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YTHDC2 inhibits rat bone mesenchymal stem cells osteogenic differentiation by accelerating RUNX2 mRNA degradation via m6A methylation

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ABSTRACT

As the most abundant internal mRNA modification, N6-methyladenosine (m6A) RNA methylation has been found to influence many biological events including bone mesenchymal stem cells (BMSCs) osteogenic differentiation. YTH N6-methyladenosine RNA binding protein C2 (YTHDC2) is an m6A reading protein with the ability to mediate the decay of combined methylated mRNA, however its role in BMSCs osteogenic differentiation remains unknown. In this study, we first found an increase of RUNX family transcription factor 2 (RUNX2) expression and a decrease of YTHDC2 expression during the process of BMSCs osteogenic differentiation. Furthermore, we transfected BMSCs with YTHDC2 interference fragment, resulting in an increased content of RUNX2 mRNA and protein inside BMSCs. Finally, through RNA Immunoprecipitation experiments, we confirmed that YTHDC2 protein can bind to RUNX2 mRNA and accelerate its decomposition. Moreover, the immunofluorescence staining also showed a negative correlation between YTHDC2 and RUNX2. In conclusion, during BMSCs osteogenic differentiation, YTHDC2 protein showed decreased expression, resulting in a higher level of RUNX2 (mRNA and protein) expression inside cells, indicating YTHDC2 as a promising molecular target for the regulation of BMSCs osteogenic differentiation.

1. Introduction

Bone is constantly undergoing physiological reconstruction, thus it needs the cooperation between osteoblasts and osteoclasts to maintain homeostasis [1]. Bone mesenchymal stem cells (BMSCs) are the most common progenitors for osteoblasts [2]. In recent years,

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there has been an increasing number of studies about the use of BMSCs to treat bone diseases, such as using silver nanoparticles or single-walled carbon nanotubes to stimulate the differentiation of BMSCs into osteoblasts [3,4]. During this process, the expression of osteogenesis-related genes such as osteocalcin (OCN), osteopontin (OPN), and RUNX family transcription factor 2 (RUNX2) will increase, but the specific epigenetic regulation underlying these changes in the gene expression process remains to be discovered [5].

RNA is the intermediate product that connects DNA and protein. The post-transcriptional modification of RNA affects many physiological activities of cells and has important regulatory effect on cell epigenetic expression [6]. Recent studies have found that there are more than 100 types of RNA modifications in eukaryotic cells, and the most abundant internal mRNA modification is N6-methyladenosine (m6A) RNA methylation, which is a reversible process regulated by 'Writers' methylases that add methyl groups on mRNA; 'Erasers' demethylases that delete methyl groups from mRNA, and 'Readers' that further recognize the m6A on mRNA to affect its stability [7–9]. Since the first discovery of m6A in 1974, it has been found to influence many biological events, such as germ cell production, neurodevelopment, human study and memory, immunoregulation, and many types of diseases and cancers [10–13]. Recent studies have shown that m6A modification also participates in osteogenic differentiation, such as the m6A 'Writer' methyltransferase 3 (METTL3) can cause changes in the ribosomal part of parathyroid hormone 1 receptor (PTH1R) mRNA, thereby affecting the parathyroid hormone (PTH)/PTH1R signal axis, which then leads to osteoporosis [14], and the m6A 'Eraser' alkB homolog 5 (ALKBH5) reduces the osteogenic differentiation of mesenchymal stem cells by accelerating the degradation of protein arginine methyltransferase 6 (PRMT6) mRNA [15]. However, there are few studies on whether or how m6A 'Readers' influence osteogenic differentiation.

YTH N6-methyladenosine RNA binding protein C2 (YTHDC2) is an m6A 'Reader'. Recent studies have shown that it has the ability to accelerate the decay of methylated mRNA. YTHDC2 can affect mammalian spermatogenesis by accelerating the degradation of methylated structural maintenance of chromosomes 3 (SMC3) mRNA [16], and in the treatment of nasopharyngeal carcinoma, YTHDC2 reduces the mRNA methylation of insulin like growth factor 1 receptor (IGF1R) , resulting in a decrease in its sensitivity to radiotherapy [17]. Therefore, we want to determine whether YTHDC2 has a regulatory function in osteogenesis and its underlying mechanism.

2. Materials and methods

2.1. Cell culture

Rat BMSCs (Cyagen Biosciences Guangzhou Inc.) were cultured with growth medium (GM) [α-MEM medium (Hyclone) containing 10% fetal bovine serum (Gibco),100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco)]. For osteogenic induction, BMSCs at a confluence of approximately 60–70% were cultured with osteogenic medium (OM) [osteogenic medium containing 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma)]. The culture plates were placed in an incubator at 37 °C with 95% relative humidity and 5% CO_2 partial pressure. The culture medium was changed every two days.

2.2. BMSCs transfection

The YTHDC2 interference fragment and negative control fragment (Synbio Technologies, China) were co-transfected with Lipofectamine 2000 reagent to downregulate the target gene expression level in BMSCs according to the manufacturer's instructions. After transfection in reduced serum medium (Gibco, Australia) for 6 h, the medium was changed into OM and the BMSCs were cultured in OM for further research. The primer sequences were synthesized by the Synbio Technologies and are showing below.

Gene	Primer type	Primer Sequence (5′-3')
YTHDC2-si	Forward	CCGACUAAGUCAAUCUCUUGGUUUATT
	Reverse	UAAACCAAGAGAUUGACUUAGUCGGTT

2.3. Alkaline phosphatase (ALP) staining

We used BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology) to evaluate the alkaline phosphatase activity of BMSCs. After two washes with PBS, the cells were fixed in 4% paraformaldehyde for 30 min and then stained with BCIP/NBT mixture solution for 10 min. The ALP activity was finally determined under an inverted phase contrast microscope (Leica, Germany), and we used ImageJ software to calculate the staining area.

2.4. Alizarin red staining

We used Alizarin Red Staining kit (Cyagen, China) to evaluate calcium mineralization. In short, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained for 15 min according to the instructions. The calcium nodules were then photographed under an inverted phase contrast microscope. The nodules were also dissolved in 10% cetylpyridinium chloride (TCI, China), and the solution obtained was measured at 562 nm using a spectrophotometer.

2.5. Quantification of overall m6A RNA methylation

We used an EpiQuik m6A RNA Methylation Quantification Kit (colorimetric) (Epigentek, USA) to quantify the overall BMSCs m6A level. First, we extracted RNA and used approximately 200 ng RNA as an initial input. Then, the RNA and standard negative control were bound to the positive control wells to assay and capture RNA. After washing, the detection antibody and enhancer solution were added, and color developing solution was added prior to measurement of the absorbance at 450 nm. The values were calculated using linear regression equations and are presented as a standard region graph.

2.6. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from BMSCs with Trizol. RNA was reverse transcribed to cDNA with reverse transcriptase (Vazyme, China) following the manufacturer's protocol, and qRT-PCR was performed using SYBR Green qRT-PCR Master Mix (GenePharma, China). β-actin was used as the internal control for mRNA expression. The primer sequences were synthesized by the Synbio Technologies and are showing below.

Gene	Primer type	Primer Sequence (5′-3')
Runx2	Forward	GCACCCAGCCCATAATAGA
	Reverse	TTGGAGCAAGGAGAACCC
Opn	Forward	CTTTCACTCCAATCGTCCCTAC
	Reverse	CTGCCCTTTCCGTTGTTGTC
Ocn	Forward	CATGAGGACCCTCTCTCTGC
	Reverse	TGGACATGAAGGCTTTGTCA
Ythdc2	Forward	GACTCAACAATGGCATACCTCAAGTTC
	Reverse	GGTCTTTCCAGACCCAGTTTCTCC
Actb	Forward	GGGAAATCGTGCGTGACATT
	Reverse	GCGGCAGTGGCCATCTC

2.7. Western blot analysis

A RIPA (Beyotime, China) buffer containing 1% Phenylmethanesulfonyl fluoride (PMSF) was used to lyse and obtain total protein from BMSCs. This process was done on ice and lasted 2 min. Then We prepared gels with PAGE Gel Fast Preparation Kit (10%) (Epizyme Biomedical Technology, China) for subsequent electrophoresis experiments. The electrophoresis conditions were 80 V for 30 min and 120 V for 1 h, followed by transfer to polyvinylidene difluoride membranes in transfer buffer at 200 mA for 1.5 h. Then, membranes were blocked in 5% skim milk at 37 °C for 1 h and incubated with YTHDC2 primary antibody (1:1000, ab220160, Abcam, USA) and RUNX2 primary antibody (1:1000, ab236639, Abcam, USA) overnight at 4 °C, and next day incubated with an HRP-labeled goat anti-rabbit secondary antibody (ab7090, Abcam, USA) for 1 h at room temperature. Immunoreactions were visualized with a chemiluminescent detection system in a dark room.

2.8. Immunoprecipitation assay

Immunoprecipitation assays of m6A and YTHDC2 were performed with an RNA Immunoprecipitation kit (BersinBio Biotech, China). In short, about $1*10^7$ BMSCs were collected, and the cells were lysed with polysome lysis buffer for 30 min at room temperature. Then, we added DNase to remove DNA impurities, the supernatant was collected after centrifugation at 16100*g* for 10 min, and then incubated with *anti*-m6A antibody (1:50, A-1801, EpigenTek, USA) and *anti*-YTHDC2 primary antibody (1:30, ab220160, Abcam, USA) overnight at 4 °C in a vertical orientation, after which magnetic beads were added and incubated at 4 °C for 1 h to enrich targets. After washing, RNA was extracted, and target gene expression was detected by qRT-PCR.

2.9. Immunofluorescence staining

To directly observe the relationship between YTHDC2 and RUNX2, we cultured BMSCs in GM and OM. First, BMSCs were fixed with 4% paraformaldehyde, treated with Triton X-100 (Sigma-Aldrich, USA) and blocked with 5% BSA (Solarbio, China) at 4 °C overnight. Then, BMSCs were stained with YTHDC2 primary antibody (1:100, 27779-1-AP, Proteintech, USA) and RUNX2 primary antibody (1:100, sc-390351, Santa Cruz, USA) at 4 °C overnight and incubated with the secondary antibody (Beyotime Institute of Biotechnology) afterwards. Finally, we stained the nuclei with DAPI and imaged the sections with Confocal Laser Scanning Microscope (Zeiss, Germany).

2.10. Statistical analysis

We made charts with GraphPad 8.0 software. For statistical analysis, two-tailed Student's t-test or Mann Whitney U tests between two groups was performed by SPSS Statistic 26 for Windows. Data are reported as the mean ± SD from three technical replicates

experiments. Results were considered to be statistically significant when the p value was <0.05.

3. Results

1 Osteogenic induction medium induces osteogenic differentiation of BMSCs

We respectively cultured BMSCs in GM and OM for seven days to detect their osteogenic differentiation ability. ALP staining and alizarin red staining showed a significant increase in ALP activity and calcium nodule deposition in OM-induced BMSCs compared with GM-induced BMSCs (Fig. 1A–B). Moreover, qRT-PCR results showed that the mRNA expression of RUNX2 was significantly increased, while the elevation of OCN/OPN was not significant (Fig. 1C). This finding may be due to that RUNX2 is expressed earlier in the process of BMSCs osteogenic differentiation [18]. Next, m6A RNA methylation quantification analysis revealed that the overall m6A level was markedly elevated in 7-day OM-induced BMSCs, indicating that m6A plays an important role during BMSCs osteogenic differentiation (Fig. 1D).

2 Decreased expression of YTHDC2 in BMSCs osteogenic differentiation

We then measured the change of YTHDC2 during BMSCs osteogenic differentiation. BMSCs were cultured in GM or OM for 7 days, qRT-PCR and Western blot results revealed that YTHDC2 expression was lower in OM-induced BMSCs (Fig. 2A–B). Meanwhile, the Western blot results showed an elevation in RUNX2 expression in OM-induced BMSCs, similar to its previous qRT-PCR results (Fig. 2C–D). Based on the previous report that the YTHDC2 protein could accelerate the degradation of mRNA after binding to its m6A [19], we speculate that the decrease of YTHDC2 is one of the explanations for the increased expression of the RUNX2 gene during BMSCs osteogenic differentiation.

3 Knockdown of YTHDC2 promotes RUNX2 expression and BMSCs osteogenic differentiation

Next, we transfected BMSCs with YTHDC2 interference fragment (YTHDC2-silenced). After OM culture for 7 days, qRT-PCR and Western blot analysis showed that compared with the negative control group, the mRNA and protein expression level of YTHDC2 was

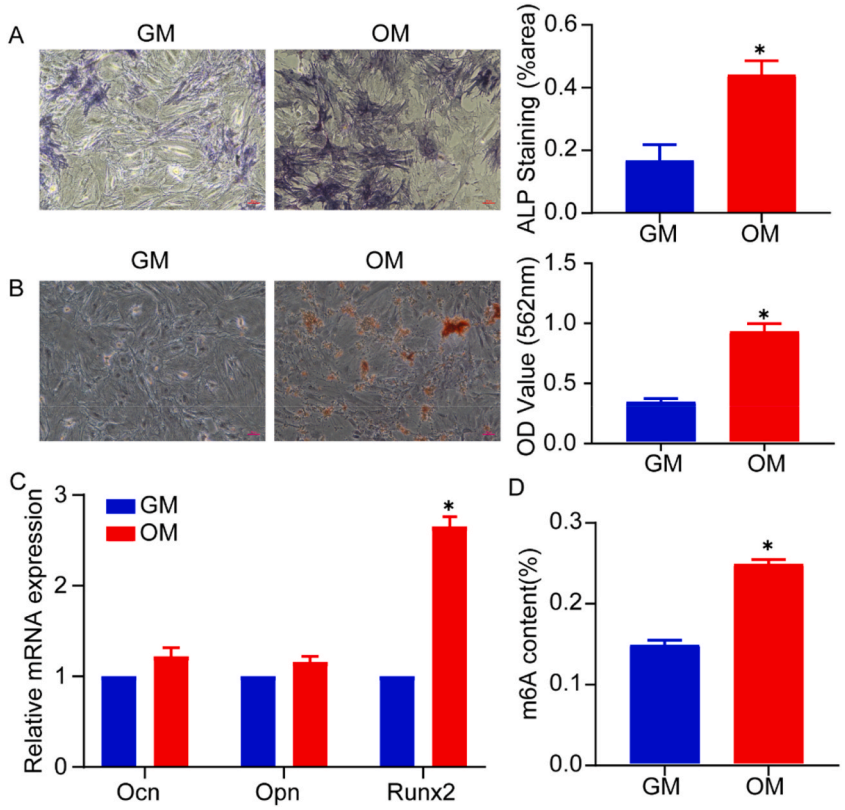

Fig. 1. Increased overall m6A level in BMSCs after osteogenic induction. BMSCs were cultured in osteogenic medium (OM) or growth medium (GM) for 7 days. **A.** ALP activity of BMSCs was analyzed using ALP staining. **B.** Formation of mineralized nodules was analyzed by alizarin red staining. **C.** The mRNA expressions of OCN/OPN/RUNX2 were quantified by qRT-PCR. **D.** Quantification result of overall m6A level in BMSCs. (*P $<$ 0.05).

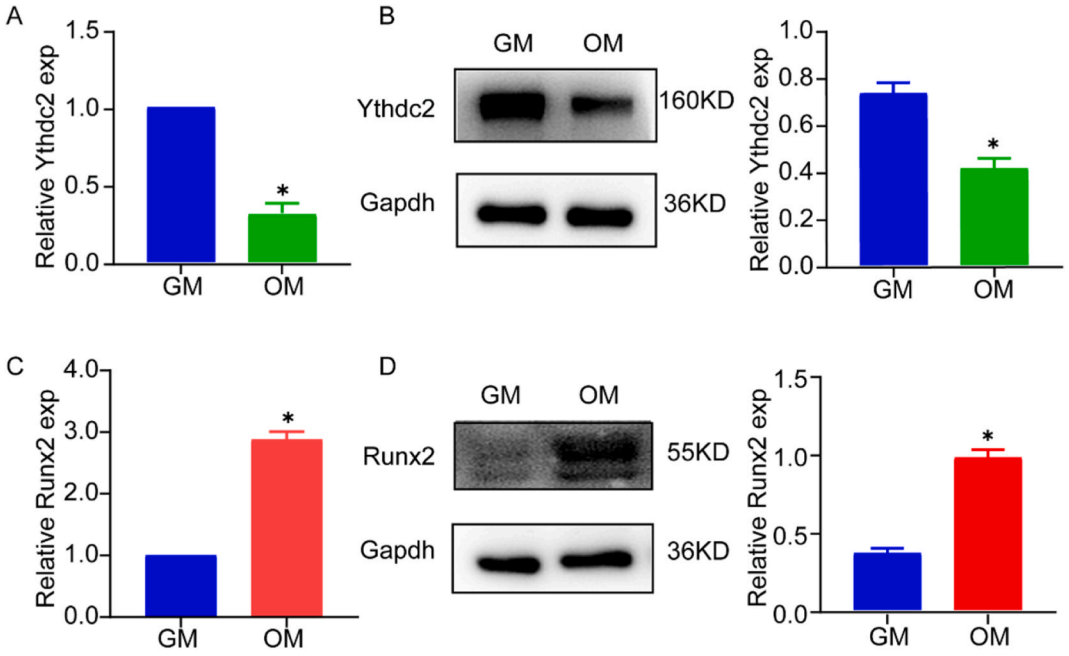

Fig. 2. Negative correlation between YTHDC2 and RUNX2 in BMSCs osteogenesis. **A-B.** The mRNA and protein expression of YTHDC2 in BMSCs quantified by qRT-PCR and Western blot. **C-D.** The mRNA and protein expression of RUNX2 in BMSCs quantified by qRT-PCR and Western blot. (*P < 0.05).

reduced after the gene was silenced (Fig. 3A–B). ALP staining assays showed that ALP activity was increased after YTHDC2 was silenced. Similarly, the alizarin red staining indicated an increase in calcium nodule formation (Fig. 3C–D). The qRT-PCR and Western blot results showed an increased expression of RUNX2 in YTHDC2- silenced BMSCs (Fig. 3E–F). These results confirmed that the reduction in YTHDC2 expression would promote osteogenic differentiation of BMSCs by increasing the cellular RUNX2 content.

4 YTHDC2 affects BMSCs osteogenic differentiation by binding to RUNX2 mRNA and accelerating its decomposition

To further confirm that the YTHDC2 protein affects BMSCs osteogenic differentiation through mediating RUNX2 mRNA expression, we conducted RNA Immunoprecipitation experiments with both m6A and YTHDC2 antibody. The qRT-PCR results of m6A Immunoprecipitation showed that OM-induced BMSCs contained more m6A-modified RUNX2 mRNA, and the qRT-PCR results of YTHDC2 Immunoprecipitation revealed that YTHDC2 was bound to the m6A of RUNX2 mRNA (Fig. 4A–B). In addition, the immunofluorescence staining showed a negative correlation between YTHDC2 and RUNX2 (Fig. 4C–D). In summary, during BMSCs osteogenic differentiation, YTHDC2 would accelerate the degradation of methylated RUNX2 mRNA through m6A modification, while silencing the YTHDC2 gene could promote RUNX2 stability (Fig. 4E).

4. Discussion

In the process of transferring genetic information, post-transcriptional modification is an important part of gene expression regulation and m6A is one of the most common internal mRNA modifications [20–22]. Since Desrosiers first discovered the m6A modification process in Novikoff hepatoma cells in 1974, an increasing number of studies have shown that m6A RNA methylation is mainly regulated by 'Writers', 'Erasers' and 'Readers' [23,24]. Methyl groups are first added to or removed from mRNA by 'Writers' [such as METTL3, methyltransferase 14 (METTL14), and WT1 associated protein (WTAP)] or 'Erasers' [such as ALKBH5 and FTO alpha-ketoglutarate dependent dioxygenase (FTO)], and then, 'Readers' [such as insulin like growth factor 2 mRNA binding protein 2 (IGF2BP2) and YTH N6-methyladenosine RNA binding protein F2 (YTHDF2)] will further bind to the m6A, which affect both the stability and translation of the mRNA. m6A plays an important role in the process of cell growth, metabolism, nerve formation, cardiovascular disease, cancer, immunity, etc [25,26]. In terms of cell osteogenic differentiation, the m6A 'Writer' METTL3 and 'Eraser' ALKBH5 can regulate the expression of RUNX2 to affect osteogenesis, and studies have summarized the methods of preventing and treating osteoporosis through m6A modification [27].

As an m6A 'Reader', YTHDC2 regulates the stability and decomposition of mRNA after its YTH domain binds to the m6A of mRNA. Studies have shown that YTHDC2 can affect many aspects of biological activities of cells. In the fetus, YTHDC2 also plays an important role in the initiation and progression of meiosis in female germ cells [28,29]. Lowering YTHDC2 expression to increase cystine uptake will promote the occurrence of lung adenocarcinoma [30]. Database analysis of patients with head and neck cell carcinoma showed increased survival with high YTHDC2 expression [31]. On the other hand, there are also some studies which found the positive effect of

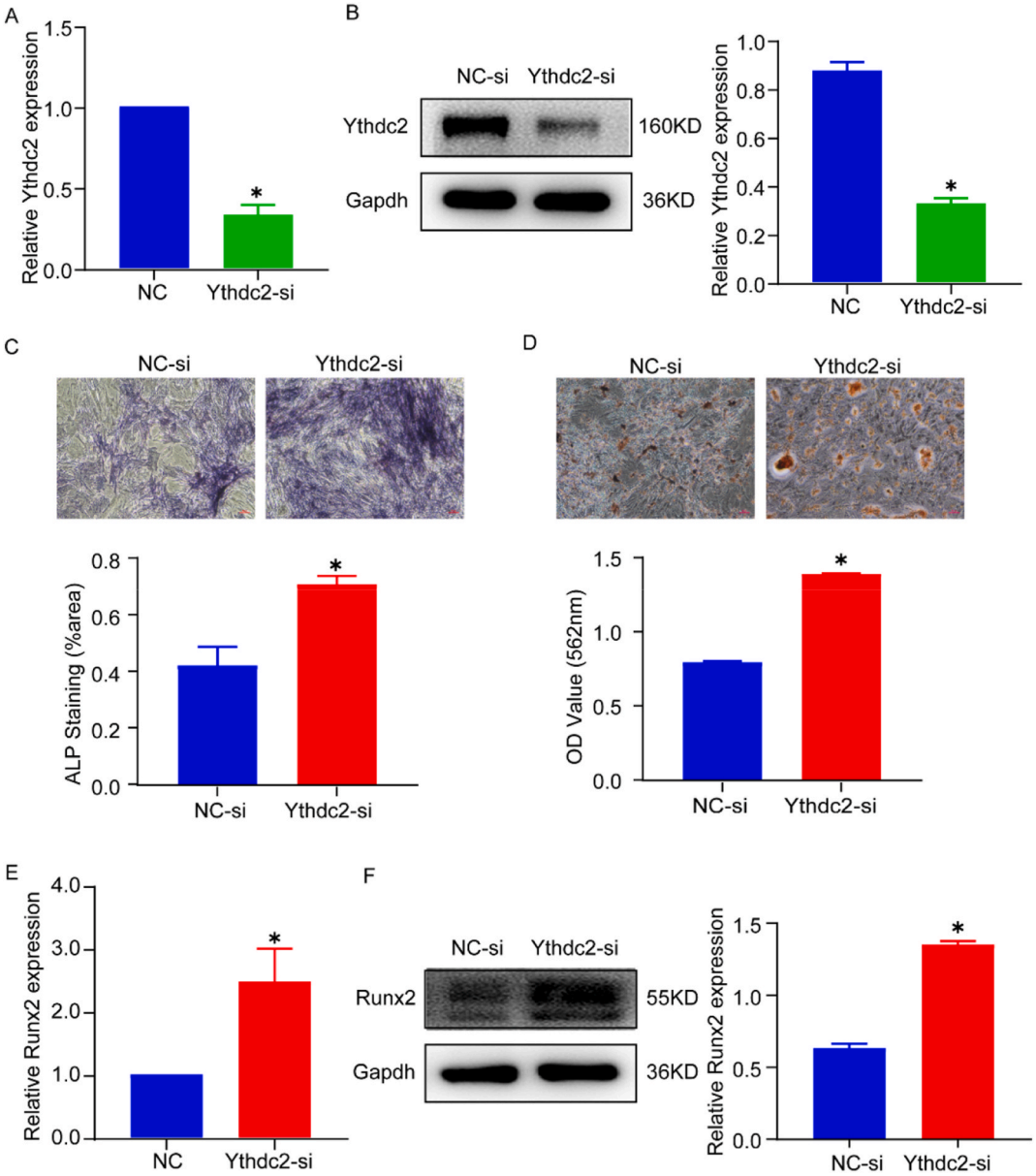

Fig. 3. Knockdown of YTHDC2 promoted BMSCs osteogenic differentiation and RUNX2 expression. BMSCs were transfected with YTHDC2-siRNA (YTHDC2-si) or negative control-siRNA (NC), after which they were cultured in OM for 7 days. **A-B**. The qRT-PCR and Western blot results of YTHDC2 expression in BMSCs. **C-D**. ALP staining and alizarin red staining of BMSCs. **E-F**. The qRT-PCR and Western blot results of YTHDC2 expression in BMSCs. (*P $<$ 0.05).

YTHDC2 in tumor progression, such as YTHDC2 promotes colon tumor metastasis by promoting the translation of HIF-1α through unwinding the 5′-untranslated region (5′UTR) of its mRNA [32]. RUNX2 is the most important transcription factor in BMSCs osteogenesis process, as one of the RUNX family, it promotes the expression of other specific osteogenic genes such as OCN, ALP, osterin, osteoprotegerin (OPG) and OPN, therefore regulates growth and development of bone [33].

In this study, we found the elevation of overall m6A level during BMSCs osteogenic differentiation. Then we silenced the YTHDC2 gene, resulting in overexpression of RUNX2. Finally, through RNA Immunoprecipitation experiments and immunofluorescence experiments, we confirmed that YTHDC2 binds to the mRNA of RUNX2 and reduces its expression. These results indicate that the m6A 'Reader' protein YTHDC2 negatively mediates RUNX2 mRNA, thereby affecting the BMSCs osteogenic differentiation process. However, there are still some limitations of our study. For example, we only found that YTHDC2 and RUNX2 bind to each other, however we speculate that there are still many unknown regulators that affect the YTHDC2-RUNX2 pathway during BMSCs osteogenesis that need to be further studied [34]. In the future, we will study the regulatory pathway of the decreased YTHDC2 expression during BMSCs

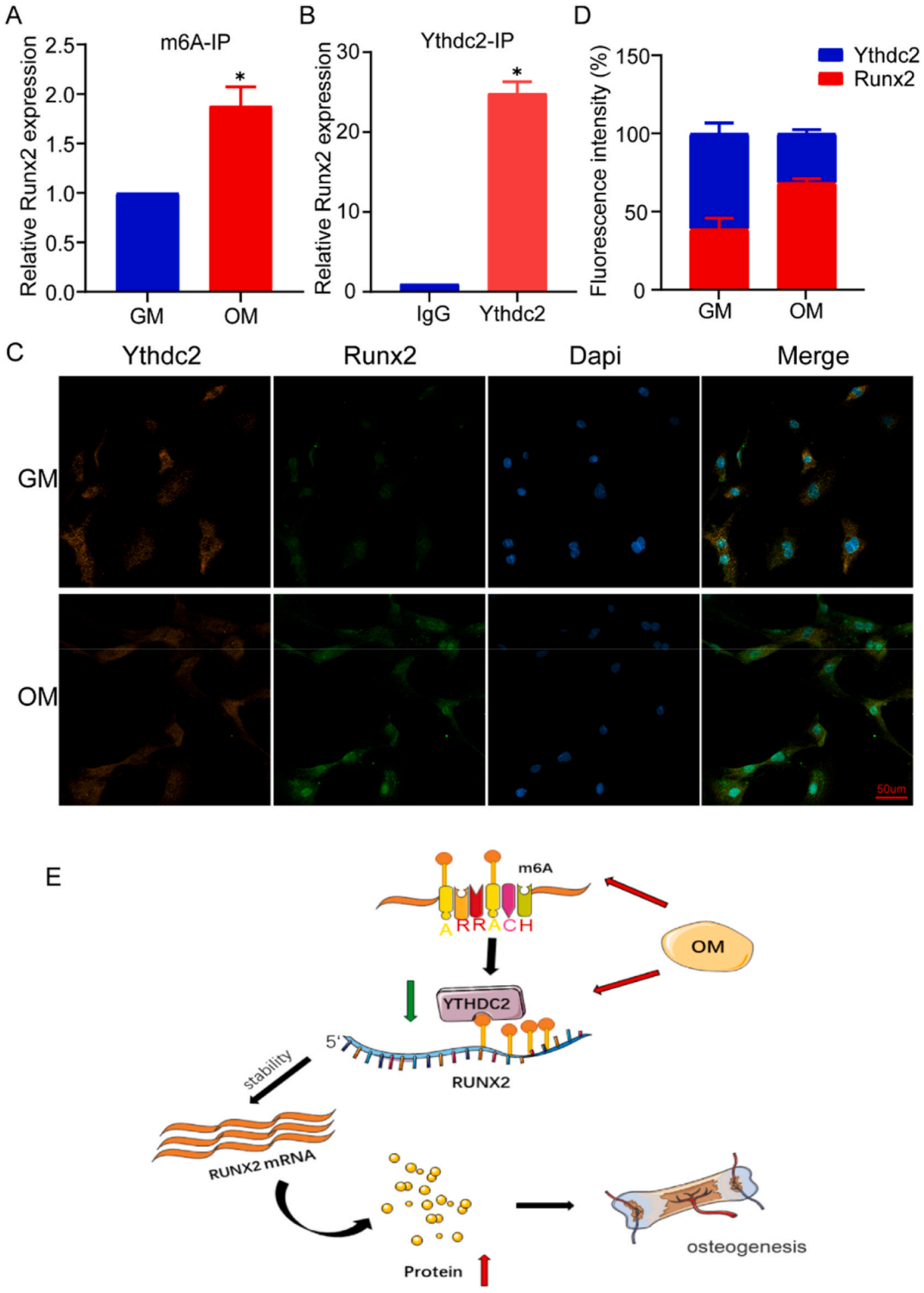

Fig. 4. YTHDC2 regulated RUNX2 expression through m6A modification. BMSCs were cultured in osteogenic medium (OM) or growth medium (GM) for 7 days. **A.** m6A-IP showed OM-induced BMSCs had more m6A modification on RUNX2 mRNA. **B.** YTHDC2-IP showed YTHDC2 tightly bound to RUNX2 m6A. **C.** Immunofluorescence staining showed negative correlation between YTHDC2 and RUNX2. **D.** Fluorescence intensity of OM-induced and GM-induced BMSCs. **E.** The mechanism of YTHDC2 potential effects on BMSCs osteogenesis through m6A modification on RUNX2 mRNA. (*P $<$ 0.05).

osteogenic differentiation and further verify this mechanism in vivo. Osteoporosis, characterized by poor bone quality and low bone mass, is caused by insufficient osteogenic differentiation of BMSCs [35]. In order to further clinically evaluate the negative correlation between YTHDC2 and RUNX2, we plan to study the BMSCs acquired from osteoporosis patients to verify whether the expression of YTHDC2 is elevated along with the reduced expression of RUNX2 compared with BMSCs from normal people. In addition, under long-term osteogenic induction, whether YTHDC2 would bind to the mRNA of OCN or OPN to regulate BMSCs osteogenesis has also aroused our interest, which needs to be further verified.

5. Conclusion

YTHDC2 binds to the RUNX2 mRNA and reduces its intracellular levels, therefore negatively regulating BMSCs osteogenic differentiation, indicating that YTHDC2 may act as a promising marker for evaluating both physiological bone development and relative pathological diseases such as osteoporosis.

Author contribution statement

Bo Ma; Pei Cao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Lichen Zhang; Hongyi Zhu; Xuwen Ye: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Lingjun Wang; Liang Chen: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18876.

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