytoprotective role by involving in the protein folding [41], while the extracellular isoform mainly involves in ECM remodeling [42]. The enigmatic mechanism of SERPINB2 for stem cell mediated-bone regeneration remains to be unveiled. Point to point, the exploration of SERPINB2 enriched the understanding of miR-196a-5p potential regulation mechanism and provided another idea

Conclusions

In conclusion, our results indicated that miR-196a-5p promoted the osteogenic differentiation potential of WJCMSCs *in vitro* and regeneration of impaired bone tissue *in vivo*. Furthermore, we also identified SERPINB2 as a key downstream gene of miR-196a-5p involved in the osteogenic differentiation. These discoveries contributed to the understanding of the function and mechanism of miR-196a and supplied a feasible target for improving MSCs-based bone regeneration therapy.

Abbreviations

ADSCs: Adipose derived stem cells; ALP: alkaline phosphatase; BMSCs: Bone marrow mesenchymal stem cells; BSP: Bone sialoprotein; DEGs: Differentially expressed genes; DPSCs: Dental pulp stem cells; DSPP: Dentin sialophosphoprotein; DMP1: Dentin matrix protein 1; ECM: Extracellular matrix; EVs: Extracellular vesicles; MiRNA : MicroRNA; MSCs: Mesenchymal Stem Cells; OCN: Osteocalcin; WJCMSCs: Wharton's jelly of umbilical cord stem cells;

Declarations

Ethics approval and consent to participate:

The animal study was approved by the animal care and use committee of Beijing Stomatological Hospital Animal laboratory (Reference number: 81,670,948). The animal care and experimental procedure were performed follow the guidelines of the Beijing Experimental Animal Management Ordinance. Human stem cells study complied with the ISSCR “guidelines for the Conduct of Human Embryonic Stem Cell Research”. Human WJCMSCs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA).

Consent for publication:

Not applicable.

Availability of data and materials:

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests:

The authors declare that they have no competing interests.

Funding:

This work was supported by grants from the General Program (Key Program, Major Research Plan) of National Natural Science Foundation of China (81670948 to Y. C.).

Authors' contributions:

WY and ZS performed the experiments. WY analyzed the data. YH, YD and ZY was responsible for collection of data and technical expertise. CY supplied experimental materials and resources. YH, CYY and CY conceived the study. WY and CYY drafted the manuscript. CYY reviewed this work. All the authors approved the final manuscript. WY and ZS are senior authors, contributed equally, and CY are co-correspondents to this work.

Acknowledgements:

Not applicable.

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Figures

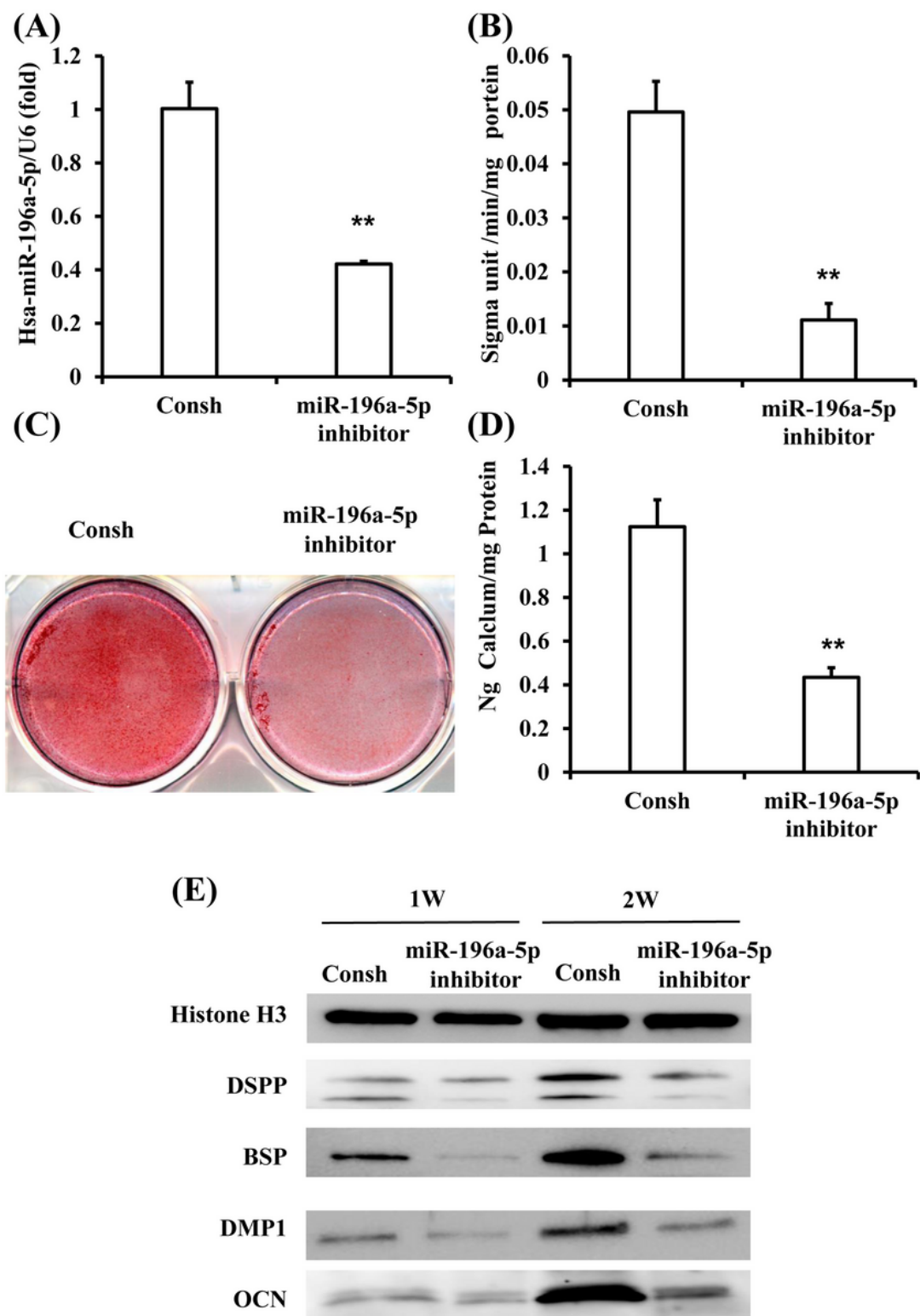


Figure 1

The inhibition of miR-196a-5p decreased the osteogenic differentiation potential of WJCMSCs in vitro. (A) Determination of miR-196a-5p inhibition efficiency by realtime-RT-PCR. (B) Inhibition of miR-196a in decreased the ALP activity of WJCMSCs. Inhibition of miR-196a-5p decreased the Alizarin red staining (C) and quantitative calcium measurements (D) of WJCMSCs. (E) The western blot results showed miR-196a-5p inhibition in WJCMSCs down-regulated the expression of BSP, DSPP, DMP1, and OCN at

osteogenic induced 1w and 2w. GAPDH or Histone H3 was as control marker; The statistical significance was evaluated by one-way analysis of variance (ANOVA) or Student's t-test; The error bar was indicated by SD (n = 3); **p ≤ 0.01.

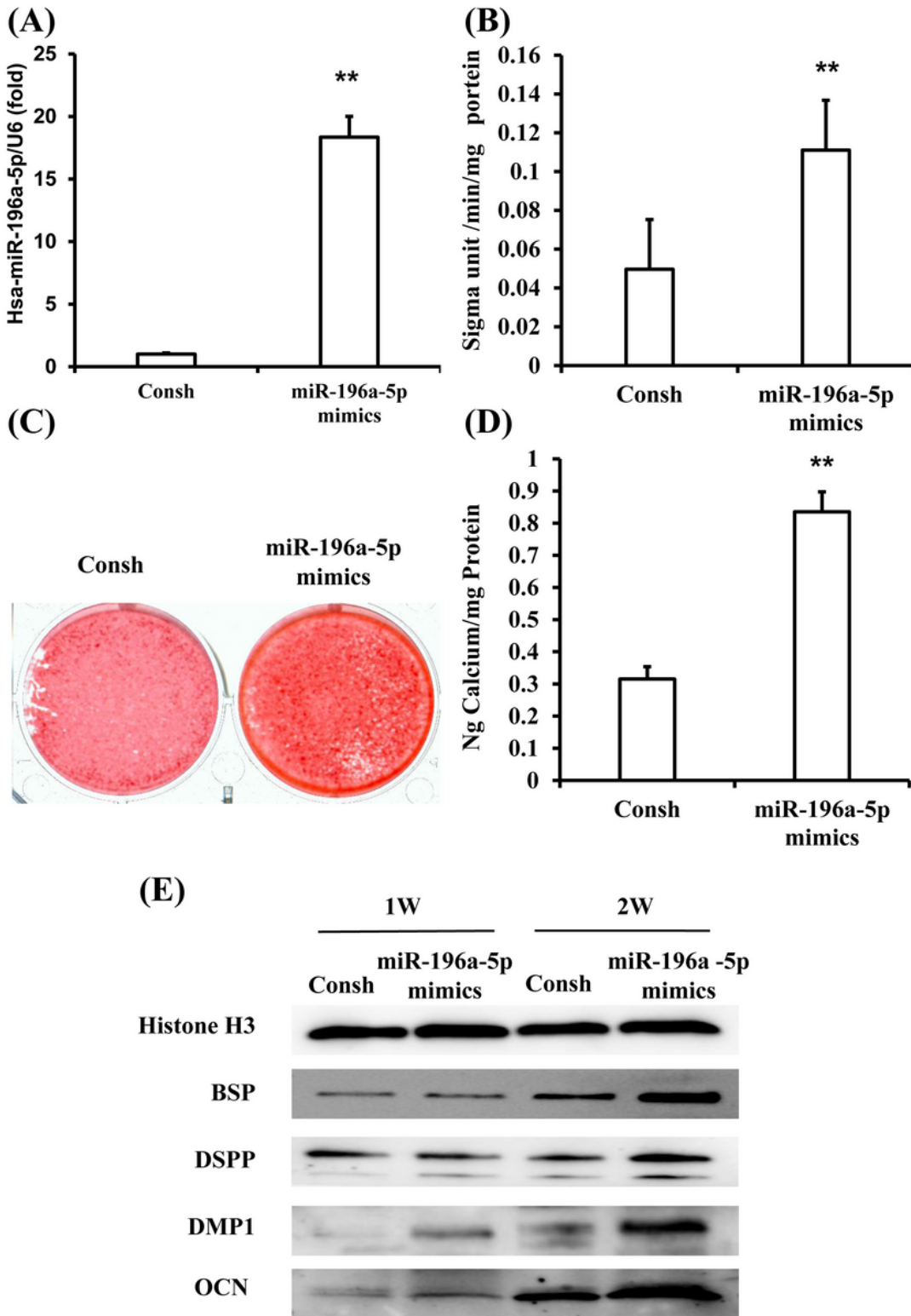


Figure 2

The overexpression of miR-196a-5p enhanced the osteogenic differentiation potential of WJCMSCs in vitro. (A) Determination of miR-196a-5p overexpression efficiency by realtime-RT-PCR. (B) miR-196a-5p

overexpression enhanced the ALP activity of WJCMSCs. Overexpression of miR-196a-5p enhanced the Alizarin red staining (C) and quantitative calcium measurements (D) of WJCMSCs. (E) The western blot results showed that miR-196a overexpression in WJCMSCs up-regulated the expression of BSP, DSPP, DMP1, and OCN at osteogenic induced 1w and 2w. GAPDH or Histone H3 was as control marker; The statistical significance was evaluated by one-way analysis of variance (ANOVA) or Student's t-test; The error bar was indicated by SD (n = 3); **p ≤ 0.01.

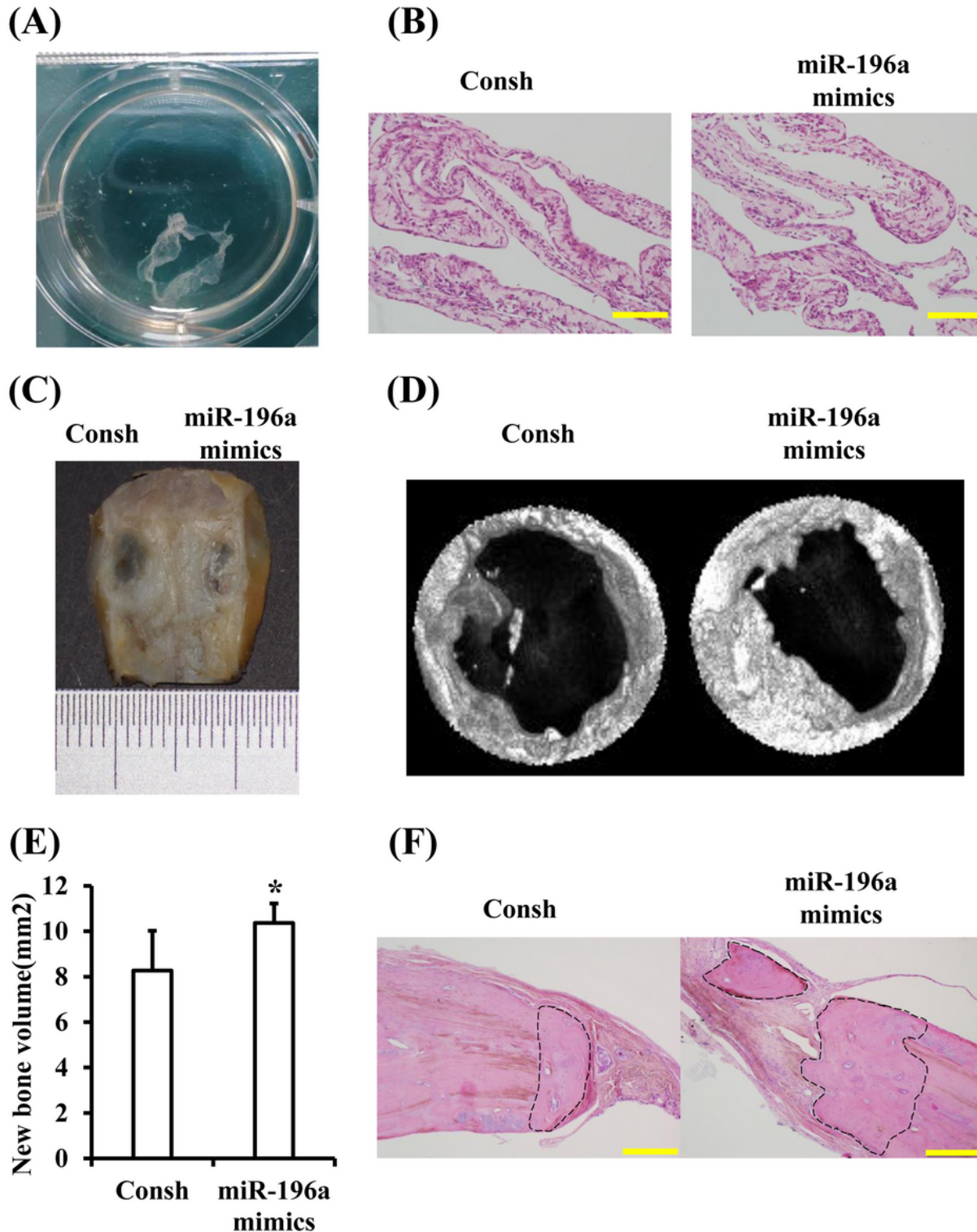


Figure 3

The miR-196a-5p-overexpressed-WJCMSCs promoted the calvarial bone defect repair in SD rat. (A) The gross morphological manifestations of WJCMSCs sheets. (B) The H&E staining showed the miR-196a-5p mimics group formed a better two- or three-layered uniformly spread structure in whole cell sheets compared to negative control group. (C) After 12 weeks of MSCs transplantation, the gross morphological manifestation showed that calvarial bone defect significantly healed in the miR-196a-5p mimics group. (D) The micro-CT reconstruction image and (E) quantitative analysis results showed formed more new bone tissue compared to negative control group. (F) The H&E staining showed more mature new bone tissue was formed in the miR-196a-5p mimics group compared to negative control group. The Yellow bar = 25 um; Black line showed new bone tissue; The statistical significance was evaluated by one-way analysis of variance (ANOVA) or Student's t-test; The error bar was indicated by SD (n = 5); *p ≤ 0.05.

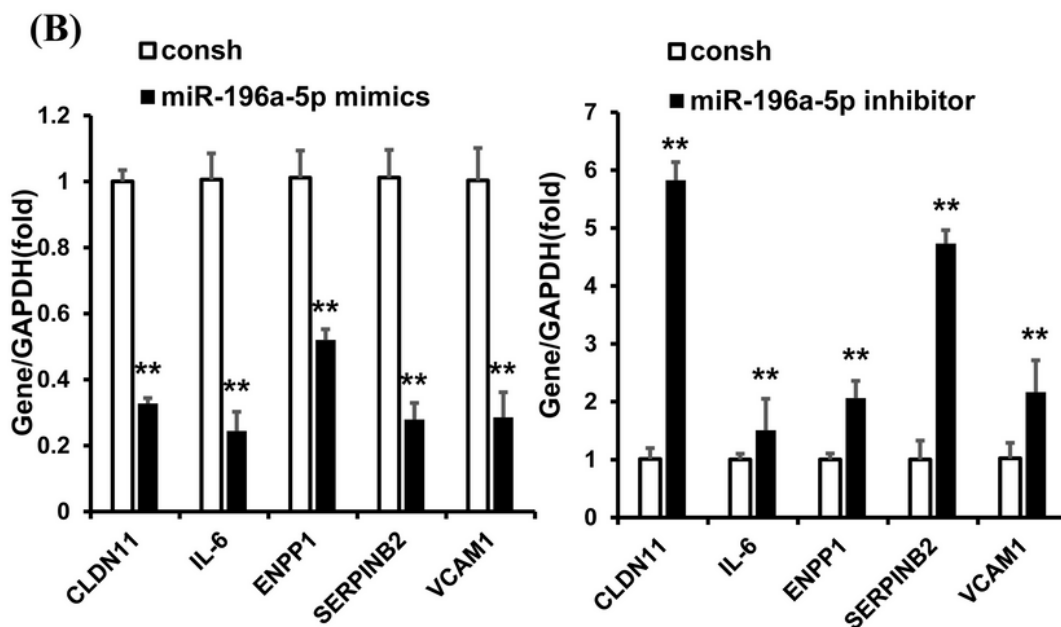
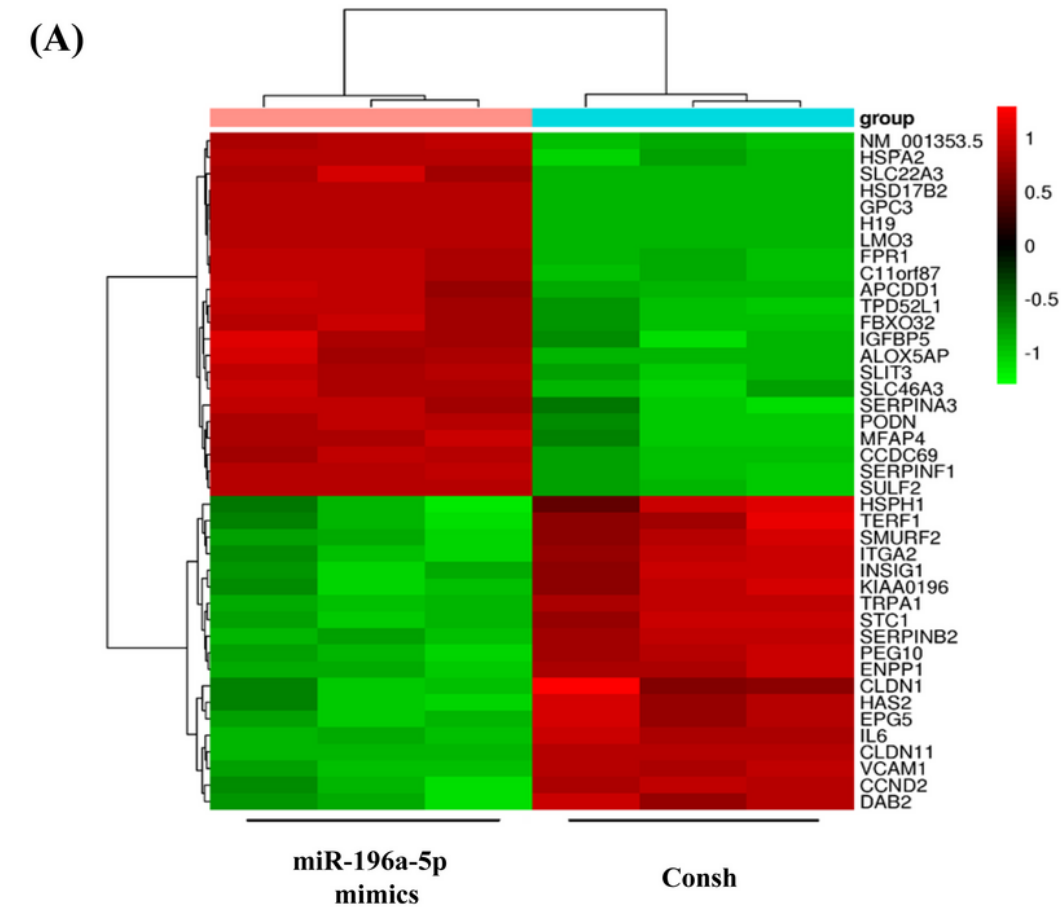


Figure 4

The profiling of differentially expressed genes regulated by miR-196a-5p in WJCMSCs. (A) The heatmap of top 20 differentially expressed genes (DEGs) regulated by miR-196a-5p in WJCMSCs. (B) Among these DEGs, CLDN11, IL-6, ENPP1, SERPINB2, and VCAM1 were chosen and the expression level were tested by realtime-RT-PCR. GAPDH was as control marker; The statistical significance was evaluated by one-way analysis of variance (ANOVA) or Student's t-test; The error bar was indicated by SD (n = 3); **p ≤ 0.01.

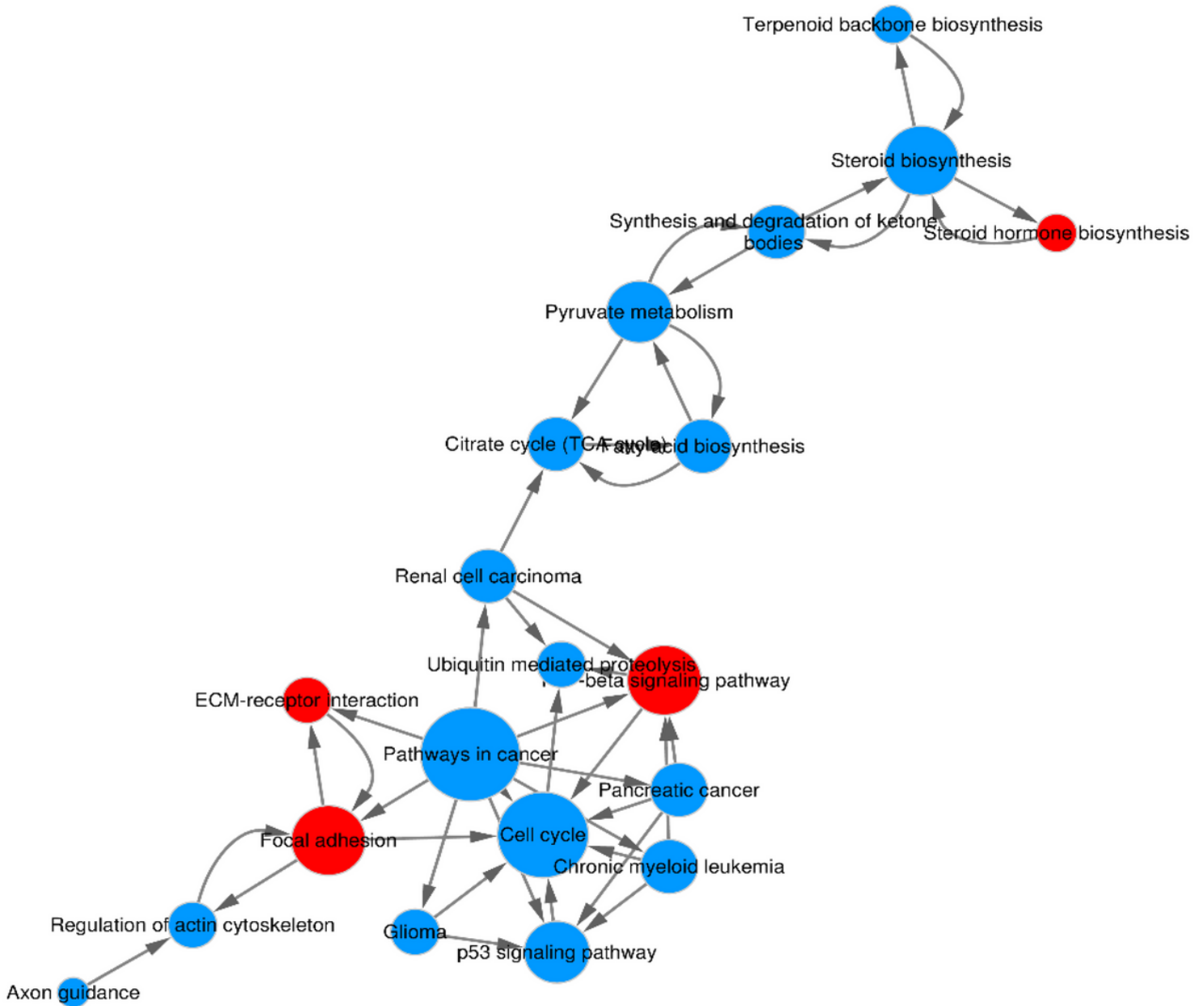


Figure 5

The significantly DEGs associated pathways Net of miR-196a-5p regulated in WJCMSCs. The red spots represent the miR-196a-5p up-regulated DEGs associated pathways and the blue spots represent the miR-196a-5p down-regulated DEGs associated pathways. The arrows indicated the relationship between DEGs associated pathways.

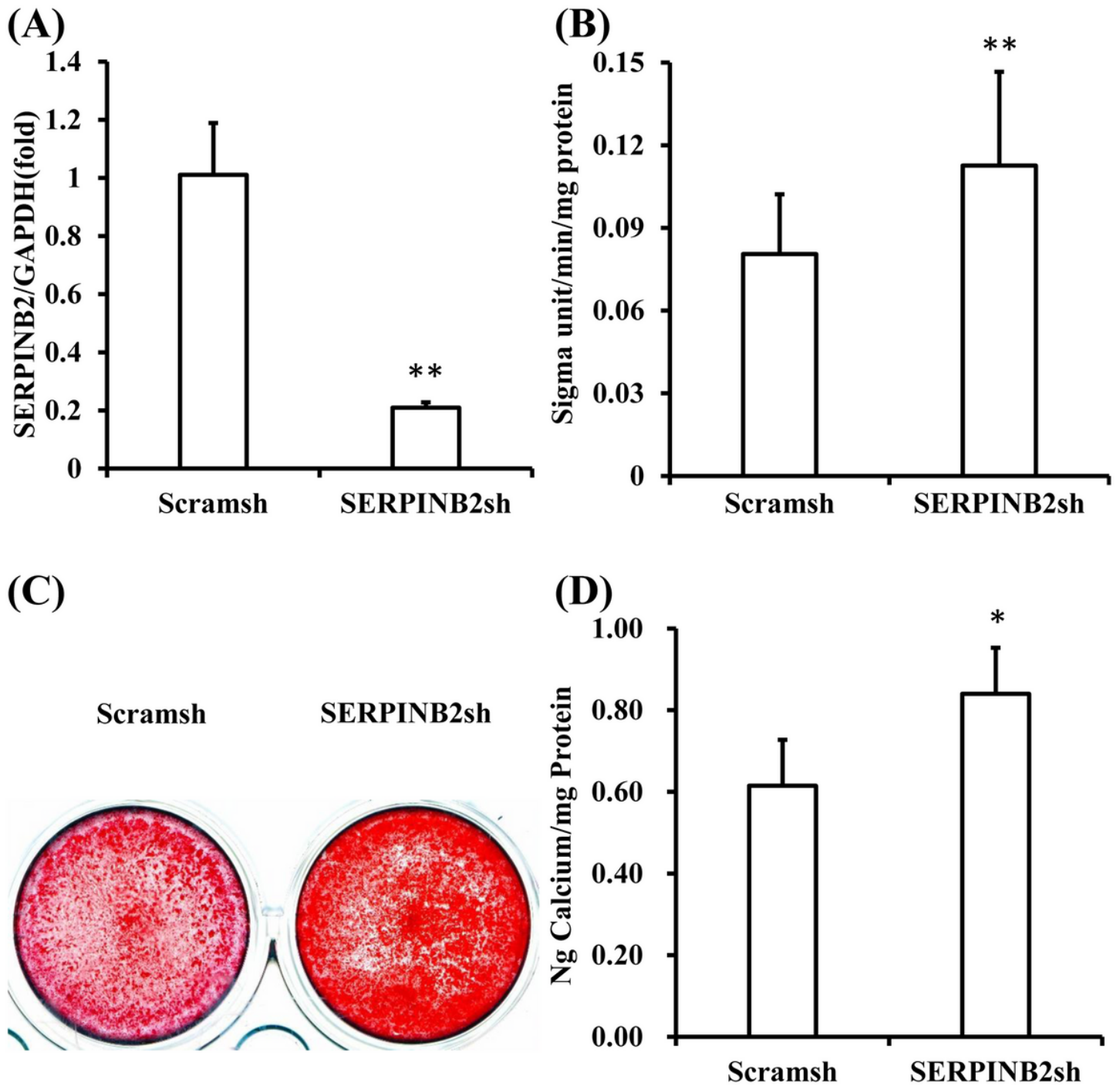


Figure 6

Deletion of SERPINB2 enhanced the osteogenic differentiation potential of WJCMSCs in vitro. (A) Determination of SERPINB2 deletion efficiency by realtime-RT-PCR. (B) Deletion of SERPINB2 increased the ALP activity in WJCMSCs at osteogenic induced 5 days. (C) The Alizarin red staining and (D) quantitative calcium results showed SERPINB2 deletion in WJCMSCs promoted the mineralization ability. GAPDH was as control marker; The statistical significance was evaluated by one-way analysis of variance (ANOVA) or Student's t-test; The error bar was indicated by SD (n = 3); *p ≤ 0.05. **p ≤ 0.01.

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