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Exploring NamiRNA networks and time-series gene expression in osteogenic differentiation of adipose-derived stem cells

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ABSTRACT

Background: Adipose-derived stem cells (ADSCs) are a type of stem cell found in adipose tissue with the capacity to differentiate into multiple lineages, including osteoblasts. The differentiation of ADSCs into osteoblasts underlies osteogenic and pathological cellular basis in osteoporosis, bone damage and repair.

Methods: Focused on ADSCs osteogenic differentiation, we conducted mRNA, microRNA expression and bioinformatics analysis, including gene differential expression, time series-based trend analysis, functional enrichment, and generates potential nuclear activating miRNAs (NamiRNA) regulatory network. The screened mRNAs in NamiRNA regulatory network were validated with correlation analysis.

Results: The NamiRNA Regulatory Network reveals 4 mRNAs (C12orf61, MIR31HG, NFE2L1, and PCYOX1L) significantly downregulated in differentiated group and may be associated with ADSCs stemness. Furthermore, the significantly upregulated 10 genes (ACTA2, TAGLN, LY6E, IFITM3, NGFRAP1, TCEAL4, ATP5C1, CAV1, RPSA, and KDELR3) were significantly enriched in osteogenic-related pathways, and negatively correlated with ADSCs cell stemness *in vitro*.

Conclusion: These findings uncover potential genes related to ADSCs osteogenic differentiation, and provide theoretical basis for underlying ADSCs osteogenic differentiation and related diseases.

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Introduction

Adipose mesenchymal stem cells (ADSCs) are derived from the mesoderm during embryonic development and belong to adipose tissue. They are one of the mesenchymal stem cells (MSCs) with the potential of self-renewal and multi-differentiation. The advantages of ADSCs over other MSCs lie in their easier acquisition, richer sources of adipose tissue, strong self-renewal ability, and more abundant secretory factors [1–3]. ADSCs can be induced and differentiated into adipose, bone, cartilage, islet beta cells, myocardial cells and other cells under specific conditions as other type of MSCs [4–6]. ADSCs are usually obtained from adipose tissue with abundant content and convenient sampling. Its immunogenicity is low and it has great potential in clinical application such as therapies for musculoskeletal disorders [7–9]. Therefore, they can provide convenience for clinical use and scientific research [10–13]. In addition, mRNAs associated with osteogenesis in adipose stem mesenchymal cells were identified through high-throughput sequencing [14,15]. Recent studies revealed its potential in bone regeneration, osteogenesis and angiogenesis [16–19]. Yet the mechanism of adipose-derived stem cells osteogenesis has not been revealed.

Bone marrow mesenchymal stem cells (BMSCs) are a type of MSCs that exist in bone marrow [20,21]. They are easy to differentiate into osteoblasts, possess robust cloning and proliferation ability *in vitro*, and are abundant *in vivo* [22,23]. As such, they are often considered as ideal seed cells for the treatment of bone nonunion or bone defects.

Osteogenic differentiation is a critical step in bone formation. It is a complex process involving the differentiation of BMSCs into osteoprogenitor cells, pre-osteoblasts, mature osteoblasts, and terminal osteocytes [24]. This process requires the signaling pathways, transcription factors, growth factors,

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microRNAs, and other factors that facilitate communication between different types of cells and within individual cells [24–26]. These factors form a complete feedback loop for bone metabolism regulation. The differentiation of mesenchymal stem cells into osteoblasts provides a powerful tool for understanding the mechanism of bone formation and has been used in the identification of genetic disorders such as osteoporosis [27], bone repair, and osteogenesis imperfecta [28]. Compared with MDSCs, ADSCs have relatively weak osteogenic ability, but their sources are wide and have potential application. Therefore, this study aims to find potential regulatory targets to enhance the osteogenic ability of cells. Although previous studies had proved the importance of ADSCs in bone regeneration, there is no systematic research unveiled the role of ADSCs in various stages of osteogenic differentiation, and the significance of adipose-derived mesenchymal stem cells in clinical diseases such as osteoporosis, bone repair or osteogenesis imperfecta remains unclear.

Current research suggests that many signaling pathways are associated with the osteogenic differentiation of ADSCs, and mechanical tension also plays a crucial role in osteoblast differentiation [29–32]. Understanding the mechanism of osteogenic differentiation of ADSCs is of great importance in exploring the pathogenesis of diseases such as osteoporosis, bone repair, and osteogenesis imperfecta.

Osteogenic differentiation is a continuous and evolving process in which specific proteins are expressed at different levels during different stages of cell development. During the sequential stages of osteogenesis, SOX9 expression is initiated in osteoprogenitor cells, followed by the induction of RUNX2 and DLX5 in the mid-stage, and culminating in the expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OCN) in the late-stage. It has been found that usually in the first stage, cell proliferation is followed by cell matrix maturation, and specific proteins related to the osteocyte phenotype such as ALP can be detected from day 10 to day 15. Late in the second stage matrix mineralization and osteogenesis markers, such as OCN in 20–30 days can be tested, therefore, the osteogenic status of cells is usually detected on a weekly basis [33,34]. The process can be divided into several steps as follows [35,36]: 1) under the stimulation of various factors and hormones, as well as other specific physicochemical factors, mesenchymal stem cells differentiate into osteoprogenitor cells; 2) subsequently, the osteoprogenitor cells further differentiate into pre-osteoblasts and enter a phase of rapid proliferation; 3) cell proliferation gradually declines and genes related to extracellular matrix (ECM) maturation (alkaline phosphatase, type I collagen, and matrix calcium-binding protein) are further activated. At this stage, osteoblasts synthesize and secrete organic matrix to form bone-like tissue primarily composed of type I collagen, with alkaline phosphatase as an early marker of osteoblast differentiation; 4) mature osteoblasts express ECM calcification-related proteins, mainly bone calcium-binding protein and osteopontin, and their mineralization activity increases significantly. Although most of the researches are based on osteoblast at different phases described, made no mention of a unified point in time. Examining and analyzing the changes in the cells over time during the osteogenic differentiation of adipose mesenchymal stem cells is important to understand the differentiation mechanism.

MicroRNA (miRNA) is a type of non-coding RNA that is approximately 19–22 nucleotides in length. The miRNA gene is transcribed by RNA polymerase II or III to generate a primary transcript (pri-miRNA) that is several thousand nucleotides in length [37–39]. Subsequently, the pri-miRNA is further processed by the protein complex Drosha-DGCR8 into a precursor miRNA (pre-miRNA) with a stem-loop structure. With the assistance of the Ran-GTP-Exportin-5 transport protein, pre-miRNA is transported from the nucleus to the cytoplasm. In the cytoplasm, pre-miRNA is recognized by Dicer-TRBP and is cut and modified to form a miRNA duplex. One of the strands is rapidly degraded, and the other is loaded into the AGO2 protein to form the RNA-induced silencing complex (RISC), which ultimately generates a mature, functional single-stranded miRNA [40]. The miRNA located in the nucleus that is associated with enhancer activation is called nuclear activating miRNA (NamiRNA) [41]. Enhancers act as cis-regulatory elements that drive cell type-specific gene expression associated with cell fate reprogramming, while miRNAs play a critical role in controlling cell fate and maintaining cell properties during development. Based on the NamiRNA-enhancer-target gene activation network model, researchers have proposed a NamiRNA-enhancer-cell fate/cell identity determination model [42,43]. That is, NamiRNA can activate gene expression by binding to target enhancers and exert regulatory effects during cell development, thereby controlling cell fate or cell identity. This can better understand the relationship between NamiRNA and enhancers, as well as the function of NamiRNA in cell differentiation and cell fate reprogramming. Recent studies have shown that miRNAs directly or indirectly regulate osteogenic differentiation of ADSCs by targeting various genes involved in self-renewal and differentiation of ADSCs, and the osteogenic differentiation is regulated by multiple transcription factors, such as RUNX2, OSTERIX [44–46], SATB2 [47,48], but there is limited research on NamiRNA in this regard.

In this study, we merged and analyzed two mRNA databases and one miRNA dataset in the Gene Expression Omnibus database to identify differentially expressed genes and pathway enrichment in osteogenic differentiation, explored dynamic changes through time-series analysis, predicted NamiRNA regulatory networks, and verified important genes *in vivo* and analyzed possible pathways related to the osteogenic differentiation of ADSCs. These findings enhance the comprehension of the molecular mechanisms of ADSCs in treating osteogenic diseases, as well as the underlying pathophysiological processes associated with osteogenesis-related conditions.

Materials and methods

Data retrieval

The relevant data was searched and downloaded from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and divided into 'osteogenic differentiation' and 'undifferentiated' subgroups: 1. mRNA dataset GSE37521 (including 91 samples), osteogenic differentiation group: cell type: Adipose Mesenchymal Stem Cells, treatment: Osteogenesis, 7 time points (n=14); undifferentiated group: cell type: Adipose Mesenchymal Stem Cells, treatment: Noninduced (n=6); 2. mRNA dataset GSE89330 (including 8 samples), divided into: osteogenic differentiation group: Osteogenesis (n=4); undifferentiated group: Undifferentiated (n=4); 3. miRNA dataset GSE72429 (including 16 samples), divided into: osteogenic differentiation: Osteogenesis (n=4); undifferentiated: Undifferentiated (n=4). For genes with multiple probes, the median value of gene expression was used in the following analyses. The subsequent analyses in each dataset were summarized in Figure 1.

Differential expression analysis

Using limma [49] (an R-based open-source software), mRNA and miRNA differential expression analyses were

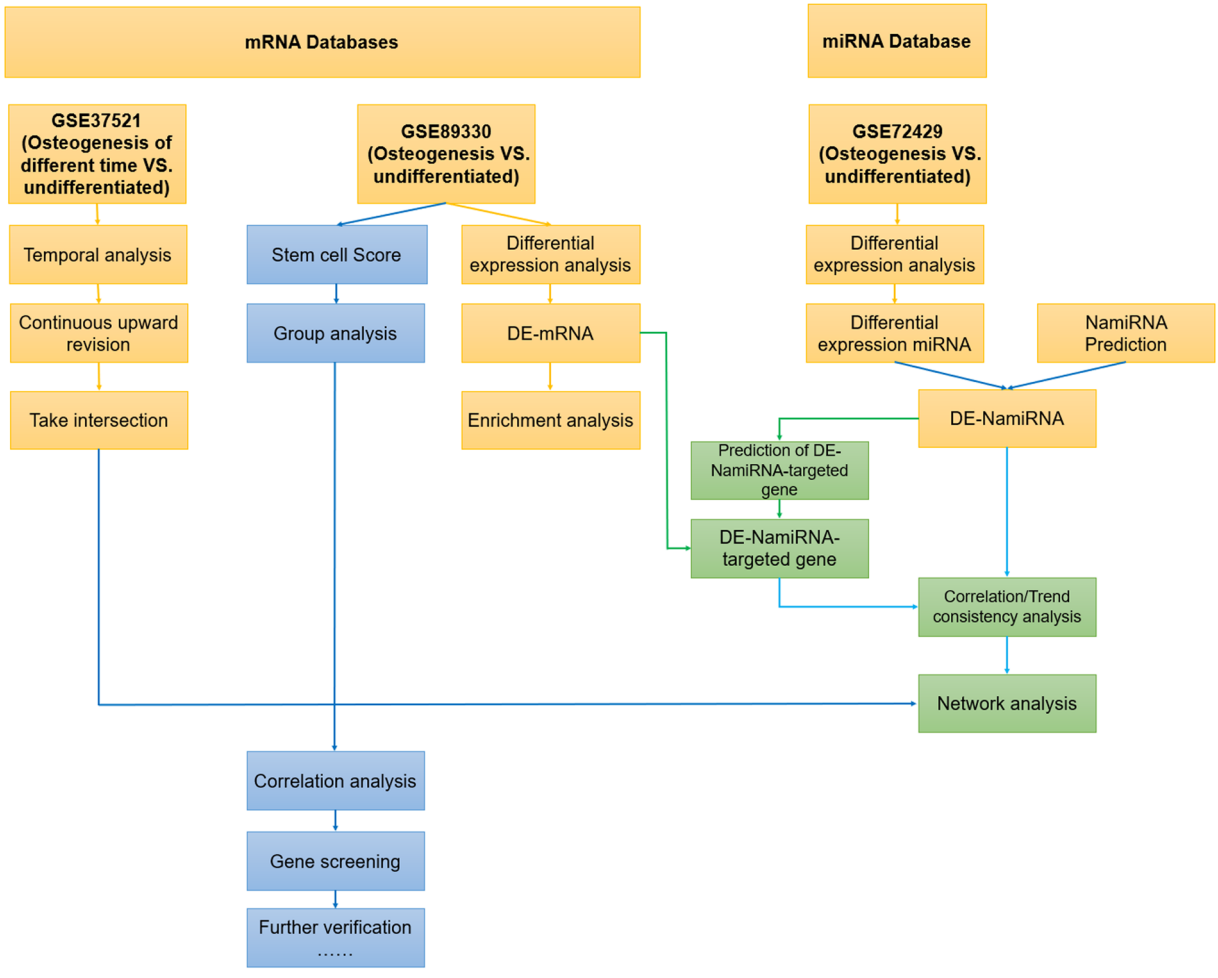

Figure 1. The workflow diagram for screening genes associated with osteogenic differentiation in adipose mesenchymal stem cells.
The yellow squares represent the difference analysis and enrichment analysis of the dataset. The blue squares represent the integrated analysis of stem cell score and correlation analysis and subsequent gene screening. Green represents NamiRNA-related analysis and its network construction. DE=Differential expression.

performed based on the different groups and expression data of the mRNA dataset. Differential expression of RNA sequencing (RNA-seq) and differential splicing analyses of RNA sequencing (RNA-seq) data were also conducted. Differential expressed genes were selected according to the corresponding parameters: p-value < 0.05, Fold change ≥ 2 and Fold change< 0.01.

Time course analysis

Using R package Mfuzz, a time course analysis was performed based on the expression levels of genes [50] (mRNAs) and the sorting of each sample in GSE37521. The GSE37521 dataset includes one undifferentiated and seven induced osteogenic differentiation time points. Genes with significantly up- or down-regulation were selected for subsequent analysis.

NamiRNA prediction

Human microRNA location information was obtained based on the miRBase [50] database (http://www.mirbase.org/) and other related databases. The human enhancer 1 location information was obtained based on the SEdb2.0 [51] database (http://www.licpathway.net/sedb/) and other relevant databases. Finally, based on the miRNA location information and enhancer location information, potential NamiRNAs in the corresponding tissue were screened.

NamiRNA regulatory network construction

Based on the DE-NamiRNA related to adipose-derived stem cells in 2.4 and the consistency of the enhancer's potential target gene expression trend in the SEdb2.0 database (up-up and down-down), a NamiRNA regulatory network was constructed.

Gene function enrichment analysis

Based on the Gene Ontology [52] database and the KEGG PATHWAY DATABASE [53], functional enrichment analysis was performed on the candidate genes. A statistical algorithm (Fisher's exact test) was used to find the most significant functional items related to a group of genes, and each item in the analysis results corresponded to a P-value to indicate significance.

Single sample gene set enrichment analysis (ssGSEA)

The 'GSVA' R package was used to perform single sample gene set enrichment analysis (ssGSEA) based on the expression levels of various genes and corresponding gene sets. The ssGSEA method in the GSVA [54] package was used for enrichment analysis, and the ssGSEA scores for each sample corresponding to each gene set were obtained. The stemness-related gene label was obtained from msigDB database (http://www.gsea-msigdb.org/gsea/msigdb/human/geneset/MALTA_CURATED_STEMNESS_MARKERS.html).

Cell culture and osteogenesis

The hADSC cell lines (HUXMD-01001) was purchased from OriCell® (Guangzhou, China). The hADSCs were induced for osteogenic differentiation after resuscitation and expansion. In short, the cell deposits were resuspended with an appropriate amount of complete culture medium, and blood cells were counted. The cells were adjusted to 1×10^5/ml, 37 °C 5% CO2 incubator for culture. The osteogenic differentiation medium(HUXMD-90021, OriCell®)was replaced on the second day, and the induction differentiation culture was continued for 14 days, during which the differentiation medium was replaced every 2–3 days.

Alizarin red staining

After 14 days of differentiation induction, the cells were washed three times with PBS after removal of the culture medium, fixed fine with 4% paraformaldehyde for 10 min, washed three times with PBS, and stained with Alizarin red (HUXMD-90021, OriCell) staining kit. Photo staining was observed under gross and microscopic scopy.

RNA extraction and quantitative real-time PCR

The cells were collected at 0, 14 days of induction and fully lysed by adding 0.5 ml TRIZOL before preparation. The total RNA extraction was done using RNAiso Plus Reagent (Takara Code No.: 9109, Dalian, China) according to the manufacturer's instructions. Around 500 ng of the total RNA was reverse transcribed to cDNA using WCGENE mRNA cDNA kit (Shanghai Wcgene Biotech, WC-SJH0001, Shanghai, China). The cDNAs were subjected to real-time PCR using gene-specific primers and WCGENE® mRNA qPCR Mix (Shanghai Wcgene Biotech, WC-SJH0002, Shanghai, China) in the StepOnePlus™ Real-time PCR system(Applied Biosystems). GAPDH was used as the internal control in the experiment. Relative mRNA expression levels were calculated by the $2-\Delta\Delta Ct$ method and were normalized to the expression of the internal control GAPDH. The experiments were performed in triplicate.

The primers were all synthesized by and purchased from Sangon Biotech (Shanghai, China); the primer sequences are listed in Supplementary Table 6.

Statistics analysis

The fold value (relative expression fold) was calculated by the method of 2-ΔCt, where ΔCt=Ct (target gene) -Ct (reference gene). The fold value of the data of each experimental group was taken, and the P value was calculated by t tests. For P value< 0.05 was marked as '*', indicating differential expression. P value < 0.01 marked '* *', expressing significant differences; P value < 0.001 is marked as '***' and represents a highly significant difference in expression. Using a Treeview software and graphpad-prism5 software to produce heat and histogram.

Results

Differential expressed mRNA and miRNA are associated with adipocyte osteogenic differentiation

To screen for mRNA and miRNA potentially associated with adipocyte osteogenic differentiation, we performed differential expression analysis using the limma package on normalized mRNA dataset GSE89330 and miRNA dataset GSE72429. Differential genes were then filtered based on the differential threshold values in the table below and statistically analyzed (see Supplementary Tables 1–3). We found that the expression trends of several genes previously reported to be positively correlated with stemness, such as CD44, HIF1A, EPAS1, CTNNB1, and KLF4 (miRNA dataset GSE72429), were significantly downregulated in the osteogenic differentiation group of this study. Furthermore, we found that the expression trends of several genes previously not reported to be associated with stemness, such as C12orf61, MIR31HG, NFE2L1, and PCYOX1L (in mRNA database GSE89330), were significantly upregulated in the undifferentiated group of this study, suggesting that these genes may be related to the maintenance of cell stemness. Similarly, we also found that several genes were significantly upregulated in the osteogenic differentiation group, such as ACTA2, TAGLN, LY6E, IFITM3, NGFRAP1, TCEAL4, ATP5C1, CAV1, RPSA, and KDELR3 which are to be negatively correlated with stemness (miRNA dataset GSE72429) (Figure 2).

Osteogenic differentiation-related genes are enriched in pathways related to stemness

To further investigate the gene function of genes related to osteogenic differentiation, we performed functional enrichment analysis on the differentially expressed genes using the Gene Ontology database and the KEGG PATHWAY DATABASE. Significant enriched pathways were selected based on the P-value. We found that pathways related to stemness, such as the PI3K-AKT pathway, Wnt signaling pathway, Hedgehog signaling pathway, TGF-β signaling pathway, and Hippo signaling pathway, were significantly enriched (see Supplementary Table 4), indicating a certain relationship between stemness and the differentiation of adipose-derived stem cells. In PI3K-AKT pathway, TLR4 and PIK3R1 are upregulated, while AKT3, CDKN1A, and CDKN1B proteins are downregulated, indicating alterations in the process of osteogenic differentiation (Figures 3 and 4).

The osteogenic differentiation genes showed significant temporal difference between differentiated and undifferentiated adipose mesenchymal stem cells

To screen genes related to osteogenic differentiation, we used the Mfuzz package to perform time series analysis based on the expression levels of each gene in GSE37521 and the time series of each sample (one undifferentiated and seven induced osteogenic differentiation time points). We found that Cluster48 was continuously upregulated during osteogenic differentiation, which included 125 genes (Figure 5A).

To further screen for genes related to osteogenic differentiation, we used Venn analysis to identify the intersection of upregulated mRNA differential expression genes and genes obtained from mRNA time series analysis, and further screened for mRNA related to osteogenic differentiation. We found that 10 genes (ACTA2, TAGLN, LY6E, IFITM3, NGFRAP1, TCEAL4, ATP5C1, CAV1, RPSA, and KDELR3) were upregulated in the osteogenic differentiation group and related to the induction time of osteogenic differentiation. We speculate that these 10 genes may participate in the process of adipose mesenchymal stem cell osteogenic differentiation (Figure 5B).

NamiRNA regulatory network reveals potential genes involved in osteogenic differentiation of adipose mesenchymal stem cells

NamiRNA regulatory mechanism is a newly discovered miRNA regulatory mode in recent years. Compared with traditional miRNAs that function in the cytoplasm, NamiRNAs located in the nucleus can regulate downstream target genes positively by regulating enhancers. To explore the upstream regulatory mechanism of

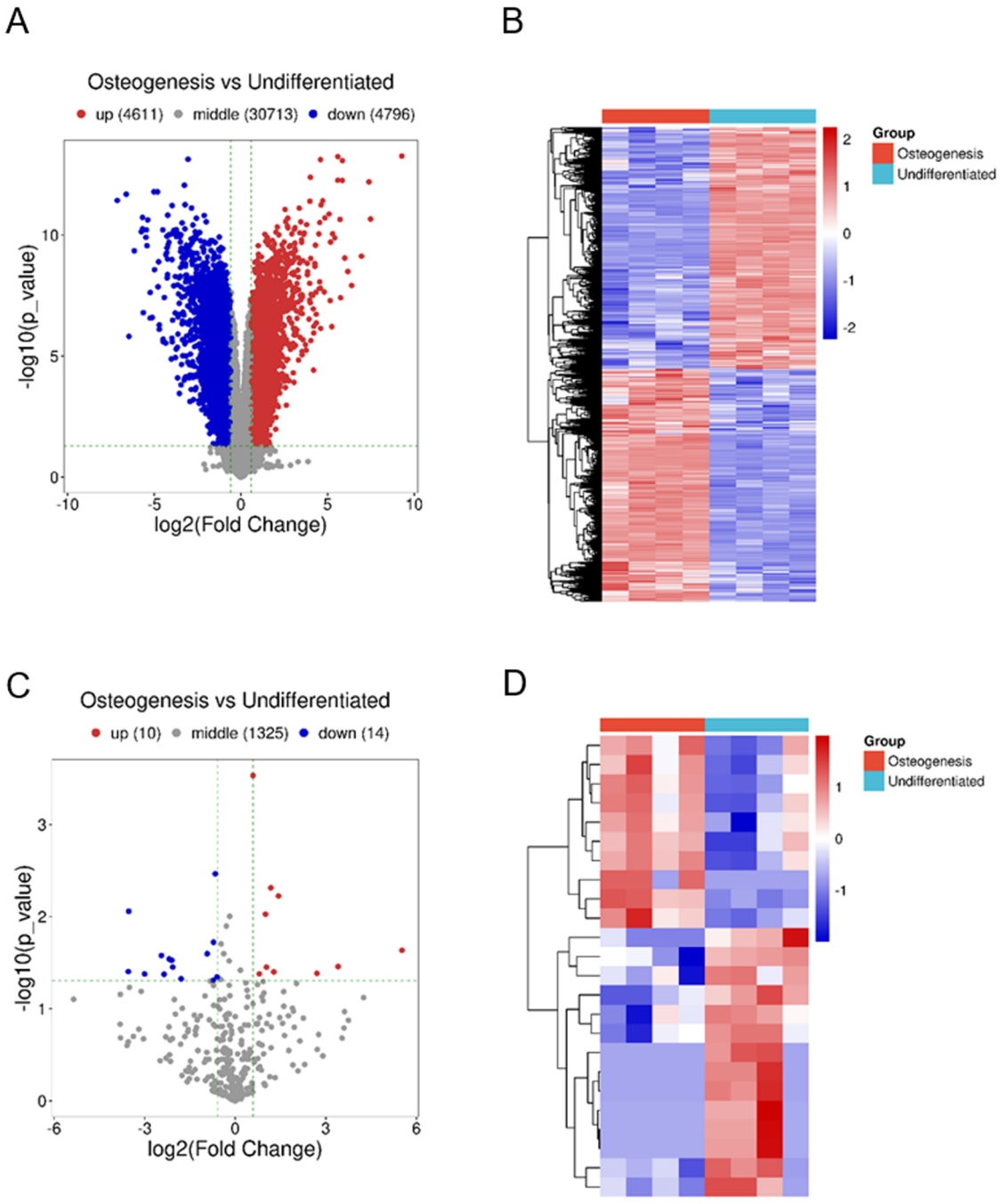

Figure 2. Differential genes between osteogenic differentiation and undifferentiation of adipose mesenchymal stem cells.
(A,B) The volcano map and heat map of osteogenic differentiation and undifferentiated related genes of mRNA database GSE89330. (C,D) The volcano map and heat map of osteogenic differentiation and undifferentiated related genes of miRNA database GSE72429.

genes related to osteogenic differentiation, this study focuses on the NamiRNA regulatory mechanism of these genes. The following information was used: first, based on the location information of the differential miRNAs in the miRBase database (http://www.mirbase.org/). Second, based on the SEdb2.0 database (http://www.licpathway.net/sedb/), the enhancer positions of human adipose-derived stem cells and their corresponding target gene information were obtained. Finally, potential NamiRNAs in adipose-derived stem cells were screened based on miRNA location information and enhancer location information. Based on the consistency between the differentially expressed NamiRNAs related to adipose-derived stem cells and the expression trend of potential target genes in the SEdb2.0 database (up-up and down-down), a NamiRNA regulatory network was constructed using Cytoscape. We found that 7 differentially expressed genes are related to NamiRNA target genes (Figure 5C). Subsequently, we conducted a NamiRNA network

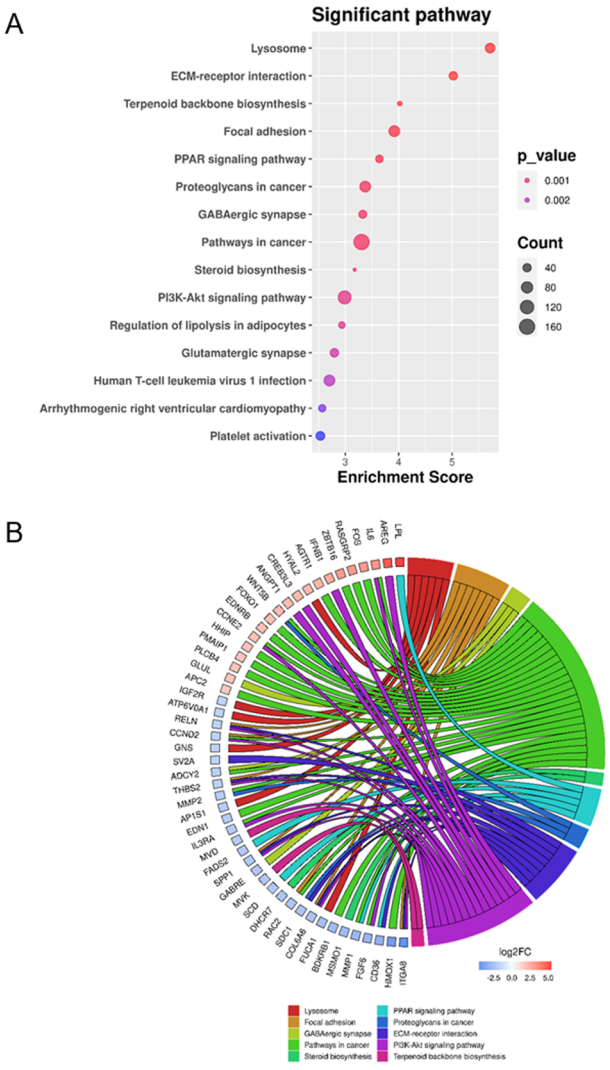

Figure 3. Enrichment analysis of differentially expressed genes in osteogenic differentiation and undifferentiation of adipose mesenchymal stem cells.
(A) Bubble plot of enrichment results. (B) The first 10 entries correspond to the gene circle map.

analysis. The results showed that the expression levels of four genes related to the maintenance of cell stemness may be regulated by NamiRNA (see Supplementary Table 5), and these four genes are C12orf61, MIR31HG, NFE2L1, and PCYOX1L (Figure 5D).

Stemness-related gene signatures distinguish the osteogenic differentiation of adipose-derived stem cells

To explore the relationship between stemness and osteogenic differentiation of adipose-derived stem cells, this study conducted ssGSEA score analysis on each sample based on the stemness-related gene signature, and statistically analyzed the differences between the two groups of samples in the GSE89330 dataset. The results showed a significant difference in stemness scores between the undifferentiated group and the osteogenic differentiation group, with the undifferentiated group having higher stemness scores. This result further supports the correlation between stemness level and mesenchymal-derived stem cell differentiation (Figure 6A).

C12orf61 and ACTA2 genes are correlated with stemness scores

To investigate the key genes related to adipose-derived stem cells osteogenic differentiation, this study further analyzed the correlation between the genes in the NamiRNA network and stemness scores. The results showed a strong correlation between these four genes and stemness scores, and in the inter-group differential analysis between the osteogenic differentiation and undifferentiated samples in the GSE89330 dataset, these four genes were downregulated in the osteogenic differentiation group. Therefore, these four genes may participate in mesenchymal-derived stem cell osteogenic differentiation and are positively correlated with stemness (Figure 6B). Subsequently, the correlation between the 10 overlapping genes (negatively correlated with stemness) in the upregulated differentially expressed genes and time-series analysis in the osteogenic differentiation group and stemness scores was investigated. The results showed a strong negative correlation between these 10 genes and stemness scores, suggesting that they may also participate in adipose-derived stem cell osteogenic differentiation (Figure 6C). Furthermore, we selected C12orf61 and ACTA2 genes for correlation analysis with stemness scores, and found that the former was positively correlated with stemness scores (Figure 7A), while the latter was negatively correlated (Figure 7B), validating the reliability of the correlation analysis.

The differential gene expression levels in human ADSC osteoblasts

We induced human ADSCs osteogenic stem cells and extracted cell RNA at 0,14 days for qPCR. The results showed that there were four genes positively correlated with cell stemness: C12orf61, NFE2L1, and PCYOX1L were up-regulated over time, but MIR31HG decreased at day 14, while there was no significant statistical difference (Figure 8A–D). Among the 10 genes (TCEAL4, TAGLN, NGFRAP1, RPSA, CAV1, KDELR3, LY6E, ACTA2 IFITM3, ATP5C1) negatively correlated with stemness, all of the genes were increased at 14 days of culture (Figure 8E–N).

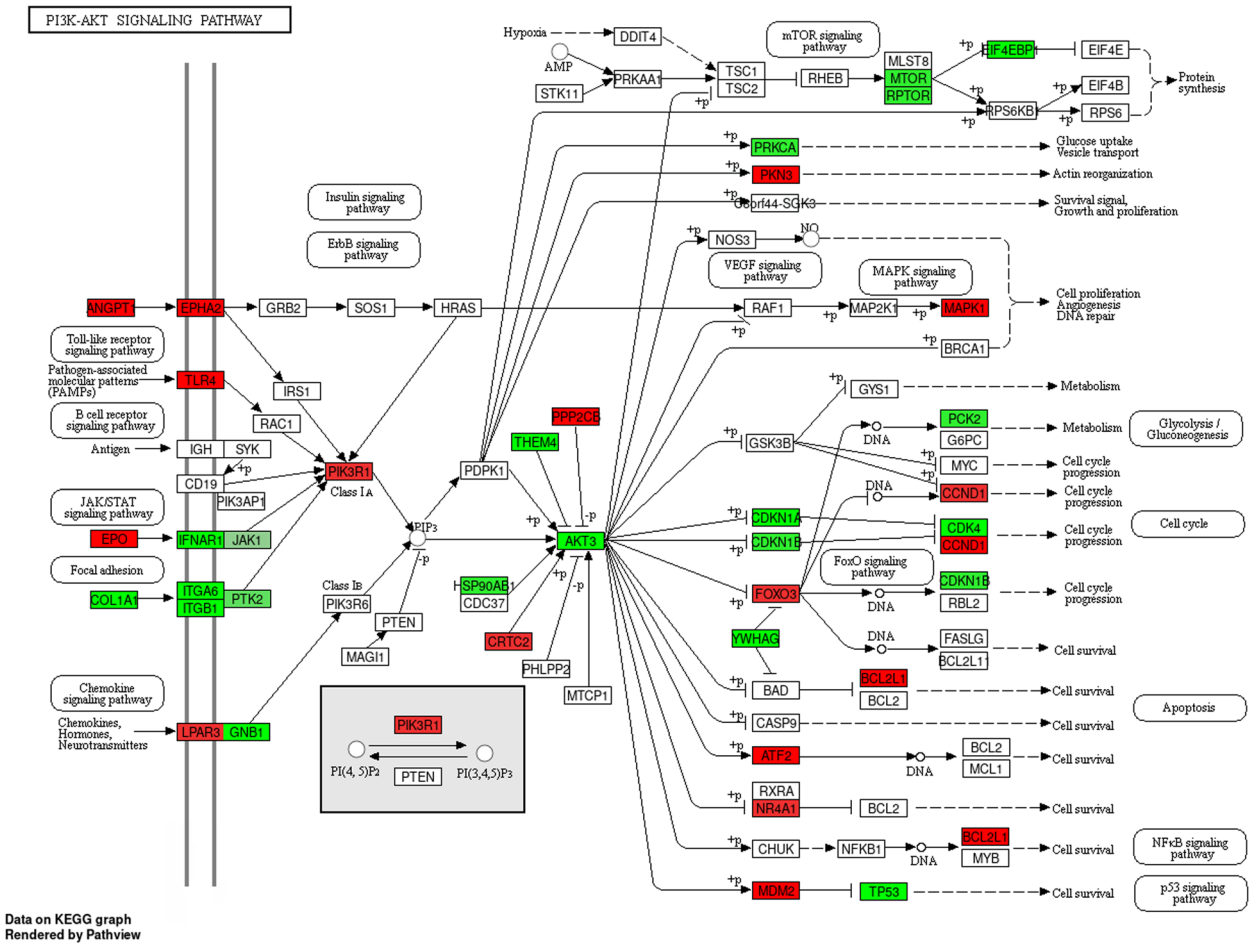

Figure 4. PI3K-AKT pathway analysis.
Red and green squares represent the positions of differentially expressed genes in KEGG pathways (red: up-regulated, green: down-regulated).

Discussion

Adipose-derived stem cells osteogenic differentiation is a critical step in bone formation, and investigating the signaling pathways involved in the intercellular and intracellular communication during osteogenic differentiation is of great significance [4,5,30,55]. In this study, we analyzed the mRNA and miRNA data sets, selected genetic variations and used human adipose mesenchymal stem cells to verify gene expression levels *in vitro*. Our purpose is to identify genes associated with ADSCs osteogenic differentiation, to provide theoretical basis for understanding the potential mechanism, and to promote the future development of targeted therapy of bone related diseases.

The research on the mechanism of human ADSCs and osteogenesis mainly focuses on the regulation of miRNAs, such as miR-154-5p and miR-26a, which regulate the osteogenic differentiation of ADSCs [56–58]. Both of them are related to Wnt pathway, and MiR-26a can also bind to SMAD1 transcription factor, which is related to the osteogenic differentiation of ADSCs. Some researchers have also reported that the methylation status of human ADSCs can affect osteogenesis. The status of ADSCs can also be affected by a variety of factors, such as serum growth factors, antioxidants, and environment, which may also affect the osteogenic capacity of ADSCs [59,60]. But there is no clear evidence reported genetic level change between people fat regulation of osteogenesis ability of mesenchymal stem cells.

The osteogenic differentiation of cells is influenced by many genes such as CD44, HIF1A, EPAS1, CTNNB1, and KLF4. CD44 is a marker for ADSCs, which can reflect the status of stem cells [61,62]. hucMSC-EV can differentiate into osteoblasts, adipocytes while positively expressing CD29, CD44, CD73, and CD90 [63,64]. miRNA-21 promotes osteogenesis and enhances bone repair through the PTEN/PI3K/Akt/HIF-1α pathway [65]. CTNNB1 encodes β-catenin, which has been shown to limit bone formation in recent years. The BMP2-dependent gene regulatory network analysis revealed that Klf4 is a new transcription factor for osteoblast differentiation [66]. Consistent with

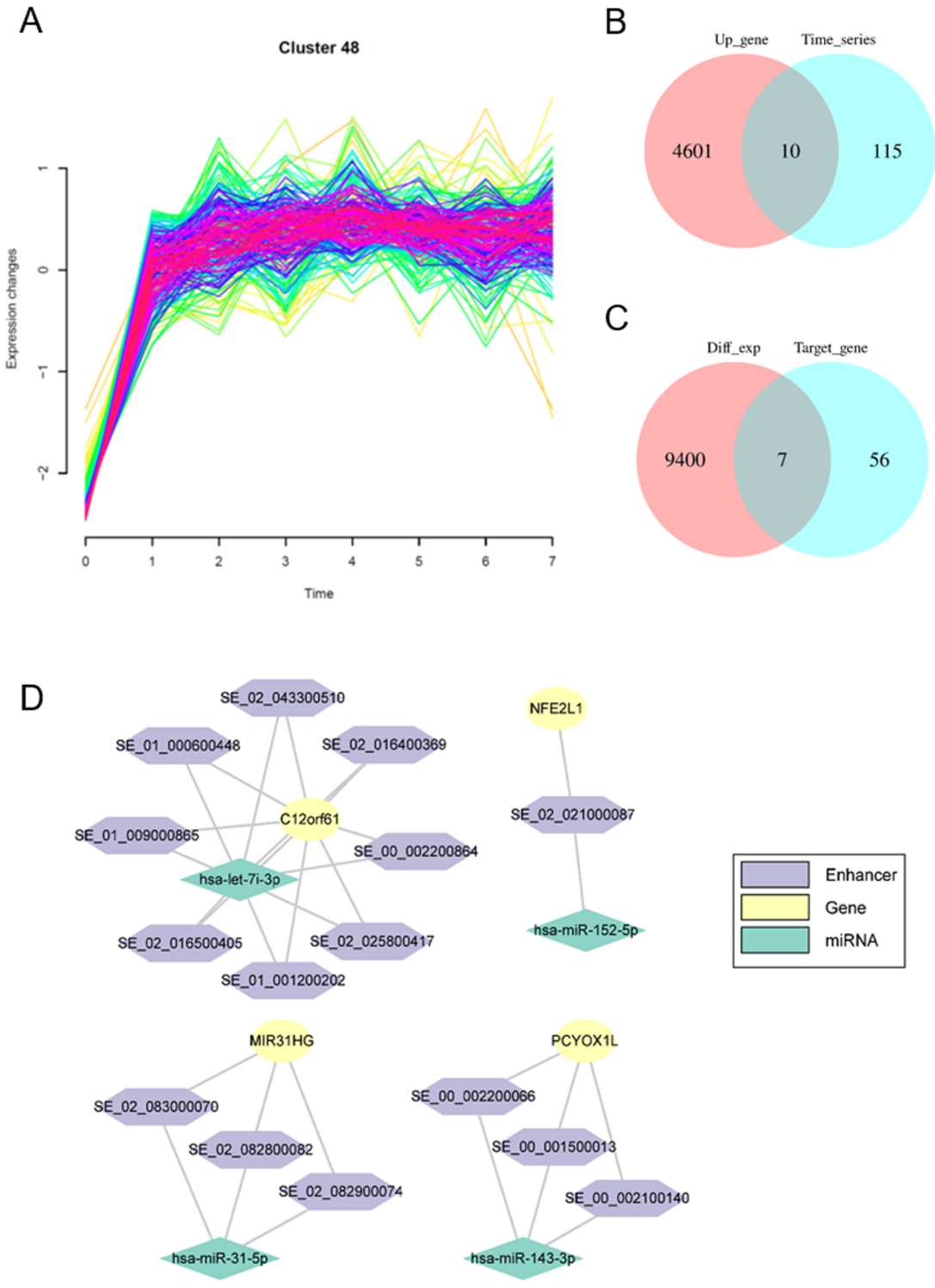

Figure 5. Time series analysis based on the corresponding gene expression of each sample in GSE37521 database.
(A) Results of Cluster48 for time-series analysis. (B) Venn diagram of genes with differentially up-regulated mRNA expression and genes obtained by mRNA time series analysis. (C) Venn diagram of intersection of differentially expressed genes and NamiRNA target genes. (D) NamiRNA network diagram (down-down) Note: There is no corresponding result for up-up.

previous literature, the expression trend of these genes in the osteogenic differentiation group in this study was significantly up regulated, indicating a positive correlation between these genes and osteogenic differentiation. In the cell experiments, we found that the levels of these genes (TCEAL4, TAGLN, NGFRAP1, RPSA, CAV1, KDELR3, LY6E) were higher on day 14.

NamiRNA is a recently reported miRNA highly associated with nuclear enhancers and enriched with enhancer marks H3K27ac and H3K4me1 [41,42].

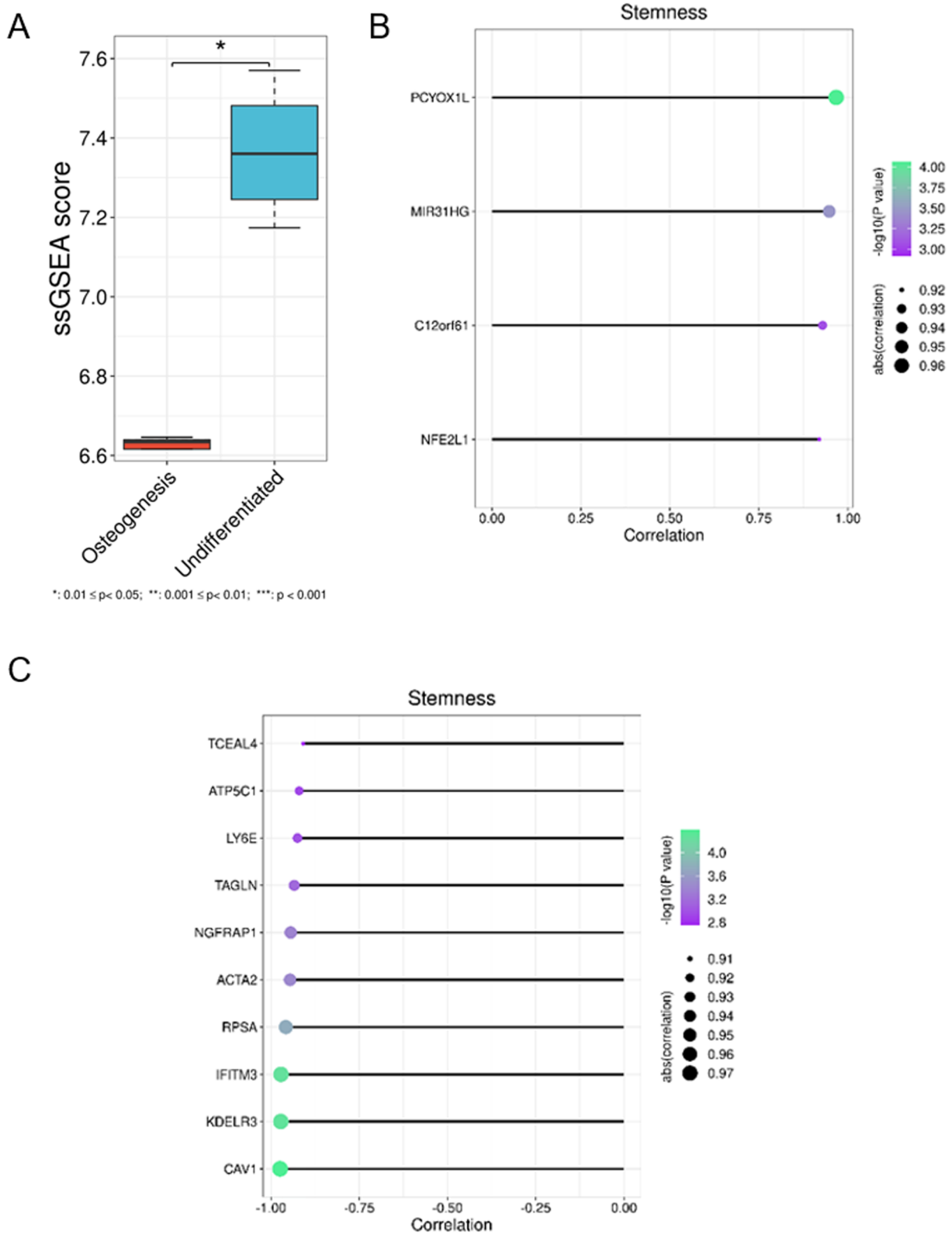

Figure 6. The ssGSEA enrichment score of stemness related gene signature and the correlation between genes in NamiRNA network and stem cell score.

(A) Differences in stemness scores between undifferentiated and osteogenic differentiated samples in database GSE89330. (B) Correlations between genes in four NamiRNA networks and stemness scores. (C) Correlation map between 10 intersection genes and cell stemness score.

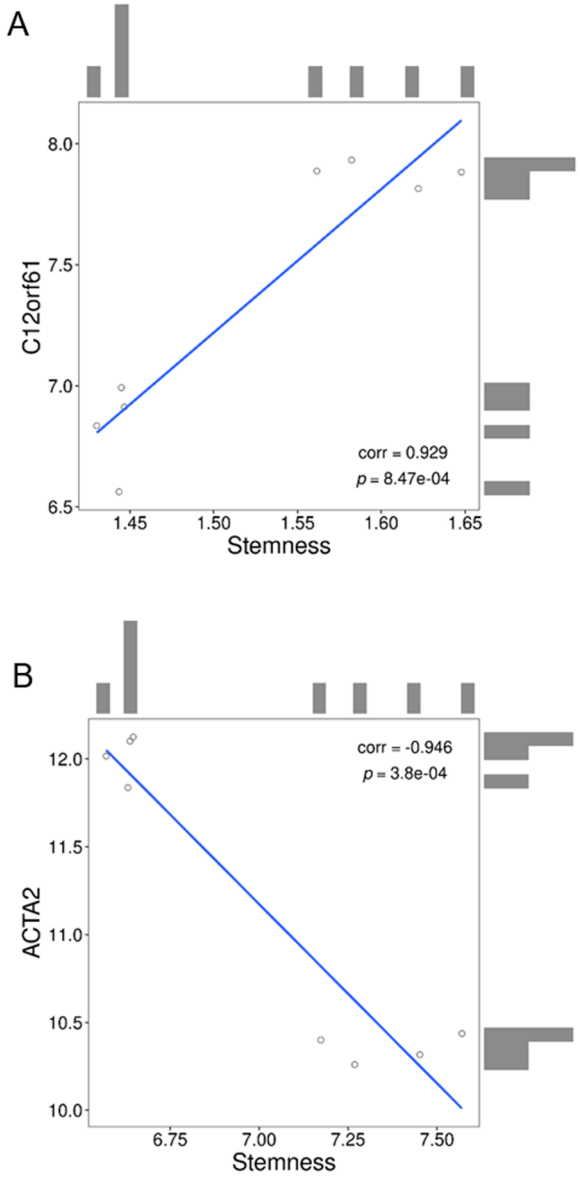

Figure 7. Correlation analysis between two genes in NamiRNA network and cell stemness scores (a). Correlation graph between C12orf61 and cell stemness scores. (B). Correlation graph between ACTA2 and cell stemness scores.

NamiRNA can serve as an enhancer trigger to regulate gene expression, such as miR-24-1 and miR-26, which are located in the cell nucleus and have activating functions on their adjacent genes [41]. In the current, most studies have focused on static time points in cells and have not observed changes dynamically, there have been few studies on the nuclear miRNA regulatory mechanism. And in this study, C12orf61, MIR31HG, NFE2L1, and PCYOX1L were identified in the NamiRNA network, which may be closely related to ASC osteogenic differentiation.

Knockdown of MIR31HG not only significantly promoted osteogenic differentiation of hADSCs, but also significantly overcame the inhibition of osteogenic differentiation induced by inflammation [67]. In another study, targeted inhibition of MIR31HG by siRNA modified Ti surface (siMIR31HG) promoted osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) [68]. In our cell experiments, we found that MIR31HG levels were decreased at day 14, which is consistent with our conclusion that MIR31HG was downregulated in the osteogenic differentiated group. But the other three genes in the osteogenic differentiation group C12orf61, NFE2L1 and PCYOX1L over time (cells osteogenesis gradually mature) increased, and expectations are not consistent. We speculate that the possible reason is that the time points of the two data sets are different, one is 14 days and the other is 21 days. Secondly, the sample size ($n=4$) was small, which caused a certain error. In addition, it is also possible that these three genes play a role earlier and have not been collected corresponding data. All these conjecture remains to be further validation.

Recent studies have shown that nuclear regulatory mechanisms are closely related to stem cell characteristics, and NamiRNA acts directly on nuclear enhancers, thus we performed NamiRNA regulatory network analysis on these genes and found that they are closely related to multiple osteogenic genes [43]. C12orf61 has been reported to promote migration in epithelial ovarian cancer cells and is associated with chemotherapy resistance [69]. Nuclear factor erythroid 2-related factor 1 (NFE2L1, also known as NRF1) belongs to the CNC-bZIP transcription factor family, and coordinates various physiological processes and stress responses. It has been reported to be associated with osteoclast differentiation: NFE2L1 can regulate osteoclast differentiation in an antioxidant-dependent and independent manner [70]. Lnc-PCYOX1L has been reported to be prognostically relevant in renal clear cell carcinoma. However, its relationship with mesenchymal stem cell osteogenic differentiation has not yet been reported. These genes have not been experimentally validated to be associated with osteogenic differentiation, and we believe that this is a potential direction for future validation of the underlying mechanisms related to adipose-derived stem cells osteogenic differentiation.

The osteogenic differentiation of ADSCs is often considered to be related to multiple pathways. Recombinant human platelet-derived growth factor related-ADSCs were proved to be promising tissue engineering structures for *in vivo* craniofacial bone regeneration in mice through EGFR pathway [71]. The CircRNA-vgll3/miR-326-5p/Integrin-α5 (Itga5) pathway has been shown to be closely related to osteogenic differentiation of adipose mesenchymal stem cells, and

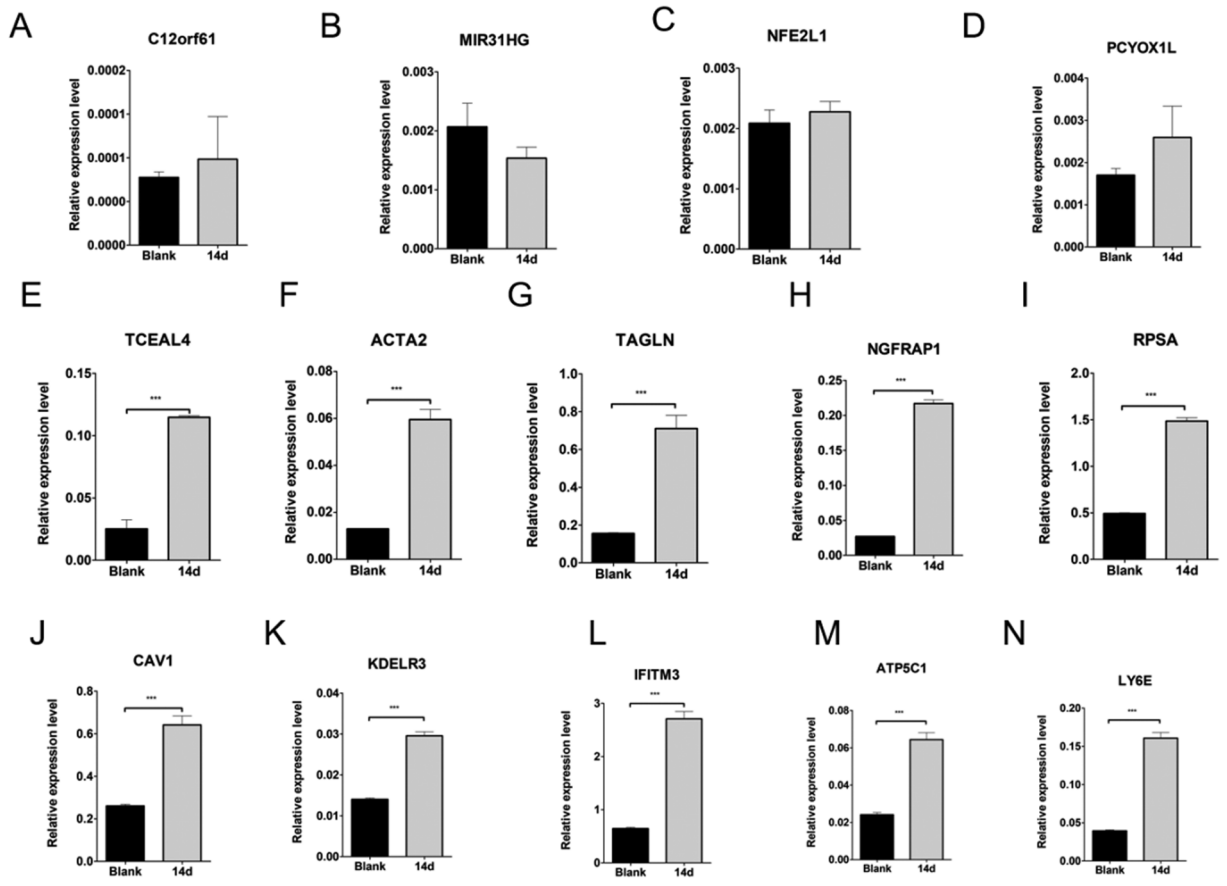

Figure 8. Gene expression levels in human adipose-derived mesenchymal cells at 0 and 14 days. (A–D) Gene expression levels of C12orf61, MIR31HG, NFE2L1, PCYOX1L. (E–N) Gene expression levels of ACTA2, TAGLN, LY6E, IFITM3, NGFRAP1, TCEAL4, ATP5C1, CAV1, RPSA, KDELR3. For P value< 0.05 was marked as '*', indicating differential expression. P value < 0.01 marked '* *', expressing significant differences; P value < 0.001 is marked as '***'.

circRNA-vgll3 increased new bone formation (enhanced bone mineral density, bone volume or tissue volume, and trabeculae number) [72]. ACTA2 and TAGLN are TGFβ-responsive genes that can be downregulated after TGFβ signaling is deactivated, thereby inhibiting the osteogenic differentiation potential of osteoblasts [73]. In our validation experiment of C12orf61, its expression level decreased but was contrary to expectations. The possible reasons include the small sample size leading to errors, and the lack of statistical significance or C12orf61 may not be involved in the stemness-related pathways in the genetic background of the cells. Therefore, the results of the bioinformatics analysis cannot be negated at present. In osteogenesis imperfecta patients, IFITM3 showed significant differential expression and functional analysis suggested dysregulated interferon signaling pathways [74]. CAV1 can promote calcium ion gate signal and bone formation, inhibit estrogen-deficient osteoporosis [75]. It can also activate the YAP-HIPPO pathway and PI3K-AKT pathway to promote osteogenic differentiation. In the PI3K-AKT pathway, AKT3 is downregulated, as well as the cell cycle proteins CDKN1A and CDKN1B. These alterations in the pathways suggest a possible downregulation of cell cycle regulatory functions, which requires further verification in subsequent studies.

The current literature on the involvement of LY6E, NGFRAP1, TCEAL4, ATP5C1, RPSA, and KDELR3 in osteogenic differentiation is limited. Furthermore, we found that pathways related to stem cell differentiation, such as Wnt signaling pathway, Hedgehog signaling pathway, TGF-β signaling pathway, and Hippo signaling pathway, were significantly enriched in our results, indicating that these pathways may be involved in stem cell differentiation and are consistent with the osteogenic differentiation-related pathways reported in current literature.

Certainly, our study has several limitations that warrant consideration. Firstly, the sample size is relatively small, which could limit the robustness of our findings. Expanding the sample size in future studies would enhance the reliability and generalizability of our results. Secondly, this study did not include multiple continuous time points in the cell experiments, nor did

it involve validation using animal models. In future work, we plan to incorporate multiple time points for repeated experiments and conduct *in vivo* validation in animal models to further substantiate our findings. Lastly, while our study primarily focuses on the correlation of newly identified genes, the intricate relationships among these genes remain to be elucidated. Future research will be necessary to explore these internal connections and verify their roles in the biological processes under investigation.

Overall, our study reveals the gene signature and NamiRNA regulatory network associated with the osteogenic differentiation of ADSCs cells. The 10 significantly upregulated genes (ACTA2, TAGLN, LY6E, IFITM3, NGFRAP1, TCEAL4, ATP5C1, CAV1, RPSA, and KDELR3) were notably enriched in osteogenic-related pathways and exhibited a significant negative correlation with the stemness of ADSCs cells *in vitro*. These findings contribute to the exploration of important genes related to the differentiation of adipose mesenchymal stem cells into osteoblasts, and provide important theoretical support for the study of the mechanism of adipose-derived stem cells osteogenic differentiation and related disease pathogenesis. Theses findings will also provide a foundation for future gene therapy in bone regeneration.

Authors' contributions

Xin Jin conceived the study design, completed data analysis and interpretation, completed experiments and manuscript writing. Yi Lu and Zhihong Fan provided suggestions for the data analysis. Zhihong Fan provided critical revisions to the paper. All authors contributed to the article and approved the submitted version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethics approval and consent to participate

This study utilized anonymized, publicly available data from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), which does not involve direct interaction with human subjects or collection of new personal data. As such, it meets the criteria for exemption from full ethical review by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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