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Hyperin up-regulates miR-7031-5P to promote osteogenic differentiation of MC3T3-E1 cells

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Summary. Objective. To investigate the effects of Hyperin (Hyp) on osteogenic differentiation of MC3T3-E1 cells.

Methods. Differentially expressed miRNA was screened by miRNA Microarray. miR-7031-5P overexpression and knockdown MC3T3-E1 cell models were constructed by transfecting miR-7031-5P mimics and inhibitor. Alizarin red staining (ARS) assay was used to observe the formation of mineralized nodules in MC3T3-E1 cells. ALP activity was detected by using ALP detection kit. Western blot assay was used to examine the changes in osteogenic differentiation-related proteins. The relationship between miR-7031-5P and Wnt7a was revealed by dual luciferase report experiments.

Results. We found that miR-7031-5P was up-regulated in MC3T3-E1 cells after Hyp treatment. The results indicated that compared with the untreated group, Hyp promoted the formation of mineralized nodules and the alkaline phosphatase (ALP) activity of MC3T3-E1 cells via overexpressing miR-7031-5P. Besides, elevated miR-7031-5P increased OPN, COL1A1, and Runx2 mRNA expression. More importantly, Wnt7a was identified as the downstream target gene of miR-7031-5P promoting osteogenic differentiation of MC3T3-E1 cells.

Conclusions. Hyp up-regulated miR-7031-5P to promote osteogenic differentiation of MC3T3-E1 cells by targeting Wnt7a.

Key words: Hyp, MC3T3-E1, Wnt7a, Osteogenic differentiation, miR-7031-5P

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Introduction

Primary osteoporosis (POP) is a natural physiological degenerative disease, which is characterized by the deterioration of bone microstructure and the decrease of bone density (Ensrud and Crandall, 2017). Studies have shown that once the role of bone resorption is greater than bone formation, the balance between bone formation and bone resorption is broken, resulting in bone loss and bone defect diseases. Bone diseases often cause short-term or long-term physical pain and even disability (Harvey et al., 2010). Besides, bone defects lead to various complications, which usually affect clinical efficacy and are a challenge for orthopedic surgeons (Pepe et al., 2019). On the other hand, promoting osteogenic differentiation has been a crucial strategy for bone loss and bone defect disease repair (Wang et al., 2016). Osteoporosis is the consequence of altered bone metabolism resulting in the systemic reduction of bone strength and increased risk of fragility fractures. MicroRNAs (miRNAs) regulate gene expression on a post-transcriptional level and are known to take part in the control of bone formation and bone resorption (Weilner et al., 2015; Pizzicannella et al., 2019). In addition, Wnt signaling disruption in osteoblastic-lineage cells leads to bone formation defects in osteoporosis (Jing et al., 2018). In recent years, herbal remedies have been found to play roles in regulating the proliferation and differentiation of human mesenchymal stromal cells leading to osteogenic differentiation (Udalamaththa et al., 2016). The whole differentiation process of osteoblasts includes mesenchymal stem cells, pre-osteoblasts, mature osteoblasts, and bone cells (Rodan, 1998; Rachner et al., 2011). MC3T3 is a C57BL/6 osteoblast derived from suckling mice. MC3T3-E1, as a subgenus of MC3T3, belongs to the pre-osteoblastic cell line (Wang et al., 2010). In addition, some studies have found that MC3T3-E1 was related to the occurrence, development, and metastasis of certain

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malignant bone tumors (Yang et al., 2016). Besides, an increasing number of documents have demonstrated that MC3T3-E1 cells have active biological properties, and their proliferation and differentiation are essential for the repair of bone loss and bone defect diseases (Choi et al., 1996).

Hyperin (Hyp) as a flavonol glycoside compound, also known as quercetin-3-O-β-D galactopyranoside, is widely present in various plants such as Garciniaceae, Leguminosae, Rhododendronaceae, and Euonymae (Wang et al., 2000). Since Nair et al. isolated Hyp from red-osier dogwood in 1960, scholars at home and abroad began to study Hyp and found that Hyp has analgesia, anti-oxidation, and protective effects on myocardium and liver (Nair and Rudloff, 1960; Wang et al., 1996). However, the roles of Hyp in osteogenic differentiation have not been reported in the published documents.

As such, in this study, we found that Hyp treatment made miR-7031-5P expression in MC3T3-E1 cells up-regulated, and then explored the mechanism of Hyp's effects on the osteogenic differentiation of MC3T3-E1 cells. The results indicated that Hyp treatment promoted the formation of mineralized nodules and the alkaline phosphatase (ALP) activity of MC3T3-E1 cells via miR-7031-5P overexpression. Besides, Wnt7a was identified as the downstream target gene of miR-7031-5P promoting osteogenic differentiation of MC3T3-E1. Our study provides a pharmacological basis for Hyp's treatment of bone loss diseases, to better improve the quality of life of patients with bone tissue damage.

Materials and methods

Cell culture

Mouse embryonic osteoblast precursor cell line (MC3T3-E1 cells) was purchased from Sciencell Corporation (Carlsbad, California, USA). Hyp was provided by Shanghai Enzyme Link Biotechnology Co., Ltd (Shanghai, China). The cells were cultured in DMEM medium (Gibco, #1868985) with Fetal Bovine Serum (Gibco, #10091-148), placing in an incubator (SANYO, Cat. No. MCO-175) with 37°C containing 5% CO_2. In terms of osteogenic differentiation, MC3T3-E1 cells were treated with osteogenic medium (containing 10% Fetal Bovine Serum, 5 mmol/L α-sodium glycerophosphate, 50 μg/ml ascorbic acid and 10^{-8} mol/L dexamethasone) for 2 to 3 days to induce osteogenic differentiation. MC3T3-E1 cells cultured in complete medium served as a negative control group.

Hyp treated MC3T3-E1 cells to screen differentially expressed miRNA

In the preliminary experiments, we treated MC3T3-E1 cells using Hyp with different concentration gradients (0, 20, 40, 80, 100, 120, 150, 200 μmol/L). The optimal concentration of Hyp was defined as 80 μmol/L by measuring the degree of cell differentiation, the ALP activity, and the formation of mineralized nodules in cells (Figs. 1-3). The experiment was divided into two groups: MC3T3-E1 cells with Hyp (80 μmol/L) and the control group (MC3T3-E1 cells without Hyp). After Hyp treatment for 7 days, the differentially expressed miRNA between the Hyp treatment group and control group was screened by Agilent Mouse miRNA Microarray Kit (Release 21.0, 8x60K).

RNA extraction and qRT-PCR

The total RNA was isolated according to the manufacturer's protocol of the Trizol reagent (Sigma, St. Louis, MO, USA), and the production was reversely transcribed to obtain cDNA using the Hiscript QRT supermix kit (Vazyme, Nanjing, Jiangsu, China). The system of qRT-PCR was 10 μL according to SYBR Green Mastermixs Kit (Vazyme, Nanjing, Jiangsu, China). U6 was used as an internal reference gene. The relative expression of mRNA was calculated by the $2^{-\triangle\triangle Ct}$ method. The primer sequences (5′-3′) are listed in Table 1.

The construction of miR-7031-5P overexpression and knockdown cell models

MC3T3-E1 cells in logarithmic growth period were seeded in a 24-well plate and cultured in serum-free α-MEM medium without dual antibodies to make the cell confluence reach 50%. 5 μL miR-7031-5P mimics and inhibitor were added into the 24-well plate at room temperature for 3-5 min. 1 μL Lipofectamine 2000 was subsequently added and cultured for 5 min at room temperature. After 24h of transfection, qRT-PCR was used to determine the transfection efficiency. The sequences of miR-7031-5P overexpression and knockdown were as follows: miR-7031-5P over-

Table 1. Primers used for qRT-PCR

Gene	Forward primer sequence	Reverse primer sequence
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MIR-7031-5P	GTGGGAGCAGCCTTGAAT	GTGCAGGGTCCGAGGT
OPN	CTATCCAGCCACCTTCACTTACA	TCGCCAGACAGACTCATCCA
COL1A1	TGCTCGTGGAAATGATGGTG	GGAGCACCATTGGCACCTTT
RUNX	TGGCTTACGGACTGAGGT	TTGGAACTCGCCTGACTG
Wnt7a	CTGGCCTTCCACTCTCAGA	AGTCGCGGGCTGTAAGTAG

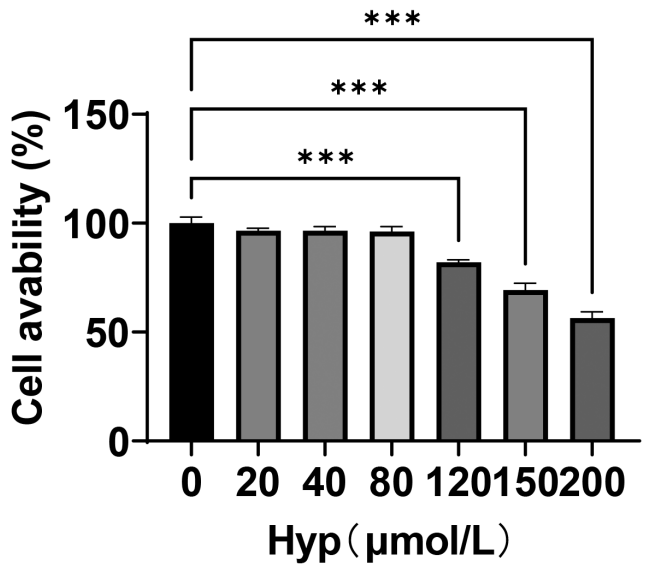

Fig. 1. The different concentrations of Hyp on MC3T3-E1 cell viability. ***P<0.001.

expression- ACATATAGGGTTCTTGCAGCTTGCC GAGAACTGTTCCCTCAGGTGGGAAGTTGTT GCAAGAGAAGGATGCTCTGTGCCTCCTTCCTCTG GAGAAACTACAGACAACCTGAGAGGCCTGAAG GGTGGGAGCAGCCTTGAATGACCAACCCTCTTG CCCTCTCCTAGAGCTCAAAGCCCAAGCTCCAAG AGGCCTGTTCCAGCATCCGGGAGGTCCGACCAT GTACACGCCTGGAGATGCCTGACAACCTCTACA CCTTTGTGTTGATTTTTT; miR-7031-5P knockdown-TCCCACCCTTCAGGCCTCTCAGGCTTCTCCCACC CTTCAGGCCTCTCAGGCTTCTCCCACCCTTCAGG CCTCTCAGGCTTCTCCCACCCTTCAGGCCTCTCA GGCTTCTCCCACCCTTCAGGCCTCTCAGGCTTCT CCCACCCTTCAGGCCTCTCAGGCTTCTTTTTT.

Alizarin red staining (ARS)

After MC3T3-E1 cells transfected with miR-7031-5P mimics and inhibitor were treated with Hyp, the culture medium with or without osteogenic induction fluid was added to culture cells for 14 or 21 days. 2 mL of 4% paraformaldehyde was added, and then discarded

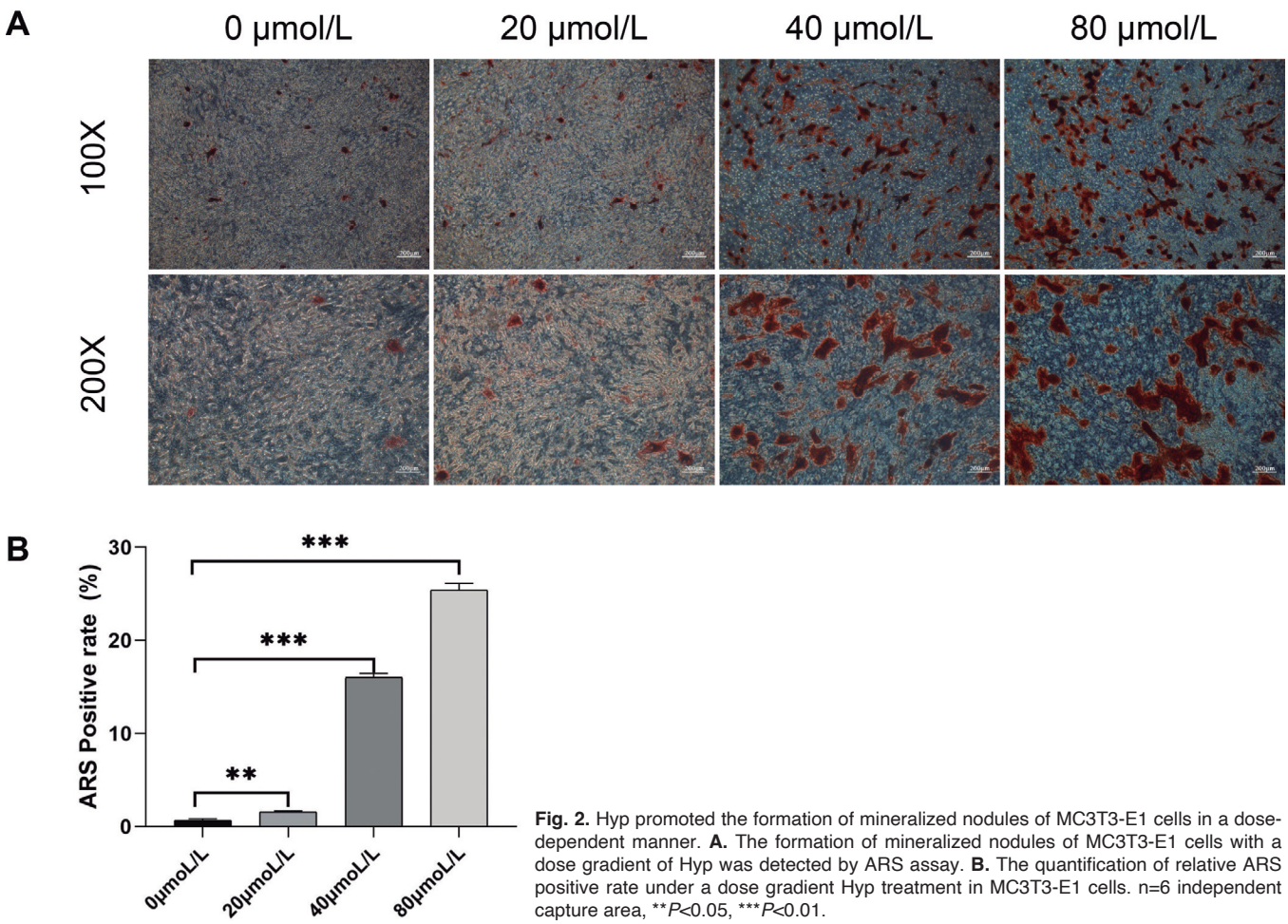

Fig. 2. Hyp promoted the formation of mineralized nodules of MC3T3-E1 cells in a dose-dependent manner. **A.** The formation of mineralized nodules of MC3T3-E1 cells with a dose gradient of Hyp was detected by ARS assay. **B.** The quantification of relative ARS positive rate under a dose gradient Hyp treatment in MC3T3-E1 cells. n=6 independent capture area, **P<0.05, ***P<0.01.

after 15 min. After that, 2 mL ARS staining solution was used to detect the formation of mineralized nodules of MC3T3-E1 cells in different groups. Finally, the cells were observed under an inverted microscope and pictures were taken.

ALP activity detection

After MC3T3-E1 cells transfected with miR-7031-5P mimics and inhibitor were treated with Hyp, the culture medium with or without osteogenic induction fluid was added to culture cells for 7 days. The ALP activity of MC3T3-E1 cells was detected using ALP activity detection kit (Jiancheng, Nanjing). After adding the chromogenic reagent to the cells, they were placed in an enzyme-linked immunoassay at 520 nm to measure the absorbance value. Finally, the ALP activity in MC3T3-E1 cells was calculated.

Dual-Luciferase report experiment

The Dual-luciferase report gene plasmid was transfected into MC3T3-E1 cells by Lipofectamine 2000. According to the manufacturer's protocol of Promega Dual-Luciferase system, a 96-well plate was prepared by adding diluted Passive Lysis Buffer, and MC3T3-E1 cells after Hyp treatment were harvested and centrifuged at 12000 rpm for 10 min to collect cell supernatant. After that, 100 μL Luciferase Assay Reagent II (LAR II) (Luciferase Assay Reagent, Progema) was added into the above 96-well plate. Then, 20 μL cell lysate was added, and the value of Firefly luciferase was measured and recorded, which was used as the internal reference value. Finally, 100 μL Stop and Glo® Reagent (Luciferase Assay Reagent, Progema) was added and the Renilla luciferase was recorded as the luminescence value of the reporter gene.

Western blot assay (WB)

After Hyp treatment, MC3T3-E1 cells were harvested to collect the total proteins. At the same time, 10% SDS-PAGE was used to segregate the total proteins and transferred into PVDF membranes followed by blocking

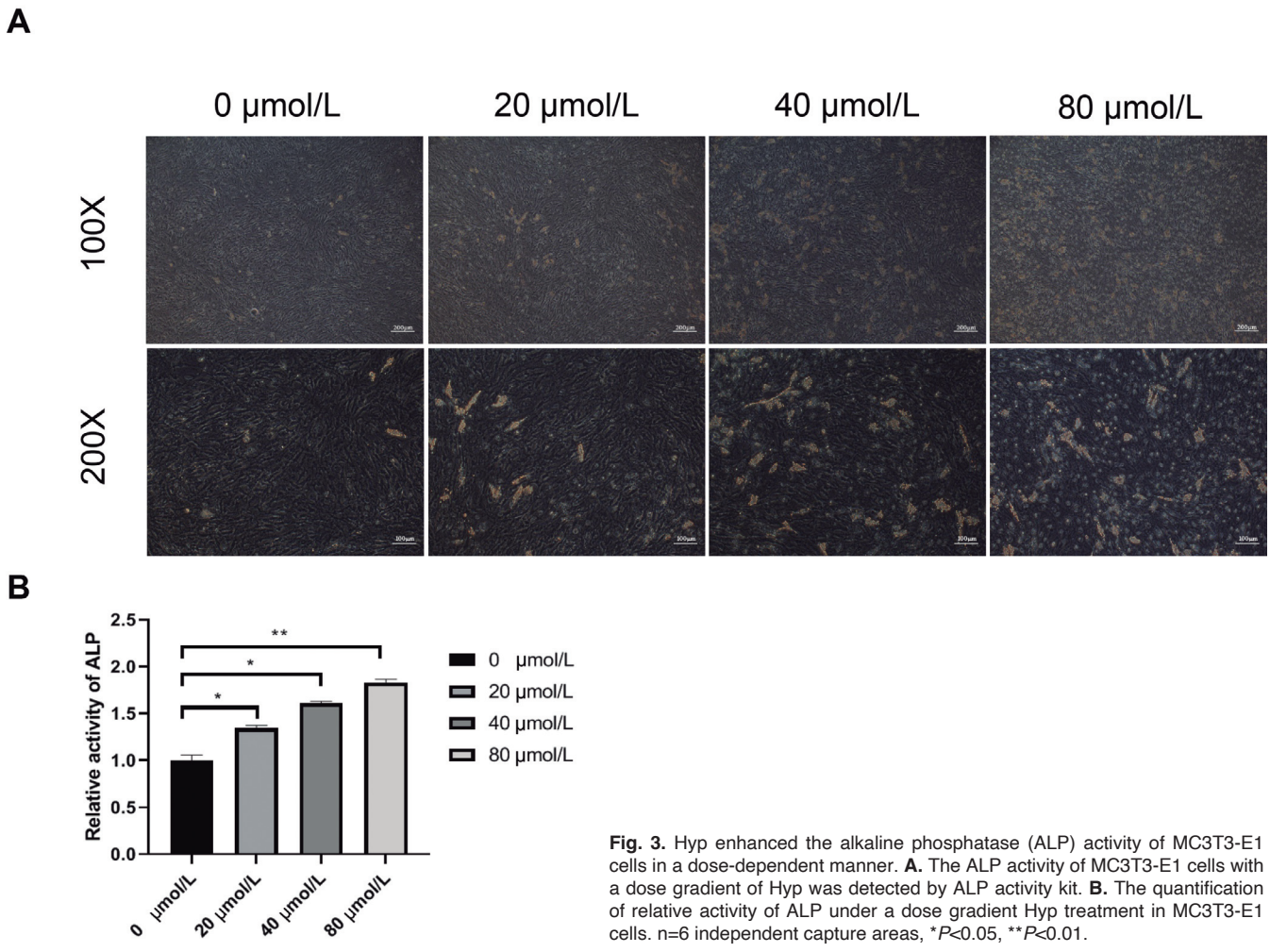

Fig. 3. Hyp enhanced the alkaline phosphatase (ALP) activity of MC3T3-E1 cells in a dose-dependent manner. **A.** The ALP activity of MC3T3-E1 cells with a dose gradient of Hyp was detected by ALP activity kit. **B.** The quantification of relative activity of ALP under a dose gradient Hyp treatment in MC3T3-E1 cells. n=6 independent capture areas, **P*<0.05, ***P*<0.01.

with TBST solution containing 5% skim milk at room temperature for 1h. Next, the membranes were incubated with primary and secondary antibodies and washed with TBST solution three times (10 min/time). Finally, the ECL+plusTM Western blotting system kit was used for color rendering and X-ray imaging was captured. The primary antibodies used in WB were as follows: OPN (1:1000, abcam, #ab11503), COL1A1 (1:1000, Beyotime, #AF1840), RUNX2 (1:1000, CST, #8486s) and GAPDH (1:3000, Bioworld, #AP0063). The secondary antibody was Goat Anti-Rabbit (1:3000, Beyotime, #A0208), Goat Anti-Mouse (1:3000, Beyotime, #A0216) and Donkey Anti-Goat (1:3000, Beyotime, #A0181).

Statistical analysis

All data were analyzed by using GraphPad Prism 8 (San Diego, CA, USA) and SPSS 19.0 (IBM, SPSS,

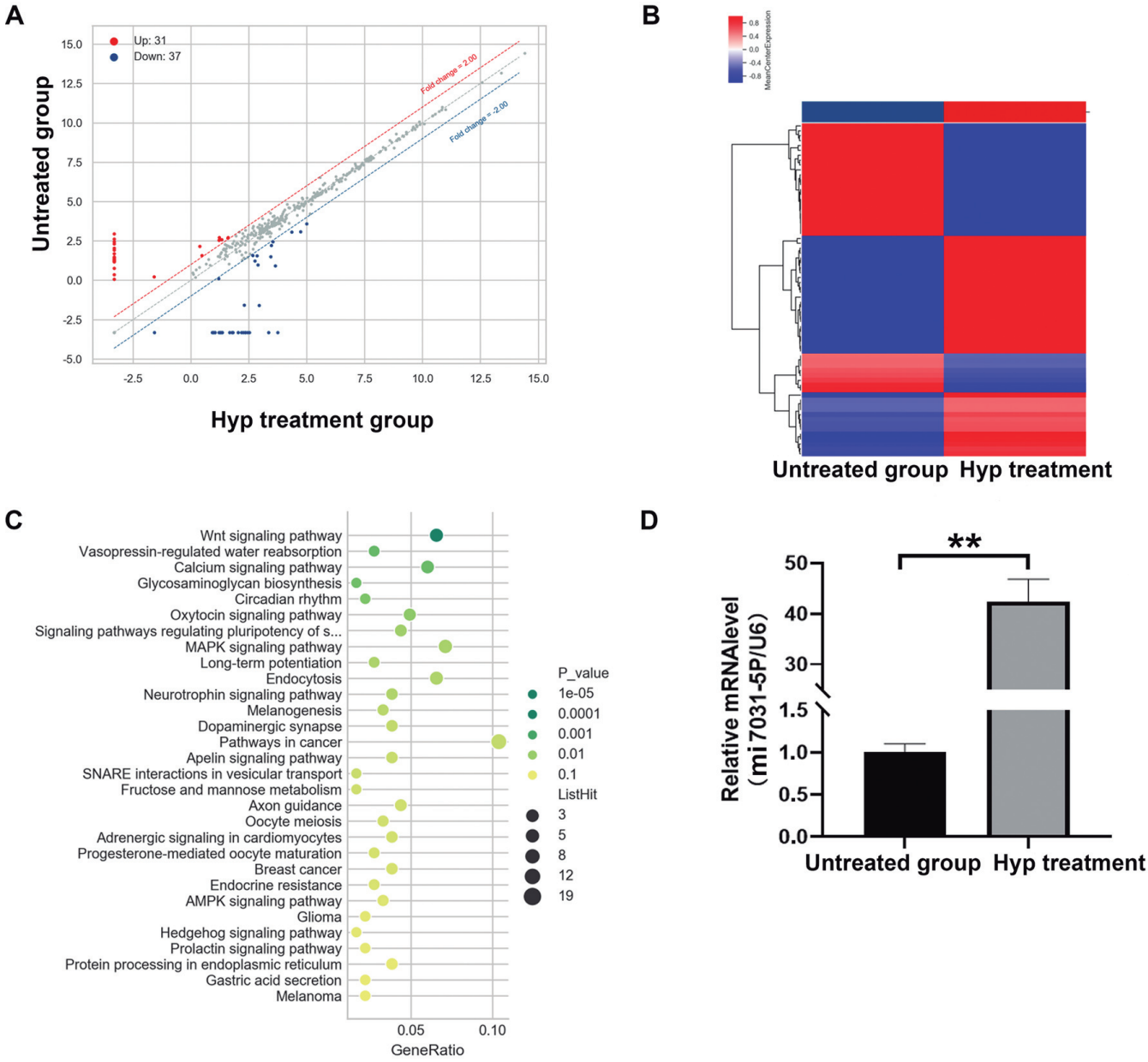

Fig. 4. Screening of differentially expressed miRNAs. **A.** The differentially expressed miRNAs were evaluated between Hyp treatment group and untreated group by scatter plots. **B.** The differentially expressed miRNAs were evaluated between Hyp treatment group and untreated group by cluster analysis. **C.** The pathways related to differentially expressed genes were evaluated by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. **D.** miR-7031-5P expression level in MC3T3-E1 cells after Hyp treatment was revealed by qRT-PCR. Results were presented as mean ± SD. ** $P<0.01$.

Chicago, IL, USA), and presented as the mean ± SD. One-way ANOVA was used to analyze the statistical significance. Pairwise comparison used Tukey's test. $P<0.05$ was considered to be significantly different. All the experiments were in triplicate.

Results

Screening of differentially expressed miRNAs

To explore Hyp regulated miRNA expression in osteoblast, the miRNA Microarray was performed in MC3T3-E1 cells from Hyp treatment groups (n=3) and untreated groups (n=3). The results identified that there were 37 up-regulated and 31 down-regulated miRNAs in Hyp treatment groups based on the threshold of absolute fold change (FC)≥2.0 and *FDR*<0.05, among which the top 5 up-regulated miRNAs with highest FC changes included miR-7031-5p, miR-7044-5p, miR-7033-5p, miR-7084-5p and miR-129-1-3p (Fig. 4A,B). Next, we used miRWalk/miRDB databases to predict the target gene relationship of miRNA-mRNA, again confirming that the differentially expressed miRNA was miR-7031-5p, and the target gene was Wnt7a. Next, combining with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, we found that most genes were enriched in the Wnt Signaling pathway, implying that it may be an action pathway of miR-7031-5p (Figs. 4C, 5). Subsequently, we detected miR-7031-5P mRNA expression level in MC3T3-E1 cells after Hyp treatment via qPCR, indicating markedly up-regulated miR-7031-5P ($P<0.01$, Fig. 4D). Collectively, these data illustrated that Hyp might promote osteogenic differentiation of MC3T3-E1 cells via up-regulating miR-7031-5P.

Hyp promoted the formation of mineralized nodules of MC3T3-E1 cells via miR-7031-5P overexpression

Hereafter, we infected miR-7031-5P mimics and inhibitor into MC3T3-E1 cells to build up miR-7031-5P overexpression and knockdown cell models. The results from qPCR manifested miR-7031-5P overexpression in the OE-miR-7031-5P group, but downregulation in the sh-miR-7031-5P group, implying both cell models were established well (Fig. 6A). We further tested the formation of mineralized nodules in MC3T3-E1 cell models by ARS staining. The data demonstrated that miR-7031-5P up-regulation in MC3T3-E1 cells was accompanied by an increase in mineralized nodules (Fig. 6B,C). Subsequently, the above knockdown cell models were treated by adding 80 μmol/L Hyp, and the representative pictures of ARS presented that the particles and distribution of mineralized nodules in sh-miR-7031-5P group were looser than those in the sh-NC group (Fig. 6D,E). Altogether, these experimental data suggested that Hyp promoted MC3T3-E1 cells to form mineralized nodules by up-regulating miR-7031-5P.

Hyp enhanced the alkaline phosphatase (ALP) activity of MC3T3-E1 cells by up-regulating miR-7031-5P

Apart from detecting the formation of mineralized nodules of MC3T3-E1 cells, the activity of ALP, a pre-osteoblastic marker protein, was measured through ALP activity detection kit. Consistently, miR-7031-5P up-regulation in MC3T3-E1 cells contributed to increase ALP activity (Fig. 7A,B). Besides, we noticed that miR-7031-5P knockdown in MC3T3-E1 cells appeared to reduce ALP activity, described in Fig. 7C,D. Therefore, the findings in this context were in agreement with the

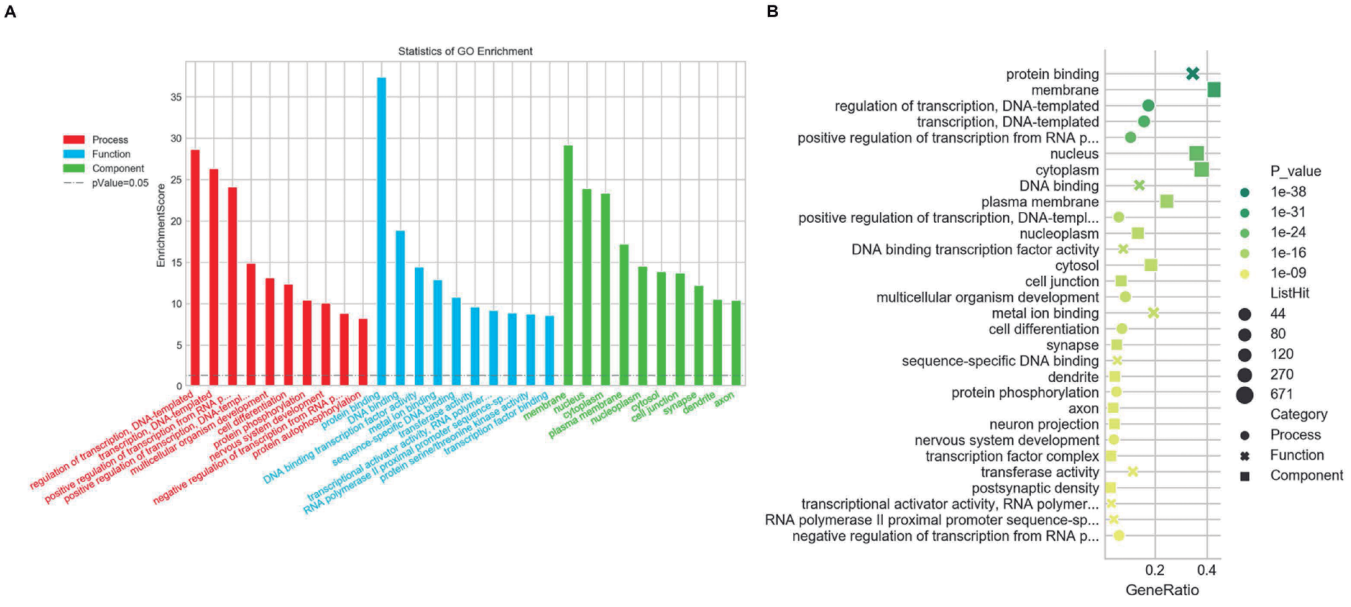

Fig. 5. The differentially expressed miRNAs were screened based on GO function classification **(A)** and GO enrichment **(B)**.

above studies, indicating that Hyp promoted ALP activity in MC3T3-E1 cells by up-regulating miR-7031-5P.

Hyp up-regulated OPN, COL1A1 and Runx2 expression in MC3T3-E1 cells via miR-7031-5P overexpression

In this section, we wanted to evaluate osteogenic differentiation-related protein levels in lentivirus infected MC3T3-E1 cells. We noticed that, from qPCR assay, OPN, COL1A1 and Runx2 mRNA expression was evidently increased in the OE-miR-7031-5P group compared with the OE-NC group, while decreased OPN, COL1A1 and Runx2 were noted in the sh-miR-7031-5P group (Fig. 8A). Finally, combining the published literature with bioinformatics online analysis (Targetscan), we again identified Wnt7a as the downstream target gene of miR-7031-5P promoting osteogenic differentiation of MC3T3-E1. Additional dual luciferase report experiments demonstrated that there was an interaction between miR-7031-5P and Wnt7a (Fig. 8B). Furthermore, we detected Wnt7a

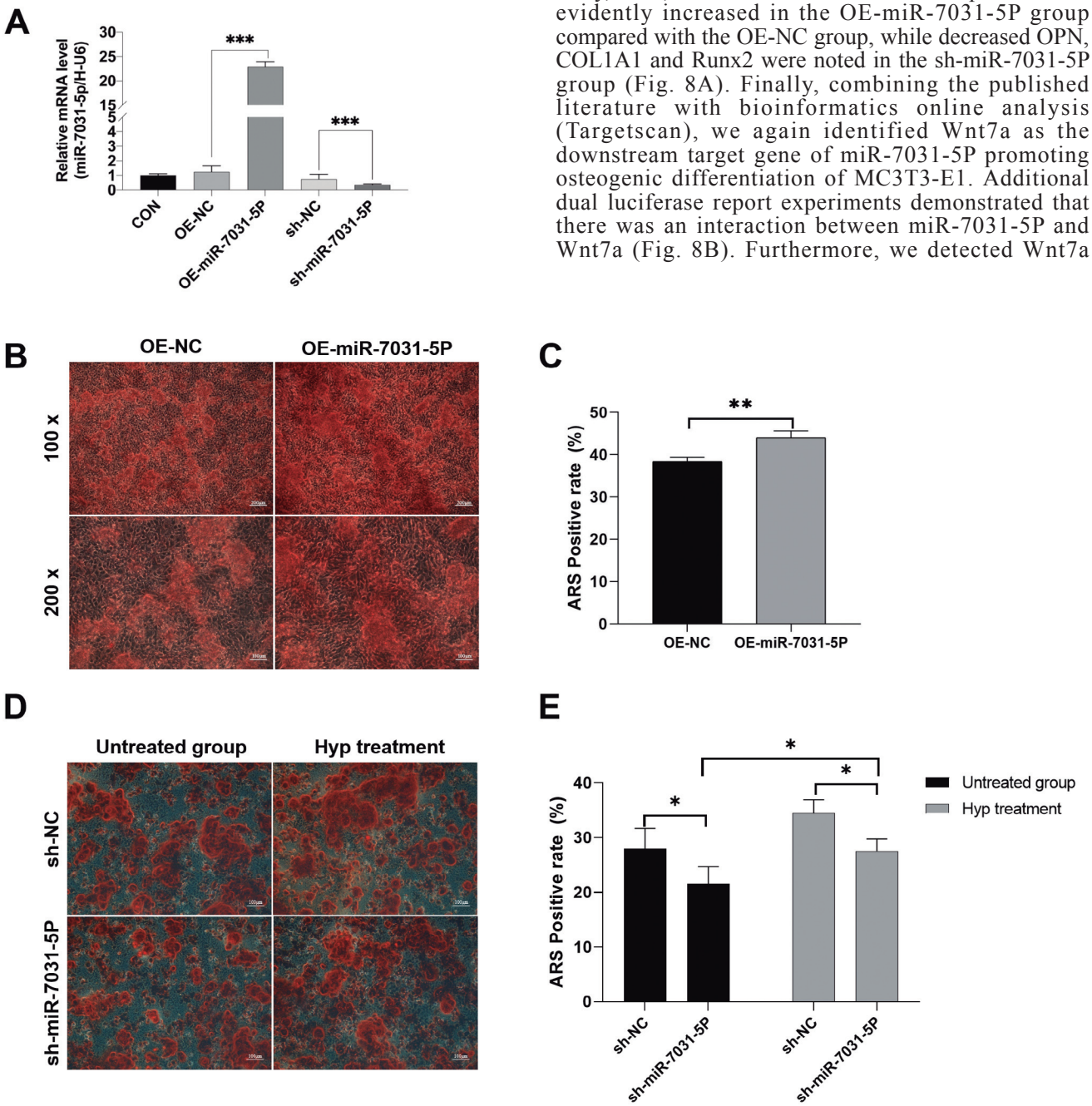

Fig. 6. Hyp promoted the formation of mineralized nodules of MC3T3-E1 cells via miR-7031-5P overexpression. **A.** The transfection efficiencies of miR-7031-5P mimics and inhibitor in MC3T3-E1 cells were evaluated through qRT-PCR. **B.** The formation of mineralized nodules of MC3T3-E1 cells with miR-7031-5P overexpression was detected by ARS assay. **C.** The quantification of relative ARS positive rate of MC3T3-E1 cells with miR-7031-5P overexpression was detected by ARS assay. $^{**}P<0.01$. **D.** The formation of mineralized nodules of MC3T3-E1 cells with miR-7031-5P down-regulation after Hyp treatment was detected by ARS assay. **E.** The quantification of relative ARS positive rate of MC3T3-E1 cells with miR-7031-5P down-regulation after Hyp treatment by ARS assay. $^{*}P<0.05$.

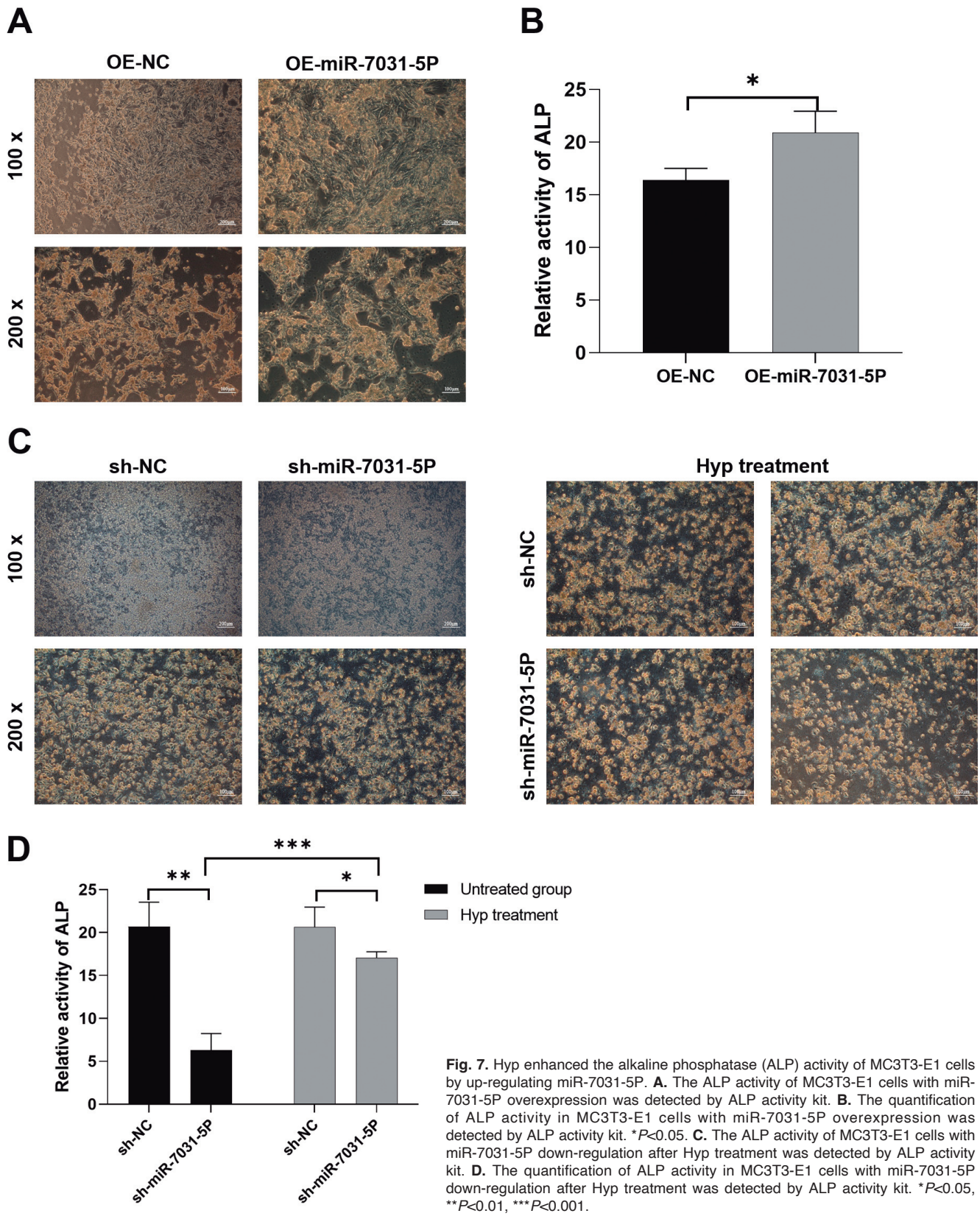

Fig. 7. Hyp enhanced the alkaline phosphatase (ALP) activity of MC3T3-E1 cells by up-regulating miR-7031-5P. **A.** The ALP activity of MC3T3-E1 cells with miR-7031-5P overexpression was detected by ALP activity kit. **B.** The quantification of ALP activity in MC3T3-E1 cells with miR-7031-5P overexpression was detected by ALP activity kit. **P*<0.05. **C.** The ALP activity of MC3T3-E1 cells with miR-7031-5P down-regulation after Hyp treatment was detected by ALP activity kit. **D.** The quantification of ALP activity in MC3T3-E1 cells with miR-7031-5P down-regulation after Hyp treatment was detected by ALP activity kit. **P*<0.05, ***P*<0.01, ****P*<0.001.

mRNA and protein levels in miR-7031-5P over-expression and knockdown MC3T3-E1 cells. As expected, elevated miR-7031-5P down-regulated Wnt7a mRNA and protein levels, while silencing miR-7031-5P up-regulated Wnt7a mRNA and protein levels (Fig. 8C,D). As per the results presented so far, we concluded that Hyp up-regulated miR-7031-5P to promote osteogenic differentiation of MC3T3-E1 cells by targeting Wnt7a.

Discussion

miRNAs are closely related to the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), and play an important regulatory role in the process of bone tissue metabolism (Long, 2011). A large number of studies have shown that a variety of intracellular and extracellular signal transmission is involved in the process of osteogenic differentiation, such as miRNA and signal pathways (Hankenson et al., 2015). For example, the level of miR-29b increases in the process of osteogenic differentiation, which can down-regulate the expression of COL4A2, COL5A3 and COL1A1, eventually promoting osteogenic differentiation (Li et al., 2009). An important cause of bone loss and bone defect diseases is that osteogenic differentiation is inhibited or weakened, which is in turn associated with the abnormal expression of miRNAs (Arfat et al., 2015; Hata and Kang, 2015). In this current study, 80 μmol·L-1 Hyp was used to treat MC3T3-E1 cells. The results indicated that miR-7031-5P expression level was markedly up-regulated in MC3T3-E1 cells after Hyp treatment. Besides, the ability of mineralized nodule formation and the ALP activity in MC3T3-E1 cells after Hyp treatment was significantly increased, suggesting that Hyp promoted the osteogenic

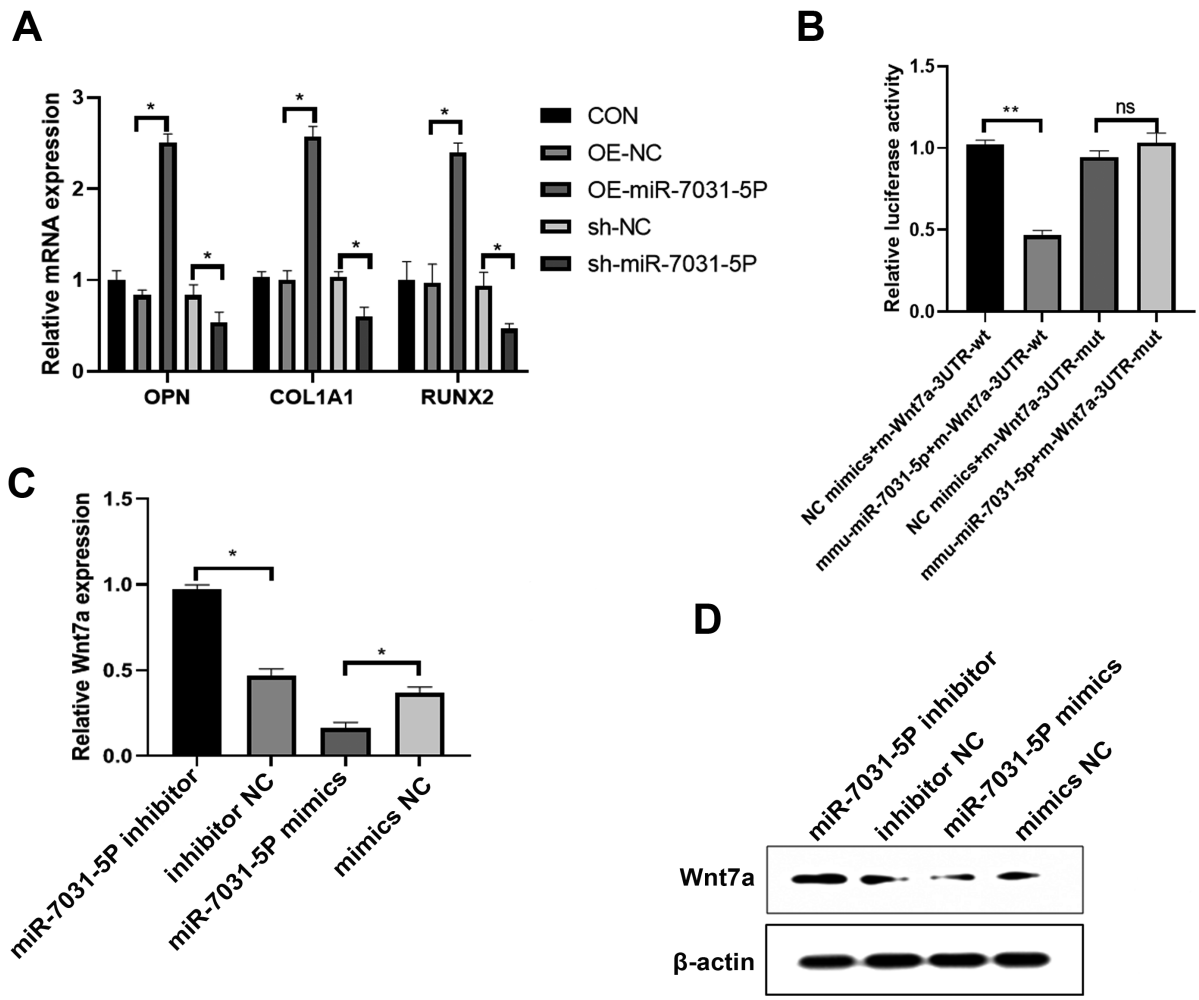

Fig. 8. Exploration of downstream mechanisms of miR-7031-5P regulating the osteogenic differentiation of MC3T3-E1 cells. **A.** Hyp up-regulated OPN, COL1A1 and Runx2Runx2 expression in MC3T3-E1 cells via miR-7031-5P overexpression **B.** The relationship between miR-7031-5P and Wnt7a was revealed by the dual-luciferase report experiments. **C, D.** Wnt7a mRNA and protein levels were determined in MC3T3-E1 cells with miR-7031-5P overexpression and down-regulation by qRT-PCR **(C)** and WB **(D)** assays. **P*<0.05, ***P*<0.01.

differentiation of MC3T3-E1 cells via up-regulating miR-7031-5P. More importantly, we found that Wnt7a was the potential downstream target gene of miR-7031-5P inhibiting osteogenic differentiation of MC3T3-E1.

Growing evidence has confirmed that Wnt plays a key role in the process of osteogenic differentiation of human mesenchymal stem cells (de Boer et al., 2004). The canonical Wnt-βcatenin signaling pathway seems to explain most of the effects of Wnt signaling in the skeleton (Canalis, 2013). The canonical Wnt signaling pathway is mediated by β-catenin (Maeda et al., 2019). The non-canonical signaling pathway is a generic term used for pathways not mediated by β-catenin. Ligands such as Wnt5a and Wnt11 activate the Wnt/Ca^{2+} and Wnt/PCP pathways without the induction of intracellular β-catenin accumulation. In the Wnt/Ca^{2+} pathway, the increased intracellular concentration of Ca^{2+} activates calmodulin-dependent protein kinase II (CaMK II) and protein kinase C (PKC). In the Wnt/planar cell polarity (PCP) pathway, small G proteins such as Rac and Rho are activated to enhance cell motility as well as determining the direction and localization of cilia (Baron and Kneissel, 2013; Karner and Long, 2017). Noncanonical Wnt signaling also regulates chondrocyte differentiation (Bradley and Drissi, 2010). Crosstalk of prototypical canonical (Wnt3a), and non-canonical (Wnt5a) Wnts leads to functional antagonism during osteogenic differentiation (Baksh et al., 2007). Furthermore, β-catenin plays a central role in regulating osteogenic differentiation of MSCs in inflammatory microenvironments through both the canonical Wnt/β-catenin pathway and the noncanonical Wnt/Ca^{2+} pathway (Liu et al., 2011). WNT7A forms a receptor complex with LRP6 and FZD5 to activate the canonical WNT signaling pathway (Lan et al., 2019). In stem cells, Wnt7a was revealed to promote differentiation of mesenchymal stem cells into bone (Yang et al., 2021) and bone mesenchymal stem cells osteogenic differentiation (Li et al., 2015). Msx2 exerts bone anabolism in part by reducing Dkk1 expression and enhancing Wnt7a and Wnt7b in primary mouse bone cells (Cheng et al., 2008). However, on the other hand, the pro-inflammatory cytokine IL-1β has been reported to induce Wnt5a and Wnt7a expression in primary culture articular chondrocytes. It was also revealed that Wnt7a induces dedifferentiation and inhibits NO-induced apoptosis of primary culture articular chondrocytes (Li et al., 2015). So the function of Wnt7a would be different in different cell types. In the present study, we identified Wnt7a as a potential target gene of miR-7031-5P. Hyp upregulated miR-7031-5P expression in MC3T3-E1 cells. We speculated that Hyp promoted osteogenic differentiation through miR-7031-5p/Wnt7a program. However, miR-7031-5p has so many potential target genes and Wnt7a was only a middle regulator that could regulate downstream genes of osteogenesis. So we still didn't know the exact intricate regulatory network involved in Hyp mediated osteogenesis. That should be researched more deeply in the future.

Many studies have shown that miRNAs are important epigenetic regulators of Wnt signaling genes during bone differentiation (Amjadi-Moheb and Akhavan-Niaki, 2019). In detail, in the process of osteogenic differentiation mediated by the Wnt signaling pathway, miRNAs bind to target genes to regulate Runx2 expression and ALP activity, thereby regulating the Wnt signaling pathway and osteogenic differentiation. For example, Zhang et al. found that miR-355-5p down-regulated DKK1, enhanced the Wnt signaling pathway, increased the expression levels of osteogenic differentiation-related factors such as Runx2, and promoted the osteogenic differentiation of BMSCs (Zhang et al., 2011). Su et al. demonstrated that miR-26a promoted osteogenic differentiation of BMSCs through the Wnt/β-catenin signaling pathway (Su et al., 2015). MiR-29a-3p had been found to inhibit proliferation and osteogenic differentiation of human BMSCs via targeting FOXO3 and repressing Wnt/β-catenin signaling pathway signaling in steroid-associated osteonecrosis (Wang et al., 2022).

In conclusion, the results of this study showed that Hyp played an important role in osteogenic differentiation of MC3T3-E1, mainly through miR-7031-5P overexpression to promote osteogenic differentiation, which may provide a promising therapy option for bone-related diseases.

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Author contributions. Dongchen Qian, Yueyue Chen, and Yixin Chen designed this research. Xusheng Qiu and Yueyue Chen performed the experiments. Baohua Zhu, Quanhong Yang, and Yifeng Yan conducted the data processing and analysis. Dongchen Qian completed the manuscript which was reviewed by Yixin Chen. All the authors have confirmed the submission of this manuscript.

Conflict of interests. The authors declare that they have no conflict of interest.

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