

# MicroRNA-4327 regulates TGF- $\beta$ 1 stimulation of matrix metalloproteinase-13 expression via CREB-binding protein-mediated Runx2 acetylation in human osteoblasts

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**Abstract.** Transforming growth factor beta 1 (TGF- $\beta$ 1), a multifunctional cytokine, induces the expression of bone remodeling gene matrix metalloproteinase-13 (MMP-13). CREB-binding protein (CBP), a co-activator and runt-related transcription factor 2 (Runx2), a bone transcription factor, play critical roles in regulating bone-remodeling genes. Recent advances in non-coding RNAs have revealed the significance of microRNAs (miRNAs) and their target genes in bone physiology. The present study hypothesized that TGF- $\beta$ 1 stimulated MMP-13 expression by downregulating CBP-targeting miRNAs and activating CBP-mediated Runx2 acetylation in human osteoblastic cells. TGF- $\beta$ 1-downregulated miRNAs that potentially target CBP were identified. Among these miRNAs, TGF- $\beta$ 1 significantly downregulated miR-4327 in these cells. TGF- $\beta$ 1 stimulated CBP, acetylated Runx2 and MMP-13 protein expression levels in human osteoblastic cells

and this effect was decreased by overexpressing miR-4327 in these cells. In human osteoblastic cells, miR-4327 was found to directly bind to the 3'-untranslated region of CBP using a dual-luciferase gene reporter assay. Thus, the present study indicated that the TGF- $\beta$ 1/miR-4327/CBP/Runx2 plays a key role in MMP-13 expression, suggesting the clinical relevance of this axis for treating bone-related disorders.

## Introduction

The skeletal system, which consists of bone and other tissues, is a living and metabolically active system that supports and protects various organs. Bones play a vital role in mineral homeostasis and hematopoiesis, with recent findings pointing to the function of bones as endocrine organs (1,2). The bone undergoes remodeling throughout life to ensure proper function and adaptation to various conditions. This process is aided by a transient anatomical structure called the basic multicellular unit, primarily composed of osteocytes, osteoblasts and osteoclasts (3,4). Bone remodeling is a multi-step process which includes osteoblastic bone formation and resorption. It often involves the activation of several signaling pathways that include fibroblast growth factors, bone morphogenetic proteins and transforming growth factor beta (TGF- $\beta$ ). Among these signaling pathways, TGF- $\beta$  is critically involved in bone remodeling (5-7). TGF- $\beta$  is a versatile cytokine that plays several roles in physiological and pathological conditions of the bone. Changes in the bone microenvironment trigger the release of active TGF- $\beta$  by proteolytic cleavage of latent peptides (8). Among the various isoforms of TGF- $\beta$ , TGF- $\beta$ 1 plays a role in bone differentiation in bone marrow mesenchymal stem cells (BMMSCs) (9). TGF- $\beta$ 1 plays a contrasting role in BMMSCs; at low concentrations, it promotes osteogenic differentiation, whereas at high concentrations, it inhibits osteogenic differentiation (10).

Several signaling pathways stimulate bone transcription factor runt-related transcription factor 2 (Runx2) that induces osteoblast differentiation (11-13). Runx2 expression or activity can be positively or negatively regulated by various co-activators or co-repressors, respectively, via post-translational modifications (14-17). Transcriptional co-activator CREB-binding

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**Abbreviations:** CBP, CREB-binding protein; cDNA, complementary DNA; HAT, histone acetyltransferase; IgG, immunoglobulin G; miRNA, microRNA; MMP-13, matrix metalloproteinase-13; MRE, microRNA responsive element; nc-miRNA, negative control miRNA; PCAF, p300/CBP-associated factor; PTH, parathyroid hormone; Runx2, runt-related transcription factor 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF- $\beta$ 1, transforming growth factor-beta 1; UTR, untranslated region; siRNA, small interfering RNA

**Key words:** transforming growth factor- $\beta$ 1, miRNA-4327, CREB-binding protein, runt-related transcription factor 2, matrix metalloproteinase-13

protein (CBP) has intrinsic histone acetyltransferase activity (HAT). The HAT domain functions as an acetyltransferase and transfers the acetyl group from acetyl-CoA to the target site. The HAT activity of p300/CBP is essential for activating several genes in the bone (18-21). CBP and p300 are closely related coactivators but differ in their substrate specificity. CBP is more selective for H3K18, while p300 shows higher specificity for H4K16 under certain conditions (22,23). Matrix metalloproteinase-13 (MMP-13), a proteolytic enzyme involved in collagen degradation (a significant component of the extracellular matrix), is an important factor that couples bone formation and resorption and is essential for bone development and healing (24-26). MMP-13 overexpression in bone leads to excessive collagen degradation, contributing to conditions such as osteoarthritis and impaired bone remodeling. Downregulation of MMP-13 can lead to delayed bone healing and impaired matrix turnover, affecting normal skeletal development. Proper regulation of MMP-13 expression is essential for maintaining bone homeostasis (27,28). TGF- $\beta$ 1 stimulates MMP-13 expression in osteoblasts, which requires p300-mediated Runx2 acetylation (15,29,30). The CBP/p300 co-activator family is required for MMP-13 expression in osteoblasts (12,13,31).

Non-coding RNAs (ncRNAs), including short microRNAs (miRNAs) and long non-coding RNAs, such as linear long ncRNAs and circular RNAs, are essential in bone physiology and pathology (32-35). miRNAs are 18-25-nucleotide long and target and regulate gene expression post-transcriptionally (36). miRNAs play roles in various bone biological functions, including proliferation and differentiation of cells (37,38).

The present study assessed the effect of TGF- $\beta$ 1 on CBP expression and its consequent effect on MMP-13 expression in human osteoblastic cells. It aimed to uncover miRNAs that putatively target CBP. The functional role of miR-4327 and the molecular mechanism of MMP-13 expression via miR-4327 under TGF- $\beta$ 1-stimulation were also determined.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM; cat. no. 11965-092), penicillin-streptomycin-amphotericin B (cat. no. 15240-062) and cell culturing reagents were procured from Lonza Group Ltd. Fetal bovine serum (FBS; cat. no. 10270-106) was sourced from Gibco (Thermo Fisher Scientific, Inc.). Human osteoblastic osteosarcoma cells (MG-63) and human bone marrow stromal cells (HS-5) were obtained from the National Center for Cell Science (Pune, India) and the American Type Culture Collection, respectively. TGF- $\beta$ 1 was obtained from R&D Systems, Inc. Antibodies against CBP (cat. no. 7389; 1:1,000), acetylated-lysine (cat. no. 9441; 1:1,000) and  $\alpha$ -Tubulin (cat. no. 2125; 1:1,000) were acquired from Cell Signaling Technology, Inc., and antibodies against Runx2 (cat. no. sc-390715; 1:100) and MMP-13 (cat. no. 18165-1-AP; 1:3,000) were purchased from Cell Signaling Technology, Inc., and Proteintech Group, Inc., respectively. Scrambled control siRNA (cat. no. Sc-37007) and CBP siRNA (cat. no. Sc-29244) were purchased from Santa Cruz Biotechnology, Inc. miR-4327 mimic (GeneGlobe ID: YM00470747) was purchased from Qiagen GmbH.

Table I. List of precursor miRNA primers used in quantitative PCR.

| Name of miRNA     | F/R | Primer sequence (5'-3')     |
|-------------------|-----|-----------------------------|
| Hsa-miR-600       | F   | CGTGCTGTGGCTCCAGCTTC        |
|                   | R   | GGCTCTTGTCTGTAAGTAACT       |
| Hsa-miR-6083      | F   | AAGGGAGCAGGAGCATCGT         |
|                   | R   | TAGGAAGCCCACAGCCTCT         |
| Hsa-miR-7-1-3p    | F   | TTGGCCTAGTTCTGTGTGG         |
|                   | R   | CAGACTGTGATTGTGTGTCG        |
| Hsa-miR-1185-1-3p | F   | GGTACTTGAAGAGAGGTACC        |
|                   | R   | GCAAATAAGAGTCTCCCCCT        |
| Hsa-miR-4327      | F   | GTAGGCTTGCATGGGGGA          |
|                   | R   | TAAAGGCTTGATGAGAACTCC       |
| Hsa-miR-3924      | F   | TAAATGAAAAAGTAGTAGTC        |
|                   | R   | TAAACAAAAAAGTAGCAGTC        |
| Hsa-miR-3133      | F   | CAGAAATTGTAAAGAACTCTT       |
|                   | R   | CAGAATATATAAAGAACTC<br>TTAA |
| Hsa-miR-4264      | F   | AAAGCTGGATACTCAGTCATG       |
|                   | R   | CTATGCAGTCTTACCCAGTAC       |
| U6                | F   | CTCGCTTCGGCAGCACA           |
|                   | R   | AACGCTTCACGAATTTGCGT        |

miRNAs/miR, microRNAs; F, forward; R, reverse

**Cell culture.** HS-5 cells were differentiated into primary osteoblasts by culturing in DMEM along with 10% FBS, 50  $\mu$ M ascorbic acid, 10 nM  $\beta$ -glycerophosphate and 0.1  $\mu$ M dexamethasone for seven days. DMEM containing 10% FBS was used to maintain MG-63 cells. Penicillin-streptomycin-amphotericin B was used in the culture media and cells were incubated in a humidified chamber with 5% CO<sub>2</sub> at 37°C. In the present study, TGF- $\beta$ 1 was used at 5 ng/ml.

**In silico analyses to determine miRNAs targeting the 3'-untranslated region (UTR) of CBP.** miRNAs that target CBP 3'-UTR were identified based on miRNA-target prediction databases. Human miRNA sequences were retrieved from miRDB (<https://mirdb.org/>). Mature miRNA sequences were analyzed and binding sites were predicted using STarMir (<https://sfold.wadsworth.org/cgi-bin/starmirWeb.pl>). STarMir provides a logistic probability score (LogitProb), signifying the confidence level of binding between miRNA and target mRNA, based on defined interaction parameters such as site type,  $\Delta G_{\text{hybrid}} \leq -14$  kcal mol<sup>-1</sup> and  $\Delta G_{\text{total}} \leq -10$  kcal mol<sup>-1</sup> (39,40). The highly probable miRNAs were classified using miRmap (<https://mirmap.ezlab.org/>). The predicted miRNAs were loaded into Venny v.2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>) to identify the miRNAs common among the three databases. The common miRNAs were shortlisted based on the LogitProb score (cut-off <0.75) and miRDB score (cut-off <50). Finally, the validated miRNAs were eliminated using TarBase (<https://dianalab.e-ce.uth.gr/tarbasev9>) and a web-based search (<https://scholar.google.com>) for obtaining unvalidated miRNAs.

Table II. The oligonucleotides with the wild and mutant 3'-untranslated region of CBP used in luciferase reporter assay.

| Gene        | Oligonucleotides (5'-3')                                     |
|-------------|--------------------------------------------------------------|
| CBP (1) W F | AAACGAAGCGGCCGCTTCAGAACTGATTCTGAAATAATGCAAGCTTATAATT         |
| CBP (1) W R | CTAGAAATTATAAGCTTGCATTATTTTCAGGAATCAGTTCTGAAGCGGCCGCTTCGTTT  |
| CBP (1) M F | AAACGAAGCGGCCGCTTCAGAACTGATTCTGAAATAATGAGAGCTTATAATT         |
| CBP (1) M R | CTAGAAATTATAAGCTTCTCATTATTTTCAGGAATCAGTTCTGAAGCGGCCGCTTCGTTT |
| CBP (2) W F | AAACGGAGCGGCCGCTCTAGTGTAATCATGCAAGCGCTCTAAT                  |
| CBP (2) W R | CTAGATTAGAGCGCTTGCATGATTACACTAGAGCGGCCGCTCCGTTT              |
| CBP (2) M F | AAACGGAGCGGCCGCTCTAGTGTAATCATGACAGCGCTCTAAT                  |
| CBP (2) M R | CTAGATTAGAGCGCTGTCATGATTACACTAGAGCGGCCGCTCCGTTT              |

CBP, CREB-binding protein; W, wild; M, mutant; F, forward; R, reverse.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using RNAiso Plus (Takara Bio, Inc.) from cells at 80% confluence. Complementary DNA (cDNA) was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.). qPCR was performed using SYBR Green (Takara Bio, Inc.) with primers for precursor miRNAs. Expression patterns of mature miRNAs were analyzed using a miRCURY LNA kit (Qiagen GmbH) with mature miRNA primers. The  $\Delta\Delta C_q$  method was used to determine the relative expression of precursor and mature miRNAs (13,30). *U6* served as an endogenous control. Table I shows the primers used to determine precursor miRNA expression in human osteoblasts. The PCR protocol consisted of denaturation at 95°C for 5 sec, followed by annealing and extension at 60°C for 34 sec, for 40 cycles. All the experiments were performed in triplicate according to the manufacturers' protocols.

**Transient transfection.** MG-63 cells (60-70% confluence) were transiently transfected with scrambled control (30 nM) or small interfering (si)RNA for CBP (30 nM) or negative control (50 nM) or miR-4327 mimic (50 nM) using X-tremeGene transfection reagent (Roche Diagnostics) or Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), as previously described (26). After 24 h of transfection at 37°C, cells were immediately left untreated (control) or subjected to TGF- $\beta$ 1 treatment. Whole-cell lysates were collected for co-immunoprecipitation and western blot analyses and total RNA was used for RT-qPCR analysis.

**Immunoprecipitation.** MG-63 cells were washed with 1X phosphate-buffered saline and lysed with immunoprecipitation lysis buffer [25 mM Tris (pH 8.0), 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid, 150 mM NaCl and protease/phosphatase inhibitors] for 10 min at 4°C. Subsequently, the whole-cell lysate was centrifuged at 12,000 x g for 10 min at 4°C. The collected supernatant (1 ml/reaction) was incubated at 4°C for overnight with 10  $\mu$ l of antibodies against immunoglobulin G (IgG; cat. no. Sc-2025) or Runx2 (cat. no. sc-390715) purchased from Santa Cruz Biotechnology, Inc. The immune complex was pulled down using protein A/G magnetic beads (Bio-Rad Laboratories, Inc.), with magnetic stacker according to the manufacturer's instructions. 1X Phosphate-buffered saline (HiMedia

Laboratories Pvt. Ltd.) was used for washing. Eluted proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17,41).

**Western blot analysis.** Protein samples were extracted using 1X radioimmunoprecipitation assay buffer (Bio Basic, Inc.) with protease and phosphatase inhibitors (MedChemExpress). Protein concentration was determined by the Bradford assay. Protein (50  $\mu$ g) was loaded into each lane and separated using SDS-PAGE (8% gel) before being transferred onto polyvinylidene difluoride membranes. Membranes were then blocked with 5% (w/v) bovine serum albumin (Sisco Research Laboratories Pvt Ltd.) for 1 h at room temperature and washed with Tris-buffered saline containing 0.1% Tween 20. The membranes were incubated with primary antibodies (1:1,000) against CBP, MMP-13, acetylated-lysine, or Runx2 overnight at 4°C.  $\alpha$ -Tubulin was used as an endogenous control. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) 1 h at room temperature, and immunoreactive bands were visualized using an Enhanced Chemiluminescence Substrate (Takara Bio, Inc.). Image Lab 6.1 (Bio-Rad Laboratories, Inc.) was used to quantify and observe band intensities (12).

**Dual-luciferase gene reporter assay.** A dual-luciferase gene reporter assay was performed as previously described (42,43). The forward and reverse primers containing the wild-type (W) or mutant (M) miRNA response elements (MREs) of the 3'-UTR of CBP were synthesized by Eurofins Genomics LLC (Table II) and cloned into an expression vector pmirGLO (Promega, Madison, WI, USA). Negative control miRNA (nc-miRNA) or miR-4327 mimics were transiently co-transfected into MG-63 cells along with the W or M constructs of CBP 3'-UTR using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h of transfection the lysates were collected and luciferase assay was performed using DLR™ Assay System (Promega Corp.). The data were normalized using *Renilla* luciferase activity. The ratio of Firefly luciferase activities to *Renilla* luciferase was calculated to determine the relative luciferase activity.

**Statistical analysis.** All experiments were carried out using biological triplicate and subjected to one-way analysis of

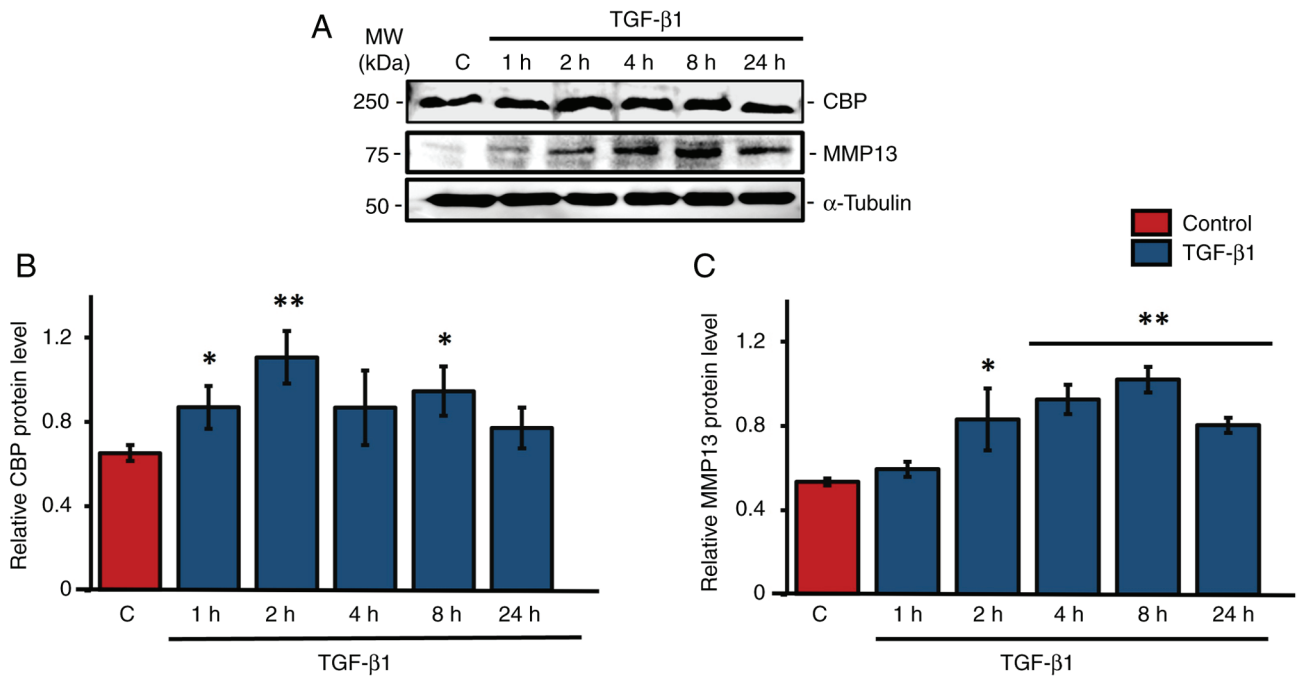


Figure 1. TGF- $\beta$ 1 treatment stimulates the expression of CBP and MMP-13 in human primary osteoblasts. (A) HS-5 cells were treated with 5 ng/ml TGF- $\beta$ 1 for 1, 2, 4, 8, or 24 h or left untreated (control). Whole-cell lysates were analyzed by western blotting using antibodies against CBP, MMP-13, or  $\alpha$ -Tubulin.  $\alpha$ -Tubulin expression was used for normalization. Relative expression of (B) CBP and (C) MMP-13 in HS-5 cells. \* $P$ <0.05; \*\* $P$ <0.01 vs. control ( $n$ =3). TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; CBP, CREB-binding protein; MMP-13, matrix metalloproteinase-13.

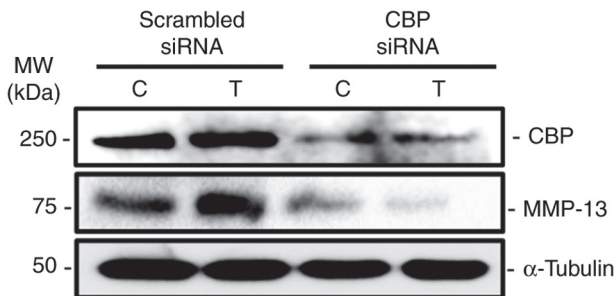


Figure 2. Knockdown of CBP decreased TGF- $\beta$ 1-stimulated MMP-13 expression in human osteoblasts. Scrambled siRNA or CBP siRNA (30 nM) was transiently transfected into MG-63 cells for 24 h. Cells were treated with 5 ng/ml TGF- $\beta$ 1 or left untreated for 8 h. Western blot analysis of the whole-cell lysates was performed using antibodies against CBP, MMP-13, or  $\alpha$ -Tubulin.  $\alpha$ -Tubulin expression was used for normalization. CBP, CREB-binding protein; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; MMP-13, matrix metalloproteinase-13; si, small interfering.

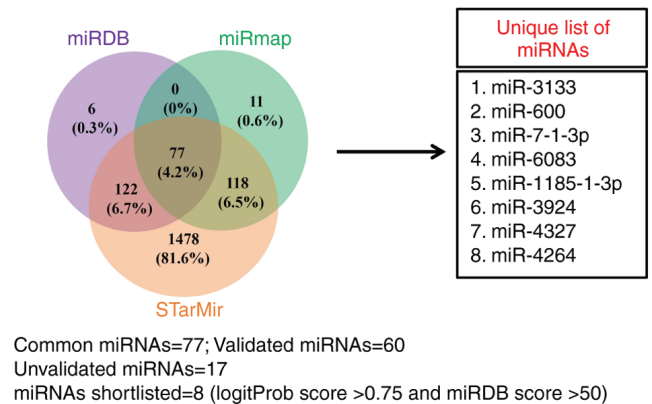


Figure 3. Identification of miRNAs that putatively target human CREB-binding protein, using miRDB, miRmap and STarMir. miRNAs, microRNAs.

variance (ANOVA) using Statistics Kingdom (<https://www.statskingdom.com/180Anovalway.html>) to verify statistical significance. Tukey's post hoc analysis was conducted to confirm the significance.  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**TGF- $\beta$ 1 stimulates CBP and MMP-13 expression in human primary osteoblastic cells.** Western blot analysis was used primarily to check the expression patterns of CBP and MMP13 under TGF- $\beta$ 1 treatment in human primary osteoblasts cells. Results showed a significant upregulation of CBP levels at 1, 2

and 8 h following TGF- $\beta$ 1 treatment compared with the control group, with the maximum expression at 2 h (Fig. 1A and B). Tukey's post hoc analysis indicated that the 2 h TGF- $\beta$ 1 treatment group showed a statistically significant difference in CBP expression compared with the control group. There was also an upregulation of CBP at 4 h of TGF- $\beta$ 1 treatment, but it was not significant. At 2, 4, 8 and 24 h after TGF- $\beta$ 1 treatment, the level of MMP-13 increased significantly. The highest level of MMP-13 expression was seen at 8 h after TGF- $\beta$ 1 treatment in HS-5 cells (Fig. 1A and C).

**CBP knockdown reduces TGF- $\beta$ 1-stimulated MMP-13 expression in human osteoblasts.** Since TGF- $\beta$ 1 treatment increased the expression of CBP and MMP-13 in human

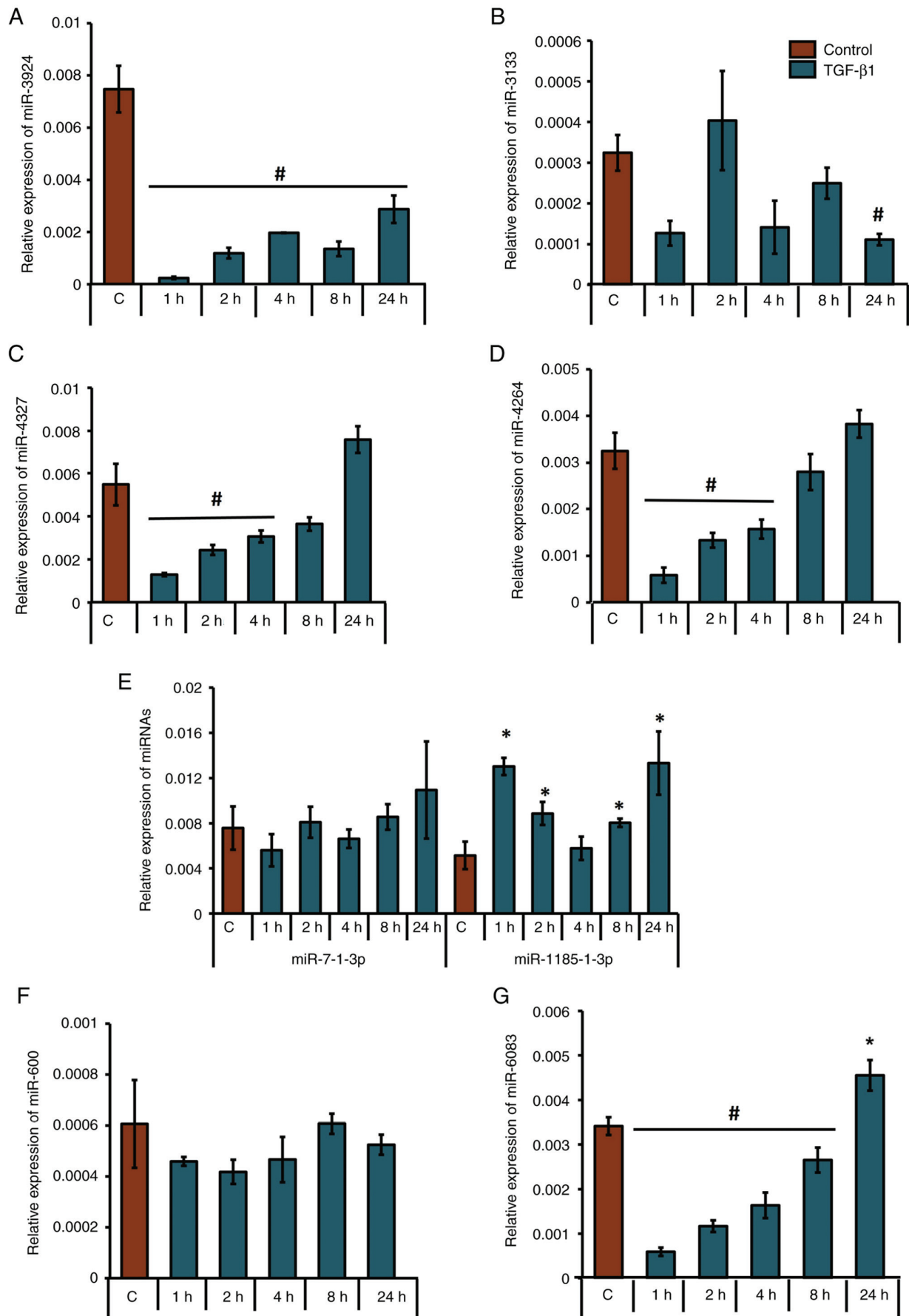


Figure 4. Differential expression patterns of precursor miRNAs in human osteoblasts upon TGF- $\beta$ 1 treatment. MG-63 cells were either treated with 5 ng/ml TGF- $\beta$ 1 for 1, 2, 4, 8, or 24 h or left untreated. Relative expression patterns of (A) miR-3924, (B) miR-3133, (C) miR-4327, (D) miR-4264, (E) miR-7-1-3p; miR-1185-1-3p, (F) miR-600 and (G) miR-6083 were analyzed using RT-qPCR. \* $P$ <0.05; # $P$ <0.05 for TGF- $\beta$ 1 treatment vs. respective control groups ( $n$ =3). miRNAs/miR, microRNAs; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

primary osteoblasts, the functional role of CBP was assessed in TGF- $\beta$ 1-stimulated MMP-13 expression in osteoblastic cells. The results revealed that CBP knockdown decreased both CBP and MMP-13 protein levels when compared with scrambled control (Fig. 2).

*Identification of TGF- $\beta$ 1-downregulates miRNAs that putatively target the 3'-UTR of CBP.* Based on the results from Figs. 1 and 2, CBP is necessary for TGF- $\beta$ 1 to stimulate MMP-13 expression in human osteoblasts; it was next investigated whether miRNAs play a role in regulating CBP expression induced by TGF- $\beta$ 1. Through *in silico* analysis, miRNAs that potentially target the 3'-UTR of CBP were obtained. A total of 77 miRNAs were identified, from which 17 were unvalidated. After scrutinizing the LogitProb score ( $>0.75$ ) and miRDB score ( $>50$ ), eight unvalidated miRNAs were shortlisted for further studies (Fig. 3).

*TGF- $\beta$ 1-downregulates miRNAs that putatively target CBP in human osteoblasts.* After shortlisting eight unique miRNAs that were predicted to target the 3'-UTR of CBP, their presence and expression patterns at the precursor level were analyzed in control or TGF- $\beta$ 1-treated MG-63 cells. Under TGF- $\beta$ 1 stimulation, miR-3924 was significantly downregulated at all time points (Fig. 4A); miR-3133 was significantly downregulated at 24 h (Fig. 4B); miR-4327 and miR-4264 were significantly downregulated at 1, 2 and 4 h (Fig. 4C and D); miR-1185-3p (the accession no. of the sequence used to design this primer: NR\_031575) was significantly upregulated at 1, 2, 8 and 24 h (Fig. 4E); miR-6083 was significantly downregulated at 1, 2, 4 and 8 h (Fig. 4G); and miR-7-1-3p and miR-600 did not show any significant upregulation or downregulation (Fig. 4E and F). Tukey's post hoc analysis showed that the groups treated with TGF- $\beta$ 1 for 1, 2, 4 and 24 h were statistically significant compared with the control with regard to the expression profile of miR-6083. Also, the 1- and 24-h TGF- $\beta$ 1-treated groups were significantly different compared with the control with regard to miR-1185-3p. Analyses of the LogitProb and miRDB scores (Fig. 3), along with expression patterns assessed by RT-qPCR (Fig. 4C), identified miR-4327 as having the most favorable characteristics for targeting CBP.

Next, the mature expression pattern of miR-4327 was analyzed in MG-63 cells. A similar pattern of significant downregulation of mature miR-4327 expression at 2, 4 and 8 h after TGF- $\beta$ 1 treatment in MG-63 cells was observed (Fig. 5A). Tukey's post hoc analysis revealed a statistical significance in miR-4327 expression at 2 and 8 h of TGF- $\beta$ 1 treatment compared with the control in MG-63 cells. In addition, miR-4327 expression was significantly downregulated at 1, 2 and 4 h after TGF- $\beta$ 1 treatment in HS-5 cells (Fig. 5B). Although a significant upregulation of miR-4327 expression was observed after 8 and 24 h of TGF- $\beta$ 1 treatment, the expression patterns of mature miR-4327 at early time points of TGF- $\beta$ 1 treatment were consistent in both MG-63 and HS-5 cells (Fig. 5). These findings supported an inverse correlation, as observed in the case of CBP expression (Fig. 1).

*Overexpression of miR-4327 downregulates CBP-mediated acetylation of RUNX2 and MMP-13 levels in human osteoblasts.*

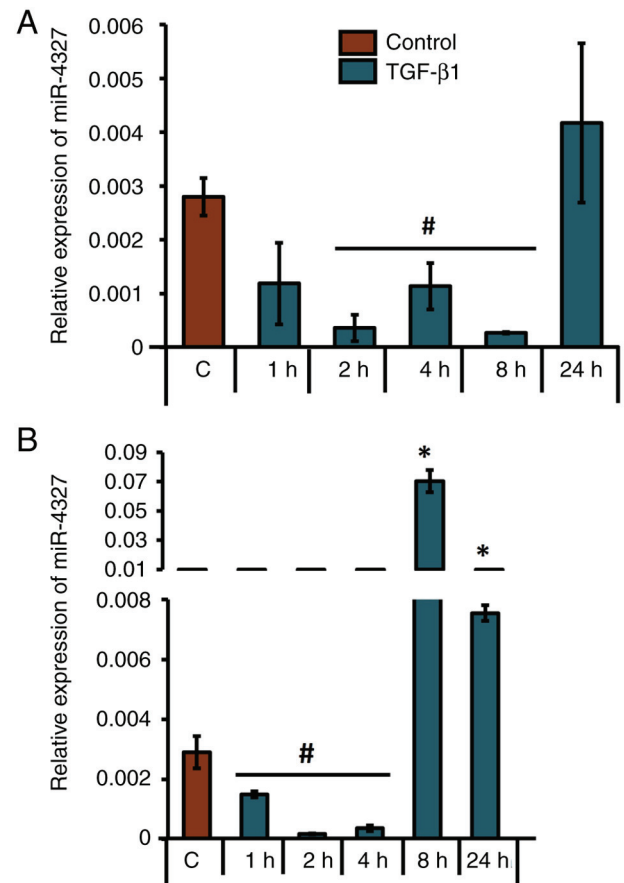


Figure 5. TGF- $\beta$ 1 regulated miR-4327 expression in human osteoblasts and primary osteoblastic cells. (A) MG-63 or (B) HS-5 cells were treated with 5 ng/ml TGF- $\beta$ 1 for 1, 2, 4, 8, or 24 h or left untreated (control). MiR-4327 expression was analyzed by RT-qPCR. \* $P < 0.05$ ; # $P < 0.05$  for TGF- $\beta$ 1 treatment vs. respective control ( $n = 3$ ). TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; miRNAs/miR, microRNAs.

As miR-4327 expression was downregulated by TGF- $\beta$ 1 stimulation, the present study aimed to analyze its functional role using miRNA overexpression studies. The negative control (nc)-miRNA (5'-UCACCGGGUGUAAAUCAGCUUG-3') or miR-4327 mimic (5'-GGCUUGCAUGGGGGACUGG-3') were transiently transfected into MG-63 cells and they were treated with TGF- $\beta$ 1 for 24 h or left untreated (control). RT-qPCR analysis showed that miR-4327 overexpression caused a substantial elevation of its endogenous expression under TGF- $\beta$ 1 treatment or control conditions (Fig. 6A). To determine the association between CBP and Runx2 and the effect of HAT activity on Runx2 expression, whole-cell lysates were collected after 2 h of TGF- $\beta$ 1 treatment and subjected to coimmunoprecipitation using IgG or Runx2 antibody, followed by immunoblotting using antibodies against Runx2, acetylated-lysine, or CBP (Fig. 6). In the miR-4327 mimic-transfected group, the levels of acetylated Runx2 (Fig. 6B and C), Runx2 (Fig. 6B and D) and CBP (Fig. 6B and E) were significantly downregulated compared with those in the nc-miRNA group. Overexpression of miR-4327 significantly downregulated the expression of CBP in human osteoblasts (Fig. 6F and G). Furthermore, western blot analysis for the aliquots of the aforementioned whole-cell lysates was performed. In the nc-miRNA group, TGF- $\beta$ 1 treatment substantially increased

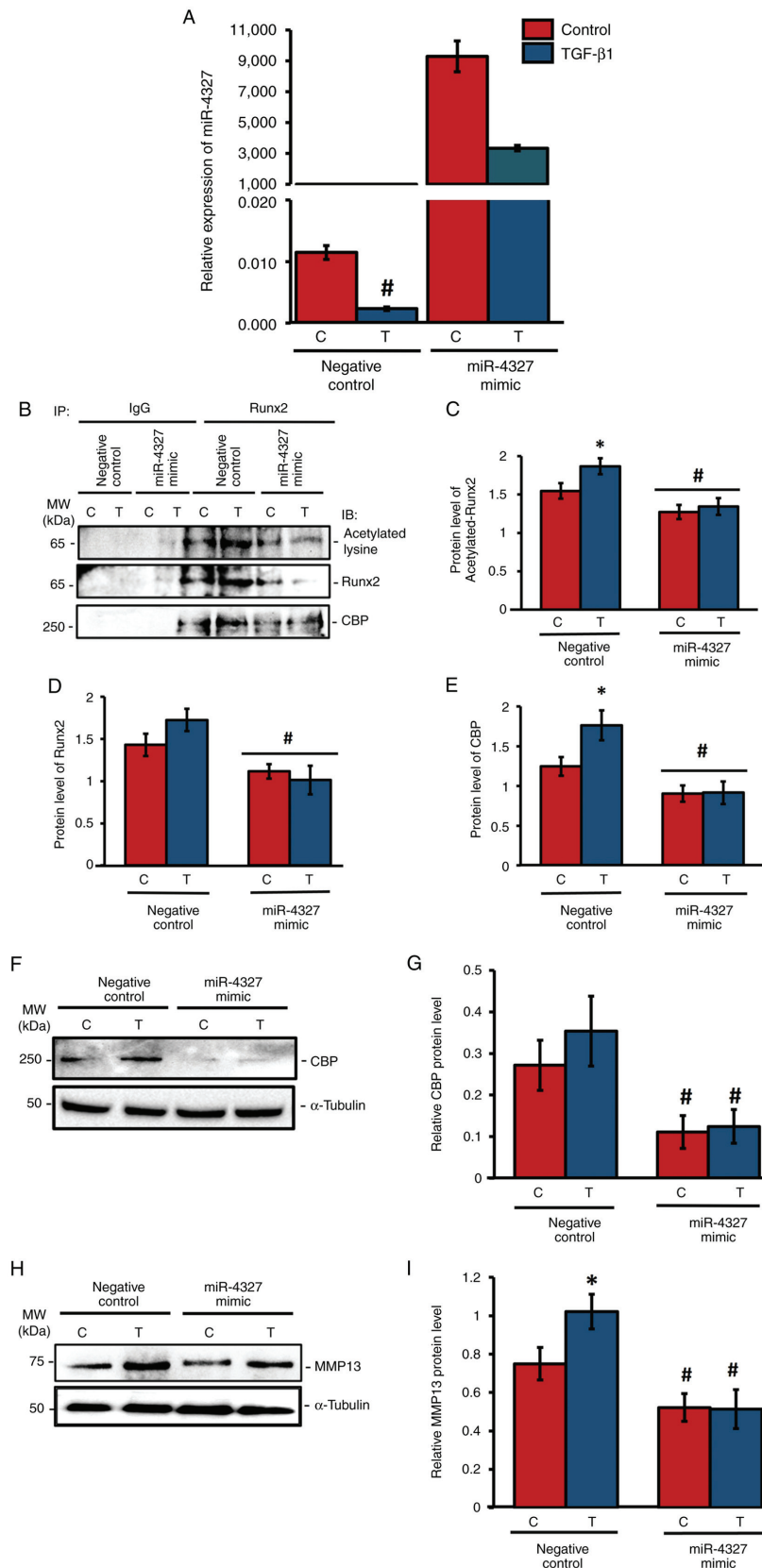


Figure 6. Runx2 acetylation and CBP protein expression were reduced by the overexpression of miR-4327 under TGF- $\beta$ 1 stimulation in human osteoblasts. Transiently transfected MG-63 cells with miR-4327 mimic or negative control miRNA (nc-miRNA) for 24 h were subjected to 5 ng/ml TGF- $\beta$ 1 treatment for 2 and 24 h. (A) The expression of miR-4327 was analyzed by reverse transcription-quantitative polymerase chain reaction.  $^{\#}P<0.05$  vs. control. (B) Whole-cell lysates were subjected to IP (2 h of TGF- $\beta$ 1 treatment) followed by IB. Representative blots of IP and IB are depicted. Protein expression was quantified using IgG expression for normalization using ImageJ software. Levels of (C) acetylated-Runx2, (D) Runx2 and (E) CBP. (F) Western blot analysis of CBP levels. (G) Relative protein expression of CBP normalized with respect to  $\alpha$ -Tubulin expression. Whole-cell lysates obtained 24 h after TGF- $\beta$ 1 treatment were analyzed by western blotting. (H) Representative blots of MMP-13 expression. (I) Relative expression of MMP-13 normalized with respect to  $\alpha$ -Tubulin expression.  $^*P<0.05$  vs. control;  $^{\#}P<0.05$  vs. negative control (n=3). Runx2, runt-related transcription factor 2; CBP, CREB-binding protein; miRNAs/miR, microRNAs; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; nc, negative control; IP, immunoprecipitation; IB, immunoblotting; MMP-13, matrix metalloproteinase-13.

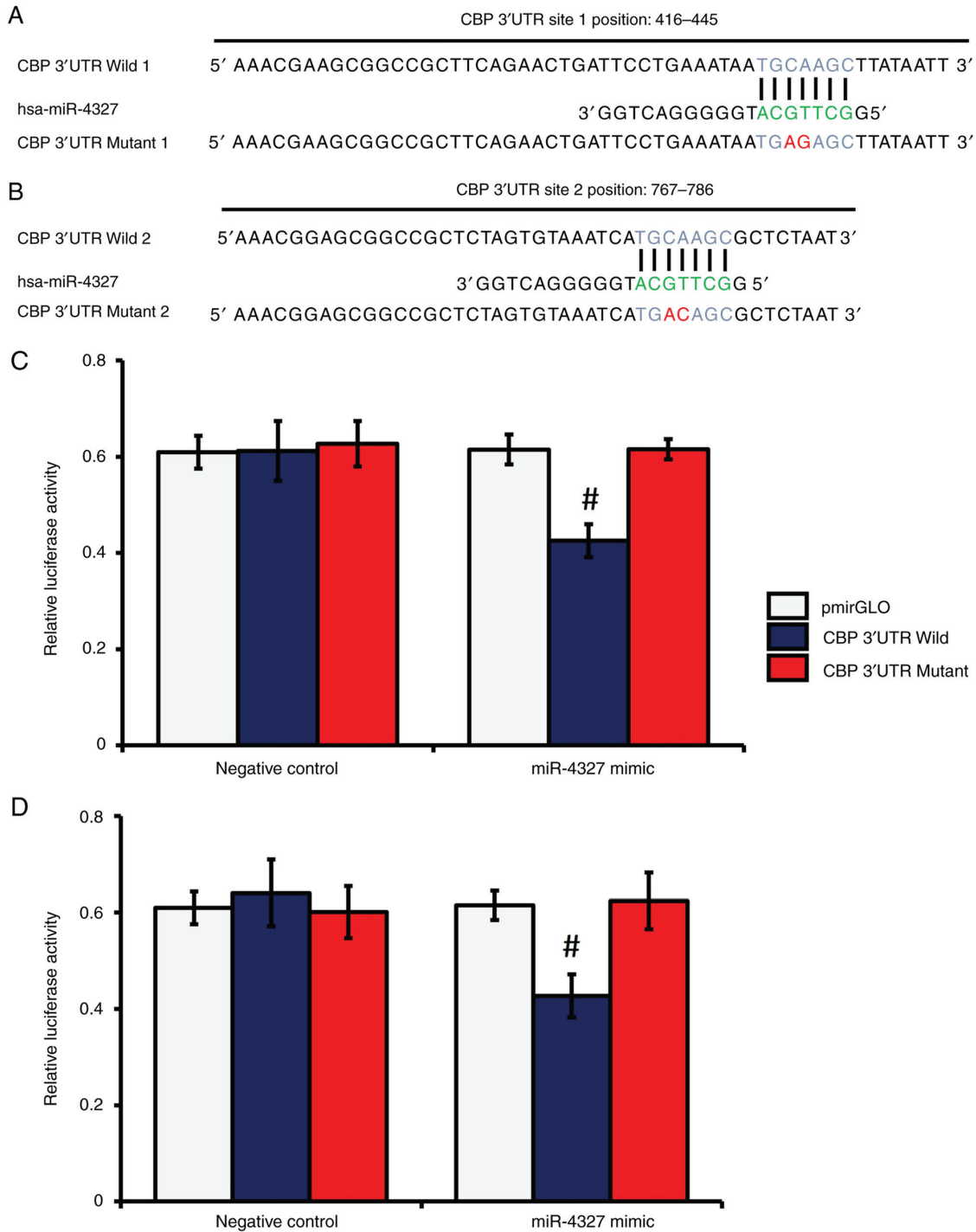


Figure 7. Direct targeting of the 3'-UTR of CBP by miR-4327 in human osteoblasts. A graphical depiction of putative interaction of miR-4327 with the (A) wild-type and mutant site 1 (416–445) or (B) wild-type and mutant site 2 (767–786) of the 3'-UTR of CBP. MG-63 cells were transiently co-transfected with the pmirGLO constructs comprising wild-type or mutant microRNA responsive elements of the 3'-UTRs of CBP and negative control miRNA (nc-miRNA) or miR-4327 mimic. After 24 h, whole-cell lysates were subjected to Firefly and Renilla luciferase assays. Relative luciferase activities of (C) site 1 and (D) site 2. <sup>#</sup>P<0.05 vs. negative control (n=3). UTR, untranslated region; CBP, CREB-binding protein; miRNAs/miR, microRNAs.

MMP-13 expression, whereas miR-4327 mimic-transfected cells significantly reduced MMP-13 expression (Fig. 6H and I). These results indicated that the interaction between CBP and Runx2 and the acetylation of Runx2 could be due to the HAT activity of CBP in human osteoblasts (Fig. 6B and C). CBP expression decreased by miR-4327 overexpression, which might have altered Runx2 stability for MMP13 expression in these cells (Fig. 6B–E).

*miR-4327 directly targets the 3'-UTR of CBP in human osteoblasts.* As miR-4327 overexpression decreased the extent of CBP-mediated Runx2 acetylation and Runx2 expression, which in turn reduced MMP-13 expression under TGF- $\beta$ 1 treatment, the present study further examined if miR-4327 directly interacted with the 3'-UTR of CBP, using a dual-luciferase reporter assay system as previously described (42,43). *In silico* analyses identified two distinct MREs in the 3'-UTR

of CBP for miR-4327 (Fig. 7A and B). A substantial decrease in luciferase activity was noticed in the samples transfected with the wild (W) CBP 3'-UTR MRE (sites 1 and 2) constructs and miR-4327 mimic, whereas no significant changes were noticed in the samples transfected with the mutant (M) CBP 3'-UTR MREs (sites 1 and 2) constructs and miR-4327 mimic or nc-miRNA (Fig. 7C and D). These results indicated the direct targeting of CBP by miR-4327 in human osteoblasts.

## Discussion

Runx2 is a critical transcription factor that orchestrates osteoblast differentiation and skeletal development (44). The proper regulation of Runx2 activity is dependent on its association with multiple signaling pathways, such as the MAPK, PI3K/Akt and Hedgehog pathways, which converge to modulate its expression (45-47). These pathways mediate the ability of Runx2 to regulate osteoblast differentiation and bone tissue formation. Runx2 is also subjected to various post-translational modifications, including phosphorylation, acetylation and ubiquitination, which influence its stability and transcriptional activity (12,47,48). Runx2 promotes bone remodeling by directly binding to the promoter region of MMP-13, a key gene responsible for collagen breakdown and bone matrix remodeling (49,50). Mice deficient in MMP-13 exhibit abnormalities in endochondral ossification and delayed bone remodeling. These defects lead to skeletal malformations and impaired fracture repair (51). This demonstrates the essential role of both Runx2 and MMP-13 in maintaining skeletal integrity.

The p300/CBP are HAT family co-activators, sharing significant structural and functional similarities and often considered interchangeable in numerous biological contexts. Overexpression or mutation of CBP/p300 is linked to various physiological and pathological conditions, including malignant bone tumors (52,53). Although p300 and CBP belong to the p300/CBP family of co-activators, they have an individual or combined HAT effect, thereby regulating various proteins involved in cellular processes, including bone remodeling (54-57). CBP facilitates transcriptional activation by acetylating histones and non-histone proteins, including Runx2, which is critical for regulating genes involved in bone formation and remodeling (17). This acetylation stabilizes Runx2, preventing its proteasomal degradation and enhancing its activity, particularly on target genes such as MMP-13, which is pivotal for ECM remodeling during bone development.

The present study demonstrated that TGF- $\beta$ 1 treatment upregulated both CBP and MMP-13 protein levels in human primary osteoblasts. Further, knockdown of CBP reduced the expression levels of CBP and MMP-13, suggesting that CBP is indispensable for TGF- $\beta$ 1-mediated MMP-13 expression. Similar to the aforementioned results, a correlation between PCAF and p300 in regulating MMP-13 expression under parathyroid hormone treatment in rat osteoblastic cells (UMR 106-01) has been reported; knockdown of *p300* and *PCAF* decreased MMP-13 levels following PTH treatment (31).

miRNAs play a crucial role in coordinating various cellular processes in the bone (58-60). They regulate target gene expression at the post-transcriptional level. miRNAs such as miR-15b (61) and miR-135-5p (62) have been implicated in

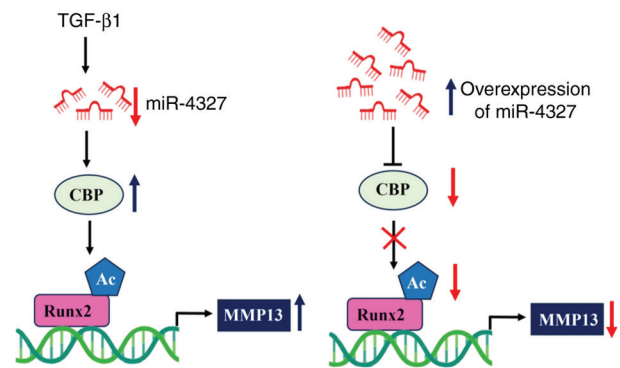


Figure 8. Schematic representation of TGF- $\beta$ 1-stimulated MMP-13 expression through the TGF- $\beta$ 1/ miR-4327/CBP/Runx2 axis. (A) TGF- $\beta$ 1 downregulated miR-4327 and upregulated CBP, increasing Runx2 acetylation, followed by an elevation in MMP-13 protein levels in human osteoblasts. (B) Overexpressed miR-4327 downregulated CBP expression, subsequently reducing Runx2 acetylation and MMP-13 expression in human osteoblasts.  $\uparrow$  Indicates upregulation;  $\downarrow$  indicates downregulation. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; MMP-13, matrix metalloproteinase-13; miRNAs/miR, microRNAs; CBP, CREB-binding protein; Runx2, runt-related transcription factor 2; Ac, acetylation.

regulating osteoblast differentiation and function via diverse pathways. In osteoblastic cells, miR-181a (59) and miR-27a (60) modulate osteoblast development under TGF- $\beta$ 1 treatment. Previous studies have shown that miR-130-5p directly targets p300, reducing its protein levels and subsequently decreasing Runx2 acetylation, which hinders osteoblast differentiation (30). However, to date, no studies have explored the role of miRNAs in regulating TGF- $\beta$ 1-induced CBP expression, Runx2 acetylation, or MMP-13 expression in osteoblasts.

*In-silico* analysis identified eight unvalidated miRNAs that putatively target CBP. Their expression was upregulated or downregulated upon TGF- $\beta$ 1 treatment in osteoblasts. Among these miRNAs, the expression of miR-4327 and miR-4327 was most effectively downregulated by TGF- $\beta$ 1 treatment in these cells. The processing of precursor miRNAs into mature miRNAs is regulated by several factors (63-65). The expression patterns of precursor miRNAs do not need to follow the expression of matured miRNAs (63,66). However, this was not the case in the present study. miR-4327 expression was consistent at both precursor and mature level in osteoblasts. The functional role of miR-4327 was determined by targeting CBP via overexpression of miR-4327 and its subsequent effects on the expression of Runx2 and its acetylation and MMP-13 expression in osteoblasts. Targeting CBP by the miR-4327 mimic caused a decrease in Runx2 acetylation, suggesting that the interaction of CBP with Runx2 is essential for Runx2 acetylation and its stability. Phosphorylation of Runx2 was previously demonstrated to be increased by TGF- $\beta$ 1 (17,48) and phosphorylated proteins may be vulnerable to proteasomal degradation (67,68). Acetylation could prevent the degradation of the phosphorylated proteins by masking their lysine residues with acetyl groups, thus preventing the attachment of the ubiquitin residues and proteasomal degradation (69). p300 and PCAF stabilize Runx2 and increase its transcriptional activity (29,70), which supports the findings of the present study. Conversely, the transcriptional activity of Runx2 could be repressed by various co-repressors, such

as histone deacetylases (71,72). The present study found that Runx2 acetylation by TGF- $\beta$ 1 treatment was mediated by the downregulation of CBP targeting miR-4327 and this effect was found to be essential for MMP-13 expression in osteoblasts. A luciferase reporter assay identified direct targeting of the 3'UTR CBP by miR-4327 in human osteoblasts. This assay system has already been used to determine direct interactions between miRNAs and their target genes (65,73).

Taken together, the data indicated that TGF- $\beta$ 1-treatment stimulated the expression of CBP via reducing the expression of miR-4327 in osteoblasts. The overexpression of miR-4327 reversed the effect of TGF- $\beta$ 1 on MMP-13 expression via CBP-mediated Runx2 acetylation in human osteoblasts (Fig. 8). The results showed that the TGF- $\beta$ 1/miR-4327/CBP axis played a pivotal role in regulating Runx2 acetylation and MMP-13 expression and has potential therapeutic application in bone and bone-related diseases. Aberrations in this regulatory axis could have profound implications for bone homeostasis. In some cases, such as cleidocranial dysplasia, an imbalance in Runx2 can stop osteoblasts from differentiating properly, which can prevent bone from forming properly and lead to structural problems (74-76). Similarly, various skeletal disorders implicate dysregulation of MMP-13, where excessive MMP activity leads to abnormal cartilage degradation and impaired bone remodeling. This can result in phenotypic features such as joint deformities and compromised skeletal integrity, underscoring the importance of MMP-13 in maintaining normal skeletal architecture (77-79).

A potential limitation of the current study was that it focused on TGF- $\beta$ 1 signaling without considering the influence of other pathways that regulate osteoblast differentiation and bone remodeling. While TGF- $\beta$ 1 predominantly signals through Smad2/3 and BMPs signal via Smad1/5/8, both pathways converge through the shared mediator Smad4. Given this convergence, it is possible that BMP signaling may also influence the regulation of miR-4327 expression, similar to TGF- $\beta$ 1. However, this potential regulatory effect of BMP on miR-4327 has not yet been investigated. To learn more about how miR-4327 is controlled in osteoblast differentiation and bone remodeling, one might look into how the BMP and TGF- $\beta$ 1 pathways work together.

The present study suggested that miR-4327 plays a significant role in regulating CBP expression, its interaction with Runx2 and MMP-13 expression under TGF- $\beta$ 1 stimulation in human osteoblasts. Although studies have shown that cytokines, growth factors and hormones regulate Runx2 post-translationally and control its expression via transcriptional co-activators such as p300, CBP and PCAF, the present study identified TGF- $\beta$ 1-induced MMP-13 expression at the post-transcriptional and post-translational regulation levels. Thus, TGF- $\beta$ 1 stimulation of the miR-4327/CBP/Runx2/MMP-13 axis significantly contributed to bone remodeling. Disruption of this axis may impair bone homeostasis, leading to altered bone structure and potentially contributing to conditions such as osteoporosis and osteoarthritis. It is also predicted that several other ncRNAs, including linear and circular lncRNAs, will target miR-4327. Future studies will aim to elucidate how these ncRNAs respond to TGF- $\beta$ 1 and their role in regulating miR-4327 and its downstream target genes. In addition,

*in vivo* studies are required to validate the clinical relevance of miR-4327 with CBP and MMP-13 in skeletal biology.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Author contribution

RK, IM, SK, MB and IS performed the experiments. RK, IM, SK, MB, IS and DP wrote the manuscript. RK, IM and DP assisted with designing and formatting the figures and analysed the data. NS designed the study and reviewed and edited the manuscript. NS secured funding for this study. RK and IS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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