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LOC646762 Is Involved in Adipogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells

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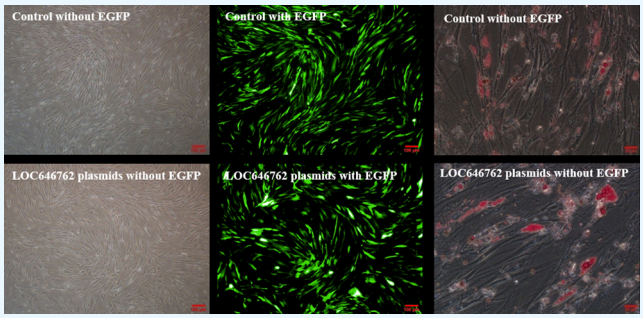
ABSTRACT: Long noncoding RNA (lncRNA) has been shown to participate in adipogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs). In this study, we aimed to investigate the role of lncRNA-LOC646762 in adipogenic differentiation of BMSCs. Transcriptome sequencing revealed a positive correlation between LOC646762 transcription and expression of adipogenic marker genes in adipogenic differentiation. Moreover, LOC646762 overexpression did not negatively impact the cell proliferation of BMSCs. Besides, LOC646762 plays a crucial role in adipogenic differentiation, as evidenced by its positive correlation with adipogenic marker gene expression. Its possible interaction with its proposed target C/EBPβ suggests its involvement in essential pathways governing adipogenesis. Collectively, our study outcomes provide valuable insights into the molecular mechanisms underlying the adipogenic differentiation of BMSCs and lay a strong foundation for further research in regenerative medicine.



1. INTRODUCTION

Adipocytes are the most common cells in the human body and serve dual roles of energy storage sites and regulators of physiological processes.[1,2] They not only maintain energy metabolism, nutritional homeostasis, and tissue homeostasis in the body but are also involved in important endocrine functions.[3,4] Adipocyte differentiation is a physiological process involving differentiation from mesenchymal stem cells (MSCs) to mature adipocytes.[5] In addition, a balance is maintained between adipogenic and osteogenic differentiation of mesenchymal bone marrow stem cells (BMSCs).[6] Adipocyte differentiation is regulated by expression of multiple functional genes, noncoding RNAs, transcription factors, hormones, and signaling pathway molecules and involves a series of biochemical reactions triggered by various hormones and cytokines.[7] Dysregulated adipocyte differentiation is closely related to several diseases such as obesity,[8] diabetes,[9] cardiovascular diseases,[10] and fatty liver;[11] however, its regulatory mechanism remains unclear. Thus, elucidating the regulatory mechanism of adipogenic differentiation is crucial for identifying new therapeutic targets for these diseases.

In recent years, functional genomics and transcriptome sequencing technologies have advanced rapidly, revealing an increasing number of factors involved in the regulation of adipogenic differentiation. Among these, long-chain noncoding RNAs (lncRNAs) have received increasing attention due to their important regulatory roles.[12,13] LncRNAs are the most abundant type of noncoding RNA and comprise >200 nt long transcripts that play important roles in cell differentiation, growth, development, and onset of many diseases.[14,15] LncRNAs exhibit multiple regulatory functions and participate in several important biological processes. They play important regulatory roles in the differentiation of MSCs into adipocytes; however, few studies have explored the role of lncRNAs in adipogenic differentiation.[16] Our previous research study based on transcriptome sequencing confirmed that various lncRNAs regulate adipogenic differentiation in BMSCs. However, the regulation of adipogenic differentiation by lncRNA in BMSCs remains largely unexplored.

To address this gap, we evaluated lncRNA expression during the adipogenic differentiation of BMSCs. Earlier transcriptome analyses revealed that LOC646762 plays a role in the adipogenic differentiation of BMSCs. Therefore, to further validate this finding in this study, we performed lentivirus-mediated stable transfection of lncRNAs to investigate the effect of LOC646762 on the adipogenic differentiation of BMSCs. Our results confirmed that LOC646762 overexpression promoted BMSC differentiation into adipocytes.

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Table 1. Gene Primers and Amplicon Sizes

gene	forward primer	reverse primer	size (bp)
AP2	5′-GGATGATAAACTGGTGGTGGAATG-3′	5′-CAGAATGTTGTAGAGTTCAATGCGA-3′	123
PPARγ	5′-GGGATGTCTCATAATGCCATCAG-3′	5′ GCCCTCGCCTTTGCTTTG 3′	97
IRS2	5′-GGCATTCCAGCCCCTATGTT-3′	5′-CGACAGCCCTCCAATCAAGT-3′	214
β-actin	5′-CGAGGACTTTGATTGCACATTG-3′	5′-AGAGAAGTGGGGTGGCTTTTAG-3′	83

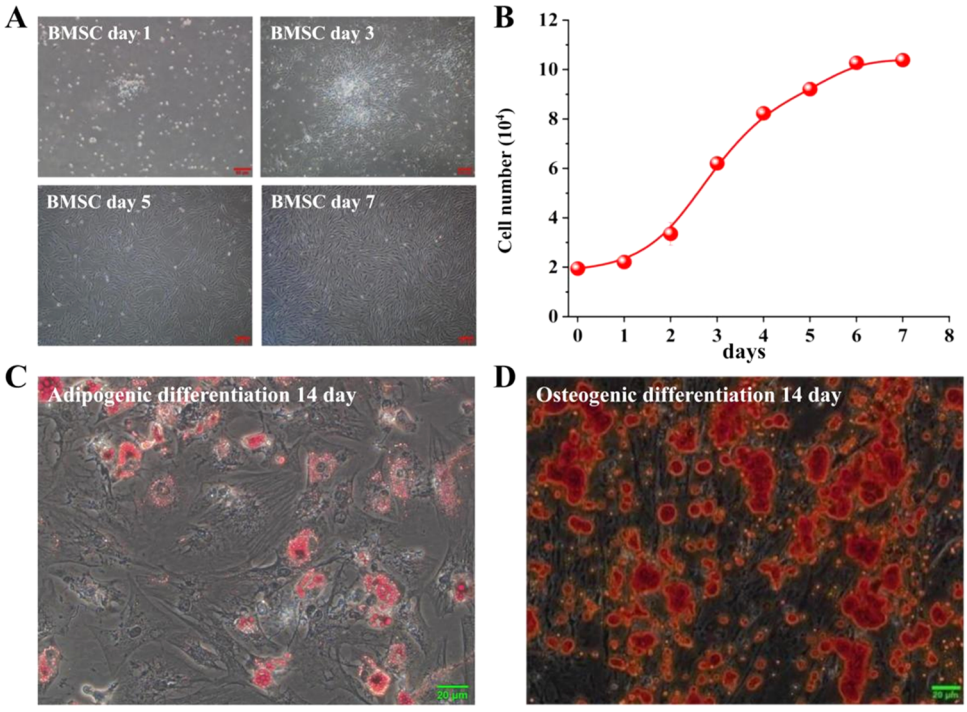

Figure 1. Isolation, culture, and functional validation of human-derived BMSCs. (A). Growth of BMSCs at different time points; (B). Proliferation curve of BMSCs; (C). Identification of BMSC adipogenic differentiation; (D). Identification of BMSC osteogenic differentiation. The scale bar denotes (A) 100 μm, (C) 20 μm, and (D) 20 μm.

In addition, we used bioinformatics tools to predict the potential target genes and signaling pathways of LOC646762. Our research findings provide new mechanistic insights into adipocyte differentiation and may help in the prevention and treatment of related diseases such as osteoporosis, insulin resistance, and type 2 diabetes in the future.

2. MATERIALS AND METHODS

2.1. Cell Isolation, Culture, and Characterization. BMSCs donated by healthy adults were approved by the Medical Ethics Committee of The First Hospital of Jiujiang City (JJSDYRMYY-YXLL-2023−235). BMSCs were isolated and cultured in DMEM containing 10% fetal bovine serum and then induced to differentiate in adipogenic differentiation induction medium comprising 1 μM dexamethasone, 0.5 mM IBMX, 10 μg/mL insulin, 100 μg/mL indomethacin, and 10% FBS added to the growth medium. Oil Red O staining was performed to detect adipogenic differentiation on 3, 7, and 14 days of differentiation.[17] Alizarin Red S staining was used to detect osteogenic differentiation. After successful induction, the cells were used for subsequent experiments. Cell proliferation was determined using the MTT assay as described earlier.[18]

2.2. SAM Dual-Vector Lentiviral Particle Construction and Transfection. GeneChem was commissioned to construct sgRNA-LOC646762 (LOC646762-sgRNA-SV40-MS2-P65-HSF1-T2A-Neo) lentiviral particles and negative control sgRNA-CON276 (U6-sgRNA-SV40-MS2-P65-HSF1-T2A-Neo) lentiviral particles. Utilizing SAM dual-vector lentiviruses, target genes were overexpressed by introducing the dCAS9-VP64 protein and sgRNA-MS2-P65-HSF1 sequence expression frames into cells using the gene expression lentivirus and the dCAS9-VP64 lentivirus. Healthy BMSCs were plated 1 day before viral infection and allowed to adhere. The next day, dCAS9-VP64 lentiviral particles were added to the cells and incubated for 3 days. Subsequently, the infected cells were subjected to G418 and puromycin double screening, and selected cells were used for functional studies.

2.3. Quantitative PCR Assay. Total RNA was extracted using a QIAGEN miRNeasy Mini Kit (cat. no. 217004), reverse-transcribed, and stored at −80 °C. For RT-qPCR, cDNA was used as a template, and target gene expression was analyzed using SYBR Prime Script RT-PCR kit. The reaction comprised 30 cycles of the following steps: 5 min at 95 °C, 15 s at 95 °C, 30 s at 60 °C, and 40 s at 72 °C. β-Actin was used as an internal control for normalizing the gene expression. The experiment was repeated 3 times for each sample. The results were analyzed by using the $2^{-\Delta\Delta Ct}$ method. Table 1 shows the sequences of the primers used in the experiment.

2.4. Western Blot Analysis. Western blotting was performed using the following antibodies: anti-AP2 (1:2000; Abcam, ab92501), anti-IRS2 (1:1000; CST 3089), anti-PPARγ (1:1000; CST 2435), and anti-β-actin (1:1000; CST 4970).

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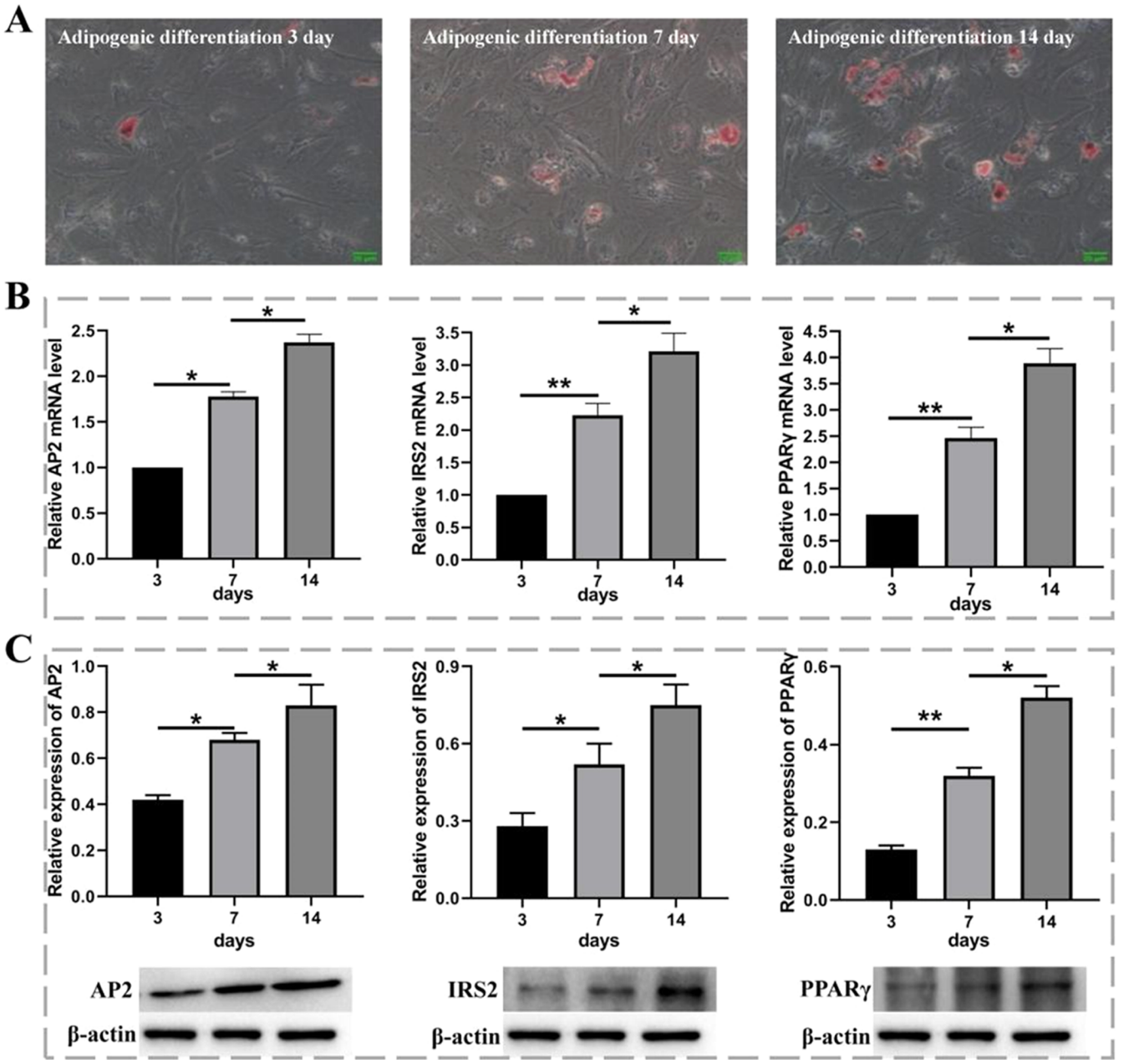

Figure 2. Adipogenic differentiation and expression of adipogenic marker genes in BMSCs on days 3, 7, and 14 of induction. (A). Oil Red O staining; (B). Expressions of adipogenic marker genes; (C). Protein levels of adipogenic markers. (*$p < 0.05$, **$p < 0.01$). The scale bar indicates 100 μm.

Target proteins were detected and quantified using Clarity Western ECL Substrate using Image Lab software (Bio-Rad Laboratories BV).

2.5. Statistical Analysis. All experiments were repeated 3 times. Results from two groups were compared using the unpaired *t*-test or Mann−Whitney *U*-test (non-normal distribution). All statistical analyses were performed using GraphPad Prism 9 statistical software. Results with $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Isolation and Culture of BMSCs, and Functional Validation. After 3 days of primary culture of BMSCs, a large number of floating cells and dead cells were observed, and cell cluster formation was observed after multiple rounds of culture media change. By 7 days, cells attained characteristic morphology and exhibited robust growth, reaching the growth plateau (Figure 1A,B). Subsequently, the cells were cultured for 14 days in adipogenic or osteogenic differentiation induction media, and Oil Red O staining and Alizarin Red S staining were used to evaluate the differentiation status. We observed lipid droplet formation after 14 days of adipogenic induction (Figure 1C). Cells subjected to osteogenic differentiation showed decreased volumes due to pleomorphism and clustered calcium nodules on their surface; these calcium nodules were stained with Alizarin Red S stain (Figure 1D), indicating significant osteogenic differentiation.

3.2. Expression of Adipogenic Marker Genes during Adipogenic Differentiation. To monitor adipogenic differentiation, the expression of adipogenic differentiation marker

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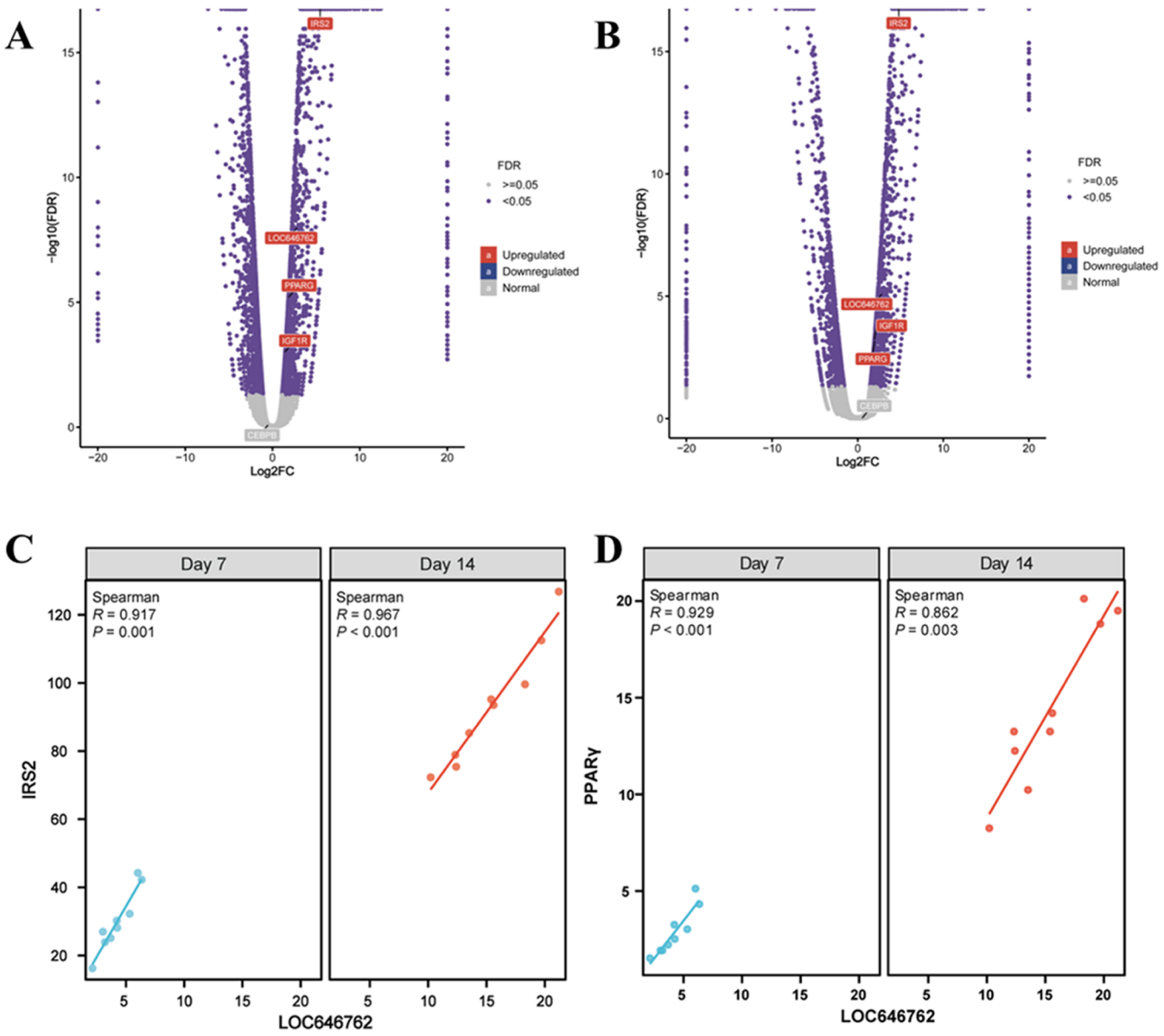

Figure 3. Volcano plot showing the lncRNA-LOC646762 transcript level and adipogenic marker genes differentially expressed on days 7 (A) and 14 (B) of differentiation. The correlations between the lncRNA-LOC646762 transcript level with *IRS2* (C) and *PPARγ* (D) expression.

genes was observed on 3, 7, and 14 days of induction. Oil Red O staining results revealed a significant increase in lipid droplet formation (Figure 2A). Furthermore, the expressions of *AP2*, *IRS2*, and *PPARγ*-the hallmark genes of adipogenic differentiation were gradually and significantly upregulated (Figure 2B). Western blot results showed that *AP2*, *IRS2*, and *PPARγ* protein levels also increased significantly (Figure 2C). All of these changes were consistently observed in a time-dependent manner.

3.3. Correlation of LOC646762 Transcript Level with Adipogenic Differentiation Markers. Cells subjected to adipogenic differentiation were used for transcriptome sequencing on days 7 and 14 of adipogenic induction. LOC646762 transcript levels were significantly and positively correlated with those of *AP2*, *IRS2*, and *PPARγ* (Figure 3A–D).

3.4. Involvement of lncRNA-LOC646762 in BMSC Adipogenic Differentiation. After 72 h of viral infection, cells displayed characteristic morphology, and green fluorescence indicated a high transfection efficiency (Figure 4A). In addition, MTT assay results confirmed comparable proliferation of cells with overexpressed LOC646762 and control cells (Figure 4B). This suggested that LOC646762 overexpression had no discernible toxic effect on cell proliferation. After 14 days of adipogenic induction, Oil Red O staining revealed that LOC646762 overexpression significantly enhanced adipogenic differentiation compared with that in the control group (Figure 4C). Moreover, mRNA and protein levels of *AP2*, *IRS2*, and *PPARγ* were significantly increased in BMSCs overexpressing LOC646762 (Figure 4D,E).

3.5. LncRNA-LOC646762 Target Analysis and Prediction. Bioinformatics analysis revealed the presence of C/EBP*β*-binding sequences in the promoter region of the LOC646762 gene, suggesting that C/EBP*β*, an important gene involved in adipogenic differentiation, may be a target gene of LncRNA-LOC646762 (Figure 5A,B).

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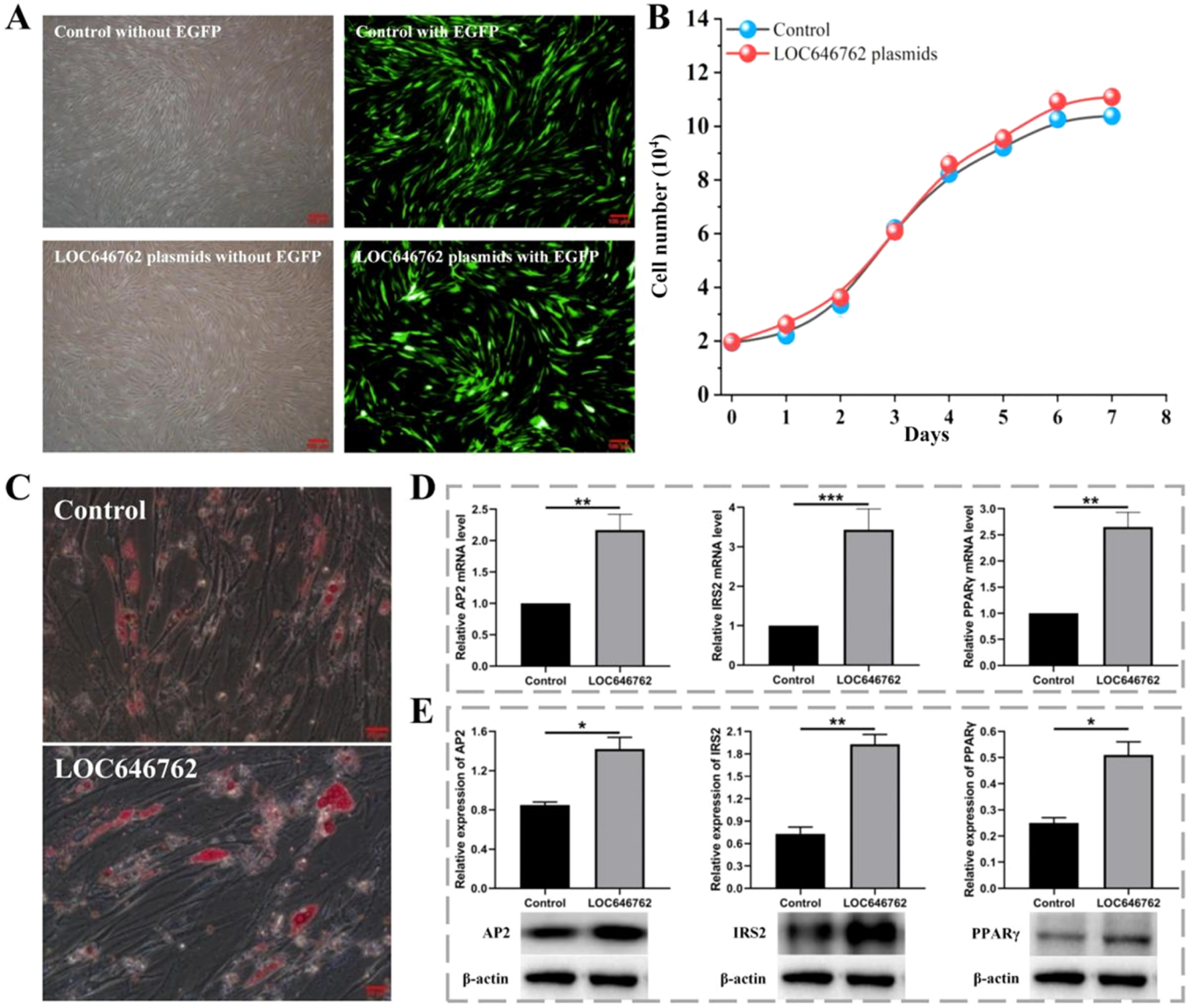

Figure 4. Effects of the lncRNA-LOC646762 overexpression in BMSCs. (A). Effect of LOC646762 overexpression on viral transfection; (B). Effect of LOC646762 overexpression on cell proliferation; (C). Effect of LOC646762 overexpression on adipogenic differentiation; (D). Effect of LOC646762 overexpression on *AP2*, *IRS2*, and *PPARγ* mRNA levels; E. Effect of LOC646762 overexpression on *AP2*, *IRS2*, and *PPARγ* protein levels. The scale bar indicates (A) 100 and (C) 20 μm.

4. DISCUSSION

In this study, adipogenic and osteogenic differentiation abilities of BMSCs were verified by performing Oil Red O and Alizarin Red S staining.[19] During adipogenic induction, the formation of lipid droplets was noted, whereas during osteogenic induction, the cells showed pleomorphism and calcium nodule formation. These results indicate that BMSCs have significant potential for adipogenic and osteogenic differentiation under various induction conditions, confirming their multidirectional differentiation properties.[20] Oil Red O staining results showed that the level of formation of lipid droplets increased significantly in a time-dependent manner. In addition, the adipogenic marker genes (*AP2*, *IRS2*, and *PPARγ*)[21,22] exhibited a gradual increase at both mRNA and protein levels during adipogenic differentiation. However, the mechanisms regulating adipogenic differentiation remain unclear.

We previously found that the LOC646762 level is significantly elevated during BMSC adipogenic differentiation. Therefore, in this study, we analyzed the effects of LOC646762 overexpression on adipogenic differentiation. The transcript level of LOC646762 was significantly and positively correlated with the expression of adipogenic marker genes, *AP2*, *IRS2*, and *PPARγ*, suggesting the involvement of LOC646762 in the adipogenic differentiation. Notably, LOC646762 overexpression had no significant cytotoxic effects on BMSC proliferation but significantly enhanced adipogenic differentiation ability. This observation was supported by the results of Oil Red O staining and the expression levels of adipogenic marker genes. These findings provide evidence that LOC646762 may support adipogenic differentiation by regulating the expression of adipogenic marker genes.

To understand the possible mechanism underlying this observation, we performed target analysis and prediction using bioinformatic tools and uncovered the presence of C/EBPβ in the promoter region of the LOC646762 gene. The presence of the binding sequence indicates that C/EBPβ may be the target gene of LOC646762. C/EBPβ is a key regulator of adipogenic

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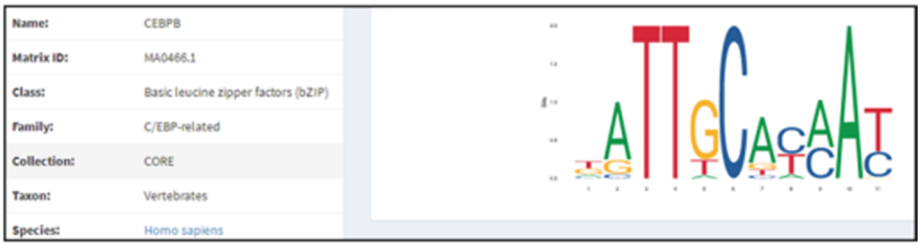
A



B

5 putative sites were predicted with these settings (80%) in sequence named LOC646762

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0466. 1	CEBPB	0.764	0.827947709769804	128	138	1	agtttaataat
MA0466. 1	CEBPB	-0.653	0.813094516410252	279	289	1	TATTTGTAAAT
MA0466. 1	CEBPB	0.208	0.822119639545449	287	297	-1	AATTTCTCATT
MA0466. 1	CEBPB	-1.651	0.802633340000348	303	313	-1	AATTTCACAGG
MA0466. 1	CEBPB	1.350	0.834090244214898	613	623	-1	TGGTGCGCCAT

Figure 5. Bioinformatic analysis predicts that the promoter region of the *LOC646762* gene has a C/EBPβ-binding sequence. (A). Predicted transcription factor-binding sequences of the C/EBPβ gene; (B). Predicted gene-binding sequences in the promoter region of *LOC646762*.

differentiation;[23] it can initiate adipogenic differentiation by acting as an early transcription factor for adipocyte differentiation.[24] Moreover, it can directly or indirectly regulate the expression of adipogenic marker genes, as well as those of several lipogenic marker genes including adipocyte-specific genes such as *AP2* and *PPAR*γ[25] and adipocyte metabolism-related genes such as *IRS2*.[26]

Expression regulation of these genes is important during adipogenic differentiation. Furthermore, C/EBPβ can form complexes with PPARγ or C/EBPα to synergistically regulate adipocyte differentiation, further driving cells toward adipocyte differentiation. Finally, C/EBPβ can also regulate adipocyte metabolic activity following adipocyte maturation, and participates in the regulation of fatty acid synthesis, lipid storage, and glucose metabolism to maintain adipose tissue function and homeostasis.[27,28] These findings collectively suggest that LOC646762 may be involved in regulating adipogenic differentiation via its target gene C/EBPβ. However, this hypothesis needs to be validated in future studies using different experimental models. Future studies should aim to elucidate the relationship between LOC646762 and C/EBPβ, providing a theoretical basis for its application in tissue repair and regeneration.

5. CONCLUSIONS

In conclusion, we preliminarily demonstrated that LOC646762 overexpression significantly enhanced adipogenic differentiation and increased the expression of adipogenic marker genes. Moreover, we propose that LOC646762 affects adipogenic differentiation by regulating C/EBPβ, which, in turn, regulates *AP2*, *IRS2*, and *PPAR*γ expression. These findings deepen our understanding of the mechanism underlying BMSC adipogenic differentiation, offer new insights for exploring the mechanistic role of LOC646762 in BMSC adipogenic differentiation, and open new avenues for identifying new therapeutic targets for adipogenic differentiation-related diseases.

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Author Contributions

‖Y.S. and M.X. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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