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MicroRNA-322 (miR-322) and Its Target Protein Tob2 Modulate Osterix (*Osx*) mRNA Stability*[S]

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Background: miRNAs exert important roles during osteoblast proliferation and differentiation.
Results: miR-322 induces expression of osteogenic genes by down-regulation of expression of *Tob2*, which binds to the 3′-UTR of *Osx* and modulates its degradation.
Conclusion: miR-322 and its target Tob2 are regulators of osteogenesis through control of *Osx* mRNA degradation.
Significance: We have identified a miRNA-transcription factor network that allows fine-tuning of the osteoblast differentiation program.

Osteogenesis depends on a coordinated network of signals and transcription factors such as Runx2 and Osterix. Recent evidence indicates that microRNAs (miRNAs) act as important post-transcriptional regulators in a large number of processes, including osteoblast differentiation. In this study, we performed miRNA expression profiling and identified miR-322, a BMP-2-down-regulated miRNA, as a regulator of osteoblast differentiation. We report miR-322 gain- and loss-of-function experiments in C2C12 and MC3T3-E1 cells and primary cultures of murine bone marrow-derived mesenchymal stem cells. We demonstrate that overexpression of miR-322 enhances BMP-2 response, increasing the expression of *Osx* and other osteogenic genes. Furthermore, we identify Tob2 as a target of miR-322, and we characterize the specific *Tob2* 3′-UTR sequence bound by miR-322 by reporter assays. We demonstrate that Tob2 is a negative regulator of osteogenesis that binds and mediates degradation of *Osx* mRNA. Our results demonstrate a new molecular mechanism controlling osteogenesis through the specific miR-322/Tob2 regulation of specific target mRNAs. This regulatory circuit provides a clear example of a complex miRNA-transcription factor network for fine-tuning the osteoblast differentiation program.

Skeletal development depends on the activity of osteoblasts that derive from condensations of mesenchymal cells. In addition, bone is constantly remodeled throughout life, and it requires stringent control of a program of gene activation and suppression in response to physiological signals. Understanding this regulatory mechanism is important for developing new strategies to treat pathological states such as osteoporosis and osteosclerosis. For the specification of mesenchymal cells to distinct terminal phenotypes, cohorts of tissue-specific transcription factors and coregulators mediate the expression of genes required for the functions of a particular differentiated tissue. Molecular and genetic research has identified Osterix (Osx), Runx2, Dlx5, Msx2, and ATF4, among others, as important transcription factors essential for osteoblast differentiation (1, 2). Osx and Runx2 have been widely accepted as master osteogenic factors because neither Runx2- nor Osx-null mice form mature osteoblasts (3, 4).

Osteoblast lineage specification and maturation of osteoprogenitors are also controlled by multiple extracellular ligands. Bone morphogenetic proteins (BMPs)[3] belong to the TGF-β superfamily and, together with Wnt proteins, are the main extracellular cues involved in the activation of skeleton-related genes for formation of cartilage and bone. BMP signals are transduced by Smad proteins and have been shown to stimulate Runx2 and Osx *in vitro* and *in vivo* (5–10).

Recently, microRNAs (miRNAs) have emerged as important regulators in various developmental, physiological, and pathological conditions, including cell differentiation and function (11). miRNAs are small noncoding RNAs that mediate translational inhibition or degradation of the transcript by binding to complementary sites in the 3′-UTRs or coding regions of target mRNAs (12). Recent studies indicate that miRNAs are important players during osteogenic differentiation, and the identification of new miRNAs characterizing genetic and metabolic abnormalities provides new approaches for treatment of diseases (13–19). It has been shown that depletion of miRNAs in the osteoblast lineage through conditional ablation of the *Dicer* gene causes evident skeletal phenotypes (20). Interestingly, previous studies have shown differential requirements of miRNA control at early and late steps of osteogenesis, suggesting that miRNAs are required for embryonic bone development as well as for bone homeostasis in the adult skeleton.

BMP and TGF-β are known to regulate miRNA biogenesis through ribonuclease 3, also known as Drosha (21). BMP-2 has

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[3] The abbreviations used are: BMP, bone morphogenetic protein; miRNA, microRNA; BM-MSC, bone marrow-derived mesenchymal stem cell; qPCR, quantitative PCR.

been shown to down-regulate a cohort of miRNAs that target inhibitors of multiple osteogenic pathways in premyogenic cells, including BMP and Wnt receptors and their ligands as well as transcriptional regulators and MAPK signaling components (15, 22–26). Interestingly, the few miRNAs that are strongly up-regulated by BMP-2 in premyogenic cells target essential factors for muscle cell differentiation, thereby repressing their differentiation to myocytes.

In this study, we characterized miR-322, a novel BMP-2-down-regulated miRNA, and investigated its effects on osteoblast differentiation. We identified Tob2 as its target and showed that Tob2 regulated *Osx* mRNA degradation. We propose a regulatory mechanism in which miR-322/Tob2 controls *Osx* degradation and allows an integrated post-transcriptional control of multiple osteogenic genes.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and Antibodies—pME18S-hTob2 was provided by Dr. T. Yamamoto. pFLAG-CMV-hCPEB4 was provided by Dr. R. Méndez. To determine the target region of miR-322, the 3′-UTR of the mouse *Tob2* cDNA was amplified from genomic DNA using primers 5′-TGCTGAAGTCTAGAGACCATCAGGCTT-3′ and 5′CTCCCATCTAGAAAAAGGATTCGCCCAGG-3′ for the wild type and primers 5′-TGCTGAAGTCTAGAGACCATCAGGCTT-3′ and 5′AGAGTGGTCTAGATGTCAATTCGTGCACC for the mutant construct and cloned between the XbaI sites of the pRL-SV40 vector (Promega, Madison, WI). Biotinylated RNA oligonucleotides were from Sigma: Osx1, 5′-AUCCUCGAGGUCUCCGAGAGUUUCUUUUUCAGUUGAGUUUUGGG-3′; Osx2, 5′-CCUGAGCUUUGUGUUUUCUUUUUUUAAACAAACACGAUGAUGAU-3′; control, 5′-CUCUCUCCGAGGGGCAGGGUUCCUCCC-3′. *Cox2*- and *Id1*-luciferase reporter constructs were described previously (27, 28). BMP-2 was from R&D Systems (Minneapolis, MN) and used at a final concentration of 2 nM. Actinomycin D (Sigma) was used at final concentration of 10 μg/ml. The following antibodies were used at 1:1000 for immunoblotting: anti-Osx (Abcam, Cambridge, UK); anti-Smad1 and anti-phospho-Smad (Cell Signaling, Beverly, MA); anti-Tob2 and anti-FLAG (Sigma); and anti-α-tubulin (Sigma). Immunocomplexes were visualized with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:10,000), followed by incubation with ECL Western blot reagent (GE Healthcare). Chemiluminescent image capture of immunoblots was performed with the Fujifilm LAS 3000 device. Quantification of protein expression was performed using Fujifilm MultiGauge software and is expressed as relative values using α-tubulin as a control.

Cell Culture, Differentiation, and Transfections—Bone marrow-derived mesenchymal stem cells (BM-MSCs) were isolated and cultured as described previously (8) and treated for 24 h with BMP-2 (2 nM) before miRNA isolation. C2C12 and HeLa cell lines were maintained as described previously (9). The MC3T3-E1 cell line was maintained in α-modified Eagle's medium supplemented with 10% FBS. To induce C2C12 cell differentiation, the medium was replaced with DMEM without serum, and BMP-2 (2 nM) was added. To induce MC3T3-E1 cell differentiation, the medium was replaced with α-modified Eagle's medium with 10% FBS, 50 μM ascorbic acid, and 5 mM β-glycerophosphate. Cells were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) for miRNAs and Lipofectamine LTX (Invitrogen) for cotransfection with plasmids. Transfection efficiency was assessed by GFP or BLOCK-iT Alexa Fluor Red fluorescent control (Ambion, Paisley, United Kingdom). Pre-miR™ and Anti-miR™ (Ambion) were used at final concentration of 20 nM.

Microarray and RT-qPCR Analysis—Total RNA was isolated from C2C12 cells, MC3T3-E1 cells, or BM-MSCs using the Ultraspec RNA isolation system (Biotecx, Houston, TX). 3 μg of total RNA was reverse-transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems). miRNAs were isolated using a mirVana miRNA isolation kit (Applied Biosystems), and reverse transcription was performed using a TaqMan microRNA reverse transcription kit (Applied Biosystems). Initial miRNA screening was performed using TaqMan Array Rodent MicroRNA B Card v3 (Applied Biosystems). miRNA qPCRs were performed using specific probes (TaqMan microRNA assays). All quantitative PCRs were carried out using the ABI Prism 7900HT fast real-time PCR system and the TaqMan 5′-nuclease probe method (Applied Biosystems). mRNA transcripts were normalized to GAPDH expression, and miRNA transcripts were normalized to U6 expression.

Luciferase Reporter Assays—Cells were transfected for 8 h without serum and treated with BMP-2 for 16–24 h. Luciferase activities were quantified using the luciferase assay system or the *Renilla* luciferase assay system (Promega). Luciferase values were normalized using β-galactosidase detection kit II (Clontech, Palo Alto, CA).

Lentiviral Transduction—LentimiRa-GFP-mmu-mir-322 and Lenti-IIII-mir-GFP control virus (ABM Inc., Richmond, British Columbia, Canada) were used for BM-MSC transduction at a multiplicity of infection of 1, and efficiency was controlled by the GFP coexpressed in the same construct. Polybrene (Sigma) was used at final concentration of 2 μg/ml to enhance viral infection. Puromycin was used to select infected cells at 2 μg/ml. Selected MSCs were cultured for 5 days in osteogenic differentiating medium (DMEM supplemented with pyruvate, glutamine, penicillin/streptomycin, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate).

RNA Pulldown Assay—HeLa cells were transfected for 4 h using Lipofectamine LTX. GFP was used as a transfection control. Cells were harvested by adding lysis buffer (50 mM Tris (pH 7), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.2% Nonidet P-40, and protease and phosphatase inhibitors). Lysates were centrifuged for 5 min at 16,000 × *g*. Yeast RNA (0.5 mg/mg of protein; Sigma) was added to the supernatant to block nonspecific binding. Biotinylated RNAs were denatured at 65 °C for 5 min and cooled for 20 min in 10 mM Tris (pH 7), 100 mM KCl, and 10 mM $MgCl_2$. 0.5 μg of each RNA was incubated with cell lysates for 60 min at 4 °C, including 200 units/ml RNasin (Promega). 25 μl of streptavidin-Sepharose (GE Healthcare) was added and further incubated at 4 °C for 60 min. Beads were washed five times with lysis buffer. Bound proteins were detected by Western blotting.

Analysis of miRNA Target Data Set—*In silico* putative targets were screened for each of the differentially expressed miRNAs

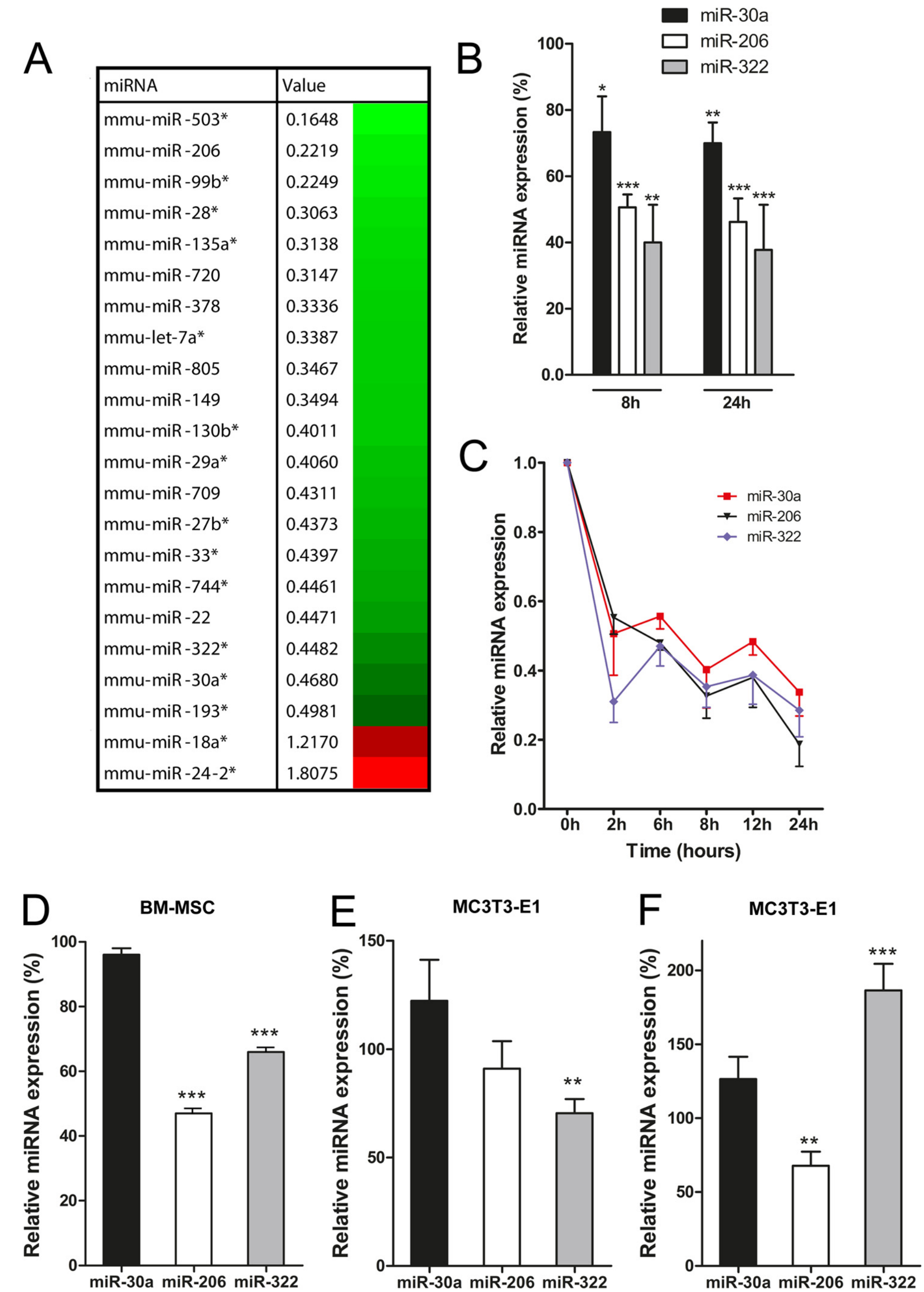

FIGURE 1. **Expression profile of miRNAs during osteoblast differentiation.** *A*, miRNA array expression data from C2C12 cells treated with BMP-2 for 8 h. *Red* denotes higher expression, and *green* denotes lower expression relative to expression in control cells. Only miRNAs with a 2-fold reduction in expression are shown. *B* and *C*, expression of miR-30a, miR-206, and miR-322 was analyzed during osteoblast differentiation. Time course experiments were performed with 2 nM BMP-2 treatment with (*B*) or without (*C*) 10% fetal bovine serum. The indicated miRNAs were isolated from C2C12 cells cultures as described under "Experimental Procedures," measured by RT-qPCR, normalized to U6 RNA, and plotted as relative expression to time 0 (means ± S.E. of five independent experiments). BM-MSCs (*D*) and MC3T3-E1 cells (*E*) were treated with BMP-2 in the absence of serum for 24 h, and the indicated miRNAs were measured by RT-qPCR. *F*, MC3T3-E1 cells were treated or not with differentiation medium for 10 days, and the indicated miRNAs were measured by RT-qPCR in five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

by three different algorithms: DIANA-microT v5.0 (29), miRanda (30), and TargetScan 6.2 (31).

Statistical Analysis—Statistical analysis was performed using Student's t test or two-way analysis of variance, followed by Bonferroni multiple-comparison tests. Quantitative data are presented as means ± S.E. Differences were considered significant at p values of <0.05.

RESULTS

miRNA Expression Profile during BMP-2-induced Osteoblast Differentiation—To identify miRNAs whose expression is altered during osteoblast differentiation, C2C12 cells were treated with BMP-2 (2 nM) in medium without serum for 8 h. Independent experiments were performed under the same conditions, and a miRNA expression profile was obtained (Fig. 1*A*).

In agreement with previous results, global analysis indicated that most of the significantly changed miRNAs were down-regulated in response to BMP-2 (13, 14). For instance, miR-206 was shown to decrease during C2C12 cell differentiation (25), and miR-30 family members are also down-regulated by BMP-2 (32).

To verify these results, miR-30a, miR-206, and miR-322 were chosen for further study. These miRNAs were expressed at relatively high levels in C2C12 cells (supplemental Table 1). To confirm the array data, kinetic assays of BMP induction in the presence and absence of serum were performed, and the expression of selected miRNAs was detected by qPCR. Time course results were consistent with array data: miR-30a, miR-206, and miR-322 all steadily decreased after BMP-2 addition (~50–60% decrease at 8 and 24 h for miR-206 and miR-322) (Fig. 1*B*). Down-regulation of miRNA expression to a similar extent was also observed in cells cultured in the absence of serum (Fig. 1*C*). We extended our observations to primary cultures of murine BM-MSCs and MC3T3-E1 cells. BMP-2 treatment of BM-MSCs or MC3T3-E1 cells for 24 h caused similar miR-322 expression down-regulation, whereas miR-30a expression remained not significantly altered with respect to the miR control (Fig. 1, *D* and *E*). We also analyzed differentiation of MC3T3-E1 cells in medium containing ascorbic acid and β-glycerophosphate. In this case, although differentiation led to similar effects as BMP-2 addition on miR-30a and miR-206 expression levels, it induced a significant increase in miR-322 levels instead (Fig. 1*F*).

Effects of miR-30a, miR-206a, and miR-322 on Osteoblast Differentiation—To determine whether the selected miRNAs affect osteoblast differentiation, C2C12 cells were transfected with mimics of miR-30a, miR-206, and miR-322 and treated with BMP-2 for 16 h. Their effects were first assessed by characterizing the mRNA expression levels of the main osteoblast-determining transcription factors. It is well known that BMP addition to C2C12 cells stimulates expression of known osteogenic markers (1, 2, 4, 9, 33). Overexpression of miRNA mimics produced a significant increase in *Osx*, *Runx2*, *Msx2*, osteocalcin, and *Ibsp* mRNA levels by miR-322 and increases in *Runx2* and *Ibsp* by miR-206 (Fig. 2*A*). Upon BMP-2 stimulation, ectopic expression of a miR-322 mimic resulted in a significant accumulation of *Osx*, *Runx2*, *Msx2*, and *Ibsp* mRNA levels compared with expression of a control miRNA mimic. miR-30a transfection reduced both basal and BMP-stimulated *Dlx5* mRNA expression. We also analyzed the effects of miRNAs at the protein level (Fig. 2*B*). Although Osx protein expression increased significantly after miR-322 transfection, miR-206 and miR-30a overexpression led to an Osx decrease, possibly indicating negative osteoblast regulation, in accordance with previous work (13, 32). C2C12 cells differentiate into myoblasts and generate multinuclear myotubes in the presence of low levels of serum. However, BMP-2 is able to transdifferentiate them from the myoblast to the osteoblast lineage (34). To investigate whether miR-322 can also influence their myogenic differentiation, C2C12 cells were transfected with miR-322 or miR control and followed up for myotube formation (Fig. 2*C*). Experiments revealed a complete lack of myotube formation up to 4 days in miR-322-overexpressing cells.

We also analyzed overexpression of miRNA mimics in MC3T3-E1 cells, an osteoblast cellular model independent of BMP activation. Similar to C2C12 cells, *Osx*, *Runx2*, and *Msx2* mRNA levels were significantly increased by miR-322 (Fig. 3*A*). Altogether, these results suggested that miR-322 might be important for osteogenic differentiation and led us to investigate its molecular mechanisms of action.

We then performed miR-322 loss-of-function experiments. Consistently, transfection of Pre-miR-322 led to increased levels of miR-322, whereas transfection of Anti-miR-322 led to a 40% decrease in expression of endogenous miR-322 (supplemental Fig. S1). Therefore, Anti-miR-322 was transfected in cells in the presence or absence of BMP-2 to assess the effect on osteogenic genes. The decrease in miR-322 levels led to a significantly lower induction of *Osx* and *Runx2* mRNAs by BMP-2 (Fig. 3*B*).

Lack of Direct Interaction between miR-322 and the Smad Pathway—Smad proteins have been found to play critical roles in osteoblast differentiation induced by BMPs (7). The above results suggested that miR-322 was able to increase expression of osteogenic genes in response to BMP-2. Thus, we decided to determine whether miR-322 directly modulates Smad phosphorylation and signaling by performing time course experiments of BMP-2 activation. Ectopic expression of miR-322 led to a slight decrease in Smad1 expression that became significant after 8 h of BMP-2 addition (supplemental Fig. S2*A*). However, immunoblotting showed no significant changes in the ratio between phosphorylated and total Smad1 levels (supplemental Fig. S2*A* , *left* panel). These results suggest that the effect of miR-322 in osteoblast differentiation is not likely related to a direct change in phospho-Smad1 levels. In parallel, Smad1 and phospho-Smad1 levels were evaluated by immunoblotting after transfection with Anti-miR-322 and BMP-2 addition. No significant changes were observed in phospho-Smad activation (supplemental Fig. S2*B*).

Tob2 Is a Target of miR-322—The essential role of Runx2 and Osx in bone development led us to analyze the mechanisms regulated by miR-322 in osteoblast differentiation. Bioinformatic target prediction was carried out using DIANA, miRanda, and TargetScan prediction software. Putative targeted genes included *HoxA10* and *Dlx5* (osteogenic transcription factors); activin receptors IIA and IIB, *Smurf1*, *Smad7*, and *Smad1* (BMP signaling); and *Cdc25A* and *Tob2* (cell proliferation and differentiation) (supplemental Table 2). These targets were experimentally validated after miRNA mimic transfection. The results showed that the expression of *Tob2* mRNA and the induction of *Tob2* and *Smad7* mRNAs by BMP-2 (6-fold increase for *Smad7*) were significantly decreased in miR-322-transfected cells, whereas no significant differences were found for miR-30a and miR-206 assays (Fig. 4*A*). *Smurf1* mRNA levels were not altered in any case. Moreover, Anti-miR-322 transfection increased *Tob2* mRNA levels, both basal levels and after BMP-2 addition (Fig. 4*B*). The results led us to focus on Tob2 regulation by miR-322. Time course experiments in C2C12 cells showed that, in correlation with the miR-322 expression decrease, BMP-2 addition promoted higher levels of *Tob2* mRNA expression (supplemental Fig. S3).We also analyzed whether changes in *Tob2* mRNA in response to miR-322 correlated with changes in Tob2 protein levels. Quantification of Western blot analysis

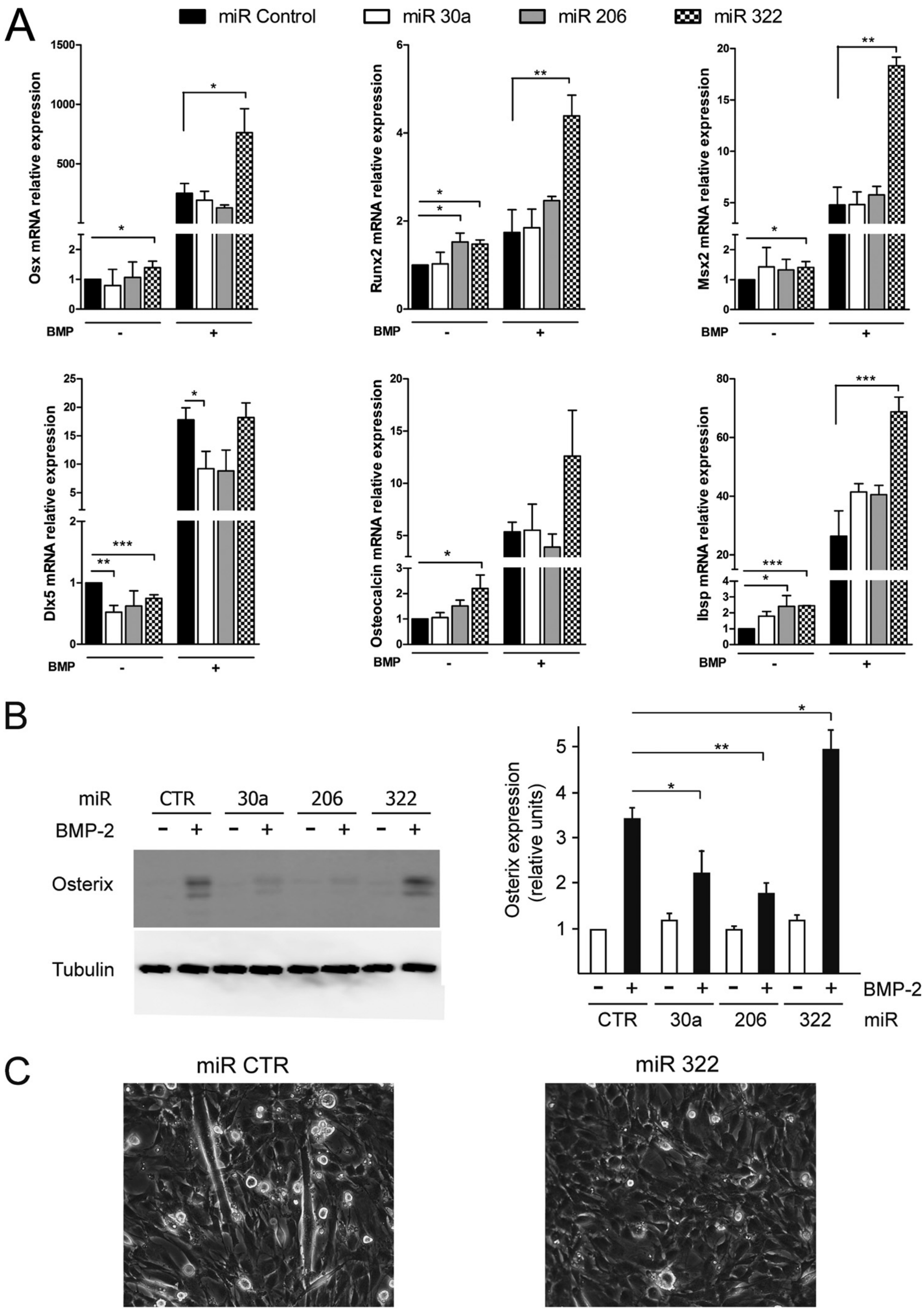

FIGURE 2. **miR-322 enhances *Osx*, *Msx2*, and *Runx2* expression.** *A*, C2C12 cells were transfected with the indicated miRNA mimics without serum for 8 h and treated with BMP-2 overnight. RT-qPCR for the indicated genes was performed, and GAPDH was used as an internal control. Results are plotted as expression relative to untreated Pre-miR control-transfected cells (means ± S.E. of four to nine independent experiments). *B*, C2C12 cells were transfected with the indicated miRNA mimics and treated with 2 nM BMP-2 for 24 h, and Osx and tubulin were detected by immunoblotting (*left panel*). Quantification of the results of three independent experiments is shown (*right panel*). *C*, miR-322 mimic-transfected C2C12 cells were maintained in medium without serum for 4 days, and myotube progression was compared with Pre-miR control-transfected cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

revealed a significant decrease in Tob2 levels after transfection of a miR-322 mimic and a significant increase after Anti-miR-322 transfection (Fig. 4*C*). To confirm our previous results, we performed lentivirus-mediated overexpression experiments in BM-MSCs. Cells were transduced with either a lentiviral construct expressing miR-322 or a control, and mRNA was extracted after 10 days of culture in osteogenic medium (Fig. 4*D*). Consistent with the data from C2C12 cell cultures, miR-322 accumulation repressed *Tob2* and *Smad7* expression and induced significantly higher *Osx* mRNA levels compared with cells transduced with a lentiviral control. Taken together, these results provide evidence that miR-322 negatively regulates *Tob2* expression in mesenchymal cells.

We attempted to confirm these observations using luciferase reporter constructs to examine whether miR-322 can directly regulate *Tob2* expression. miRNAs are able to repress gene expression by binding to seed site sequences located within the 3′-UTR of mRNA (12). We determined the presence of a puta-

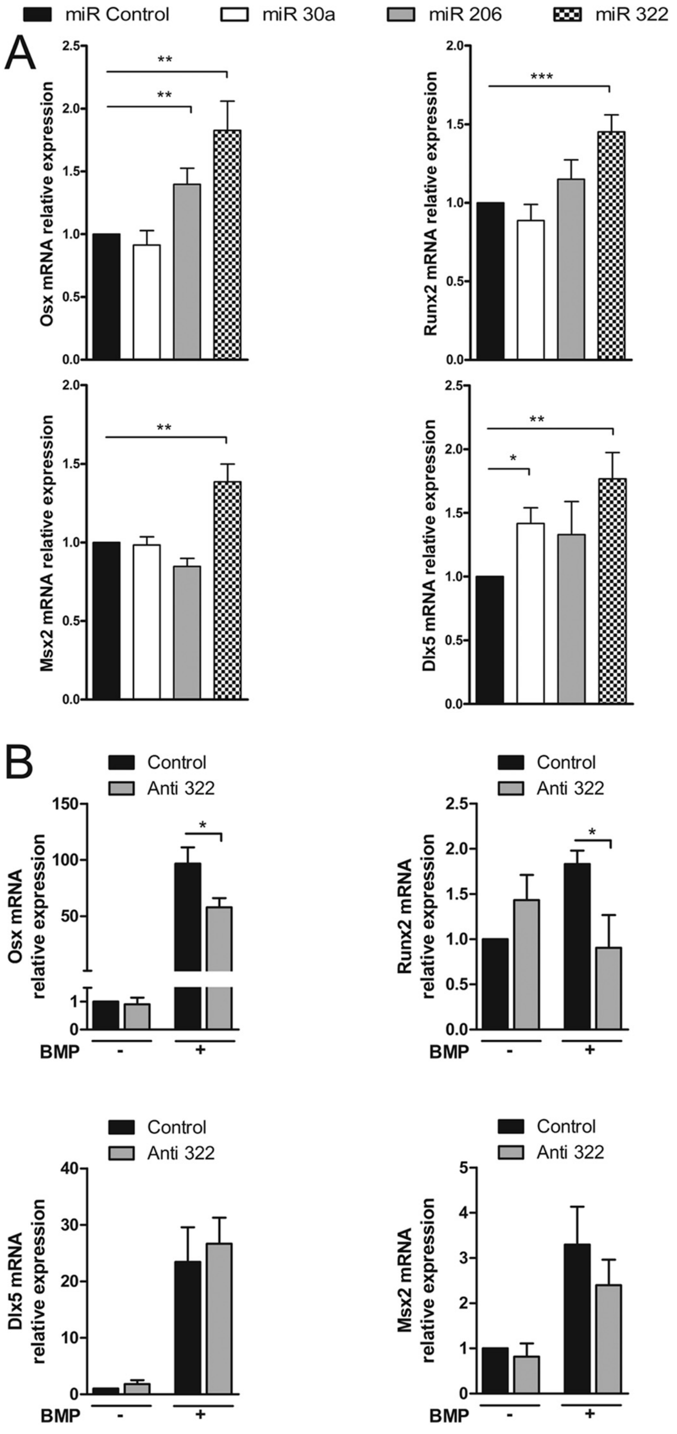

FIGURE 3. **Effects of miR-322 in MC3T3-E1 cells and antisense inhibitor for miR-322 in C2C12 cells.** *A*, MC3T3-E1 cells were transfected with the indicated miRNA mimics for 8 h and treated with differentiation medium overnight. RT-qPCR for the indicated genes was performed, and GAPDH was used as an internal control. Results are plotted as expression relative to untreated Pre-miR control-transfected cells (means ± S.E. of four to six independent experiments). *B*, C2C12 cells were transfected for 8 h with Anti-miR-322 and treated with BMP-2 overnight. The indicated mRNAs were measured by RT-qPCR, normalized to GAPDH, and expressed as relative expression (means ± S.E. of four to five independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

tive target region of miR-322 at position +1769 of *Tob2* mRNA using miRNA target prediction tools. We generated *Renilla* luciferase reporter constructs containing the wild-type *Tob2* 3′-UTR or a construct with the miR-322-binding sequence mutated (Fig. 5*A*). The luciferase activity of the wild-type *Tob2* 3′-UTR construct was significantly inhibited after cotransfection with miR-322 in C2C12 or MC3T3-E1 cells (Fig. 5, *B* and *C*). Furthermore, the mutant *Tob2* 3′-UTR construct lacking the possible binding region of miR-322 was refractory to the decrease in luciferase activity after miR-322 mimic cotransfection. These results provide evidence that miR-322 acts as an inhibitor of *Tob2* mRNA expression through binding to a region located in its 3′-UTR.

Tob2 Accelerates Decay of Osx mRNA Levels—Tob1-deficient mice have a higher bone mass due to an increased number of osteoblasts and an accelerated bone formation rate (35). Thus, Tob proteins act as negative regulators of bone formation, although the mechanisms of this regulatory role are mostly unknown. We then further examined how miR-322/Tob2 increases osteoblast-related genes and their interaction with the BMP-Smad pathway. First, we analyzed luciferase activity using *Id1* and *Cox2* promoter constructs with strong responsiveness to BMP signaling, previously generated in our group (27, 28). Luciferase reporter constructs were cotransfected with constructs overexpressing Tob2, miR-322, or Anti-miR-322. No major differences in luciferase activities were observed (supplemental Fig. S4).

Previous studies identified Tob not only as a general regulator of mRNA decay (36, 37) but also as a specific regulator by binding to CPEB2–4 (cytoplasmic polyadenylation element-binding protein) and recruiting Cnot7 deadenylase to the target mRNAs (38). To analyze the ability of Tob2 to regulate the mRNA degradation of specific genes, we performed mRNA decay assays. The mRNA levels of C2C12 cells overexpressing Tob2, miR-322, or miR control were quantified by qPCR at different time points after treatment with actinomycin D (a known inhibitor of mRNA synthesis). mRNA expression of the genes analyzed was already modified by the transfections before actinomycin D treatment (Fig. 6*A*). Overexpression of Tob2 reduced the basal levels of expression of *Osx*, *Runx2*, *Smad7*, and *Dlx5*. Moreover, overexpression of miR-322 also led to increased levels of *Osx* and *Runx2* mRNAs. In the mRNA decay assays after actinomycin D addition, overexpression of Tob2 reduced the half-live of *Osx* transcripts (significant reduction after 1 h). Moreover, accumulation of miR-322 led to significant stabilization of *Osx* mRNA and *Runx2* to a lesser extent (Fig. 6*B*). *Smad7*, *Dlx5*, and *Msx2* mRNA degradation was not significantly altered in any case. These data indicate that Tob2 modifies the mRNA decay of specific target genes. To further our observations, we performed the same experiments in MC3T3-E1 cells. Expression of the genes analyzed was also already modified by transfection of the miR-322 mimic. *Osx*, *Runx2*, *Dlx5*, and *Msx2* mRNA expression was significantly increased (Fig. 7*A*). mRNA decay assays after actinomycin D addition further demonstrated that overexpression of miR-322 led to a significantly slower rate of *Osx* mRNA decay (Fig. 7*B*). In view of our results, we hypothesized that *Osx* mRNA could be a direct target bound by Tob2 and CPEB2–4 and, as a consequence, specifically decayed. We analyzed 3′-UTR sequences, and interestingly, *Osx* has compatible secondary structures containing a similar stem-loop structure bound by CPEB2–4 (supplemental Fig. S5) (39). We performed RNA

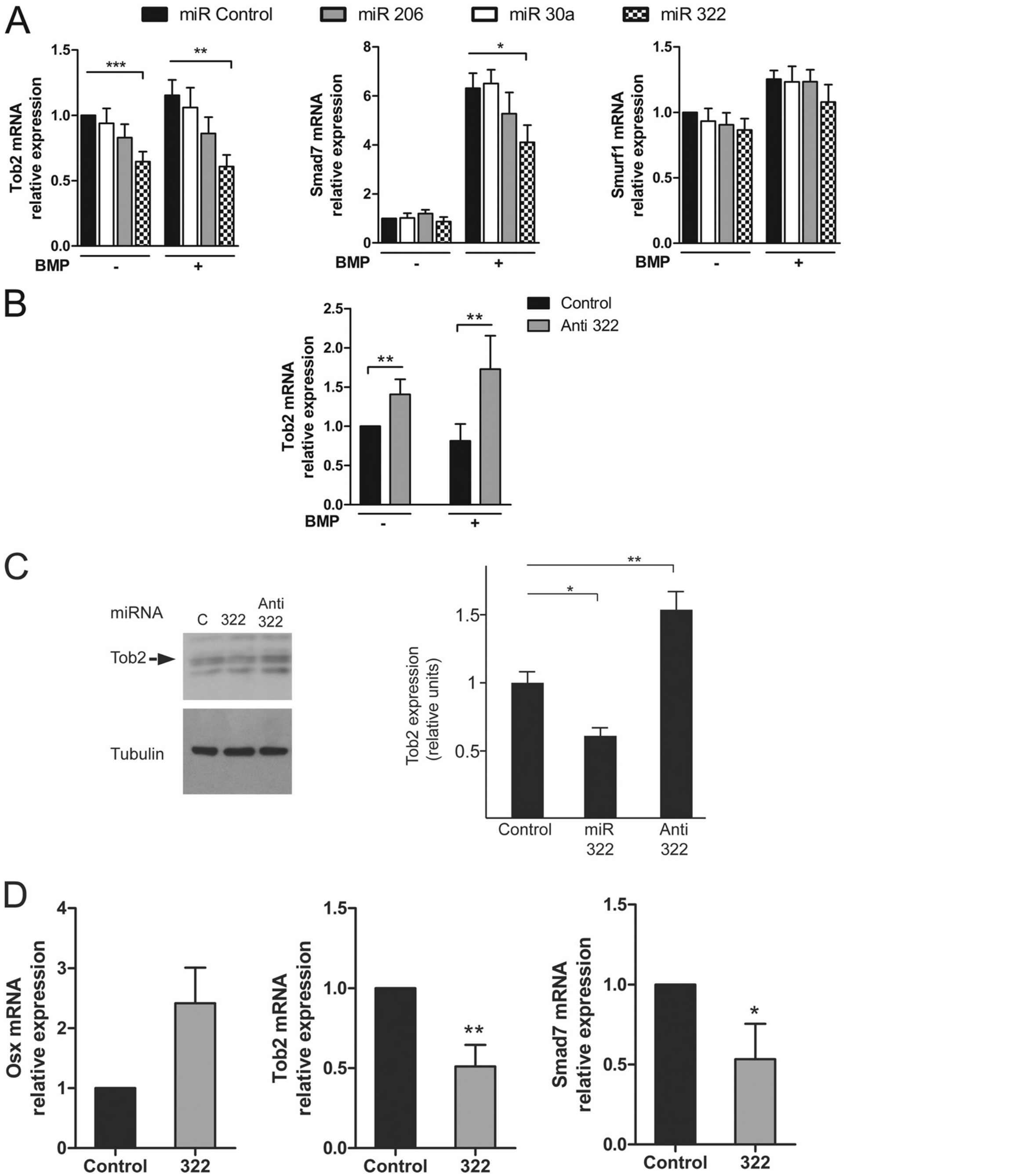

FIGURE 4. **miR-322 inhibits *Tob2* expression.** C2C12 cells were transfected with selected miRNA mimics (*A*) or miR-322 inhibitor (*B*) without serum for 8 h and treated with BMP-2 overnight. *Tob2*, *Smurf1*, and *Smad7* expression was detected by RT-qPCR, normalized to GAPDH, and expressed relative to the miR control transfection. All values represent the means ± S.E. of four to seven independent experiments. *C*, C2C12 cells were transfected with 20 nM miRNA mimic or inhibitor, and Tob2 and tubulin were detected by Western blotting (*left panel*). The *right panel* shows the relative Tob2 levels normalized to tubulin expression. *D*, BM-MSCs were infected with viral particles containing mmu-miR-322 or miR control overnight. Transduced cells were selected with puromycin for 4 days as described under "Experimental Procedures." Cells were cultured until confluence with osteogenic medium and collected to analyze the indicated mRNAs by RT-qPCR. Results were normalized to GAPDH and plotted as relative expression (means ± S.E. of three to four independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

pulldown assays using two oligonucleotides corresponding to distinct stem-loop sequences found in the 3′-UTR of *Osx* mRNA and a control RNA. Either expressed alone or in combination, CPEB4 and Tob2 were able to bind specifically the *Osx* 3′-UTR sequences (Fig. 7*C*).

Taken together, these results strongly suggest that miR-322 increases *Osx* mRNA expression via inhibition of *Tob2* mRNA. Furthermore, our data suggest that the well known effects of Tob proteins on bone development and homeostasis could derive, at least partly, from the role of Tob in the specific degradation and decay of *Osx* mRNA.

DISCUSSION

In our study, we identified miR-322 as a miRNA down-regulated by BMP-2. We have demonstrated for the first time that miR-322 is involved in osteoblast differentiation by targeting

A
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  48 3' agGUUUUGUACUUAACGACGAc 5' 25    mmu-mir-322
           | ||||||||||||||||
1769 5' guCCAGAUGUGAAUUGCUGCUc 3' 1790  Tob2 wt


  48 3' agGUUUUGUACUUAACGACGAc 5' 25    mmu-mir-322
           | |||||| ||||  |||
1769 5' guCUAGAUGUCAAUUCGUGCAc 3' 1790  Tob2 mut
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B

C

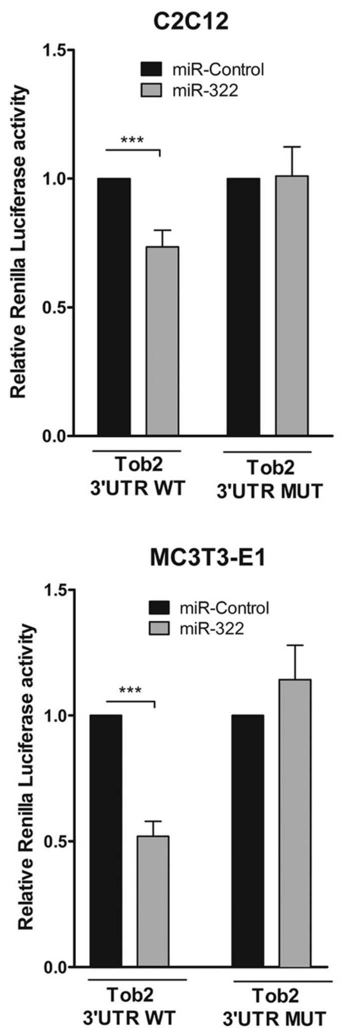

FIGURE 5. ***Tob2* is a direct target of miR-322.** *A*, schematic representation of the alignment of the mouse *Tob2* mRNA 3′-UTR predicted to be targeted by miR-322. Mutations generated within the region corresponding to the seed sequence were included in the mutant *Tob2* 3′-UTR construct. C2C12 (*B*) or MC3T3-E1 (*C*) cells were cotransfected with the *Renilla* luciferase reporter plasmid carrying the wild-type or mutant (*MUT*) *Tob2* 3′-UTR and miR-322 or miR control, respectively. After 8 h, cells were cultured until confluence for 16 h, and luciferase activity was analyzed and normalized to β-galactosidase expression. Relative luciferase activities are expressed as means ± S.E. for triplicates from four to six independent experiments. ***, $p < 0.001$.

Tob2. We reported that overexpression of miR-322 decreases *Tob2* mRNA and protein expression and leads to increased levels of the transcription factor Osx. Furthermore, we have demonstrated direct evidence of the binding site of miR-322 in the *Tob2* 3′-UTR. We propose a molecular description of the mechanism whereby the osteogenic master gene *Osx* is controlled post-transcriptionally through a mechanism driven by miR-322/Tob2. More importantly, our findings strongly suggest that control of specific mRNA decay is relevant in bone development and homeostasis.

miR-322 has been previously studied together with miR-503 in myogenesis as promoters of cell cycle quiescence and differentiation by down-regulation of Cdc25A (40). Expression of miR-322 (which is clustered with miR-503, miR-351, and miR-450) is highly enriched in fibroblasts and cells of the mesenchymal lineage (microRNA.org). Our findings add a new role and a novel target of miR-322 during osteoblast differentiation. Our results also show that, after differentiation by BMP-2, the miR-322 level progressively decreases in C2C12 and MC3T3-E1 cells and primary cultures of BM-MSCs. We have mentioned the up-regulated expression of osteogenic transcription factors during BMP-2 treatment. Then, miR-322, by means of *Tob2* down-regulation, adjusts the expression levels of some of these factors, particularly *Osx*. This profile seems to allow the transcriptional up-regulation of the osteogenic transcription factors, whereas miR-322 may later exert a regulatory mechanism that allows fine-tuning of bone homeostasis.

Our results suggest that the miR-322/miR-351/miR-450/miR-503 cluster is repressed by the addition of BMP-2 (miR-503 was also highly down-regulated in our screen). Smad proteins, the signal transducers of the BMP pathway, have been found to regulate miRNA expression through both transcriptional and post-transcriptional mechanisms (41). Like mRNAs, miRNAs are transcribed by RNA polymerase II and have been shown to be subjected to either positive or negative transcriptional regulation through Smad-containing transcriptional enhancers located in the specific miRNA promoter (41). In addition, Smad proteins have been shown to regulate specific miRNA processing and maturation through interaction with the Drosha complex (21). These miRNAs require a CAGAC sequence at the 3′-end of their mature miRNA sequence to bind and be regulated by the Smad-Drosha complex (42). Because none of the mature miRNAs corresponding to the miR-322 cluster contain this CAGAC sequence, it is likely that regulation of the miR-322 cluster depends on direct transcriptional repression by Smad proteins, as has been demonstrated in other miRNAs (43, 44).

The cellular levels of phosphorylated Smad1 were not significantly modified by the addition of either miR-322 or Anti-miR-322. Notably, we found that Smad7 is also a target of miR-322 in C2C12 cells and BM-MSCs, and it is well known that *Smad7* is induced by BMP-2 as a negative feedback loop. *Smad7* belongs to the inhibitory Smad (I-Smad) family, the main inhibitory mechanism of the cooperative signaling of R-Smad and Co-Smad proteins (45). Moreover, Tob proteins have been shown to associate with the MH2 domains of Smad proteins and to repress Smad transcriptional complexes (35). These two mechanisms may contribute, in an additive way, to a lower BMP response. Nevertheless, analysis of the quantitative role of Smad7 and Tob2 repression in the induction of *Osx* and *Runx2* transcripts by miR-322 also awaits further study.

Although mRNA decay has been shown to be highly relevant in a number of physiological processes (46), there are few examples of this post-transcriptional regulatory mechanism in bone development. For instance, polymorphisms in the 3′-UTR of

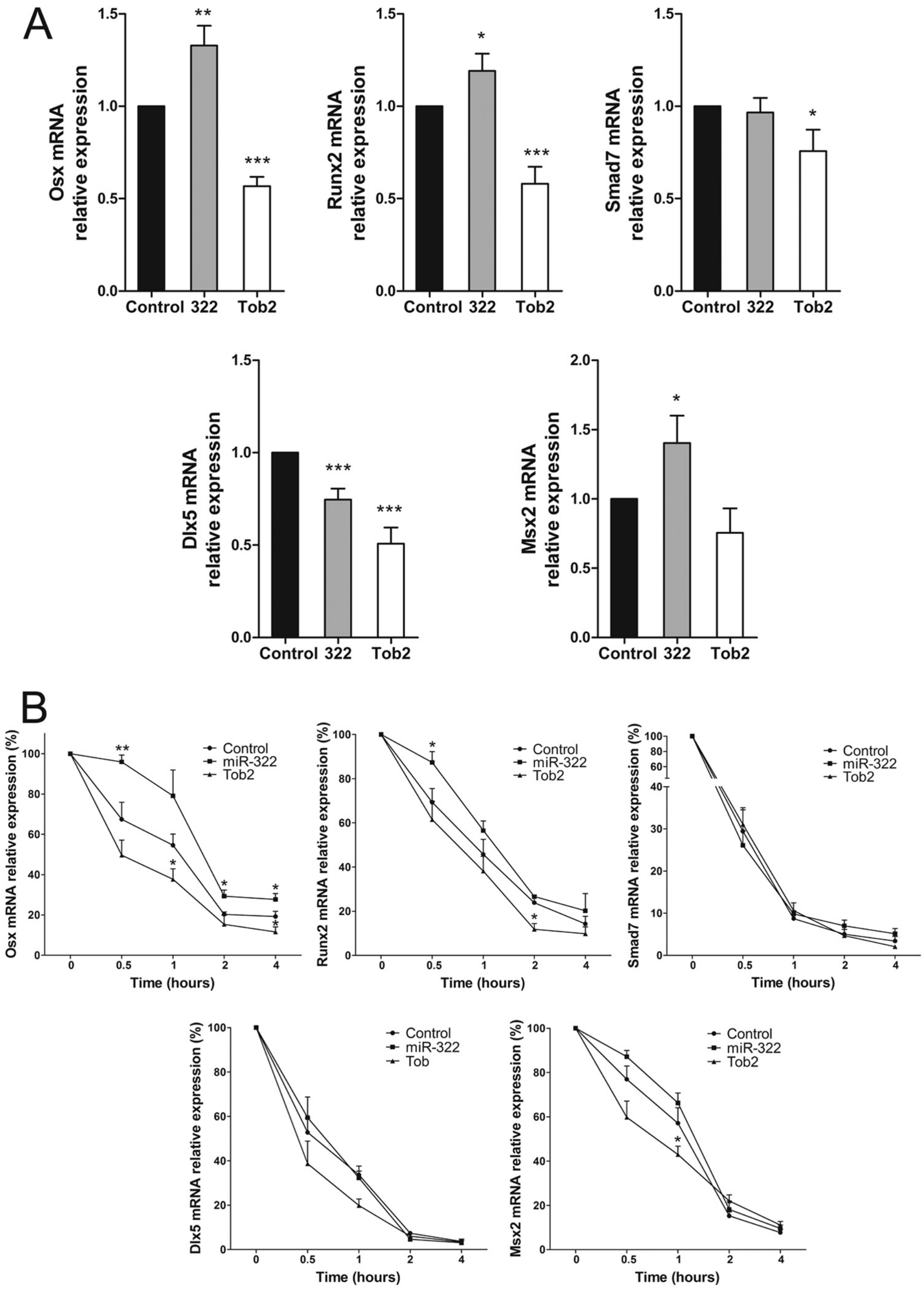

FIGURE 6. **miR-322 stabilizes *Osx* mRNA via Tob2 repression in C2C12 cells.** *A* and *B*, C2C12 cells were transfected with Tob2, miR-322, or miR control for 8 h, cultured overnight, and treated with BMP-2 for 2 h before actinomycin D addition. *A*, expression of the indicated mRNAs at time 0. Expression of selected genes is plotted relative to miR control transfection (means ± S.E. of six independent experiments). *B*, actinomycin D was added, and mRNAs were collected at the indicated times. RT-qPCR was performed using GAPDH as the endogenous control. Data are expressed relative to control transfection mRNA levels at time 0 (means ± S.E. of seven to eight independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

VDR lead to differential mRNA decay and increased risk of osteoporosis (47). In addition, it has also been shown that AU-rich elements mediate stabilization of collagenase-3 mRNA in osteoblasts (48). Tob1 and Tob2 proteins constitute a Tob subfamily and belong to the BTG/Tob antiproliferative factor protein family (49, 50). *Tob* genes have emerged as key players in mediating post-transcriptional gene expression by regulating mRNA deadenylation and therefore cytoplasmic mRNA levels (51). Our results suggest that new target genes displaying compatible Tob-interacting secondary structures at their 3′-UTR could be subjected to specific mRNA repression by Tob family members, as we suggest here for *Osx*. These data are in agreement with previous research showing that, although Tob1 knock-out mice are born without apparent phenotypic abnormalities, Tob1-deficient adult mice were shown to have higher bone mass compared with wild-type mice (35). These data also explain why Tob deficiency enhances osteoblast activity blocking osteoporosis induced by ovariectomy (52). Furthermore, mice deficient in Cnot7, the Tob-interacting deadenylase, also exhibit a high bone mass phenotype and increased responses to BMPs (53).

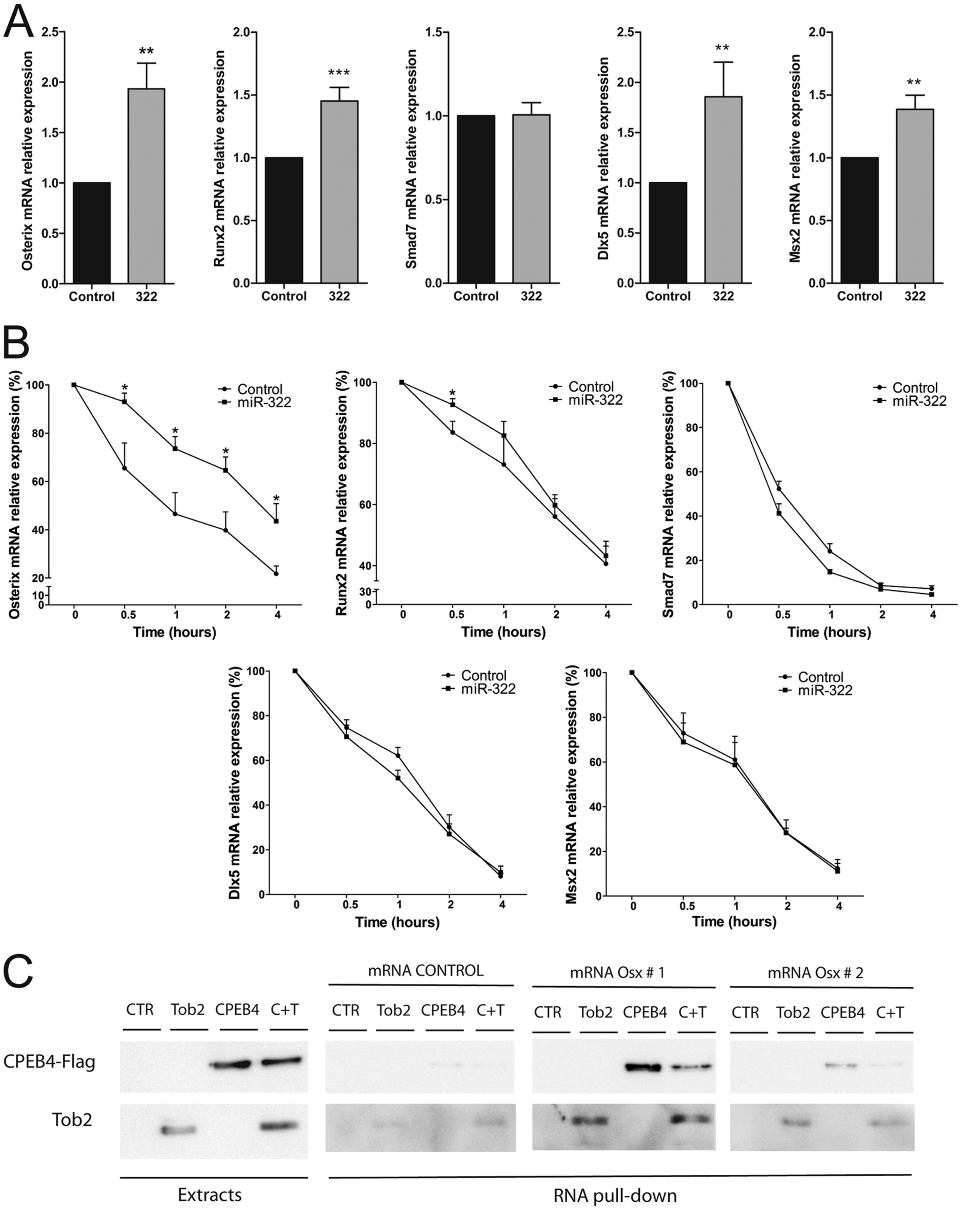

FIGURE 7. **miR-322 stabilizes *Osx* mRNA in MC3T3-E1 cells, and Tob2 binds to the *Osx* 3′-UTR.** *A* and *B*, MC3T3-E1 cells were transfected with miR-322 or miR control for 8 h and cultured overnight. *A*, expression of the indicated mRNAs at time 0. Expression of selected genes is plotted relative to miR control transfection (means ± S.E. of four independent experiments). *B*, actinomycin D was added, and mRNAs were collected at the indicated times. RT-qPCR was performed using GAPDH as the endogenous control. Data are expressed relative to control transfection mRNA levels at time 0 (means ± S.E. of four independent experiments). *C*, HeLa cells were transfected with GFP (control (*CTR*)), Tob2, CPEB4, or CPEB4 and Tob2 (*C+T*). RNA pulldown assay was performed as described under "Experimental Procedures," and the presence of Tob2 and CPEB4 was confirmed by Western blotting. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Long C-terminal regions of both Tob1 and Tob2 include short conserved PAM2 motifs, which are not found in the other BTG/Tob family members. These motifs mediate direct interactions with the poly(A)-binding protein PABPC1 and Cnot7 simultaneously, contributing to the role of Tob1 and Tob2 in general mRNA deadenylation and mRNA turnover (37). Moreover, it has been shown that Tob proteins can interact with CPEB2–4 and specifically intensify the rapid decay of particular transcripts (51). Whereas CPEB1 binds to the cytoplasmic polyadenylation elements (UUUUUAU) in the 3′-UTR of mRNAs, promoting polyadenylation, CPEB2–4 also bind a sequence other than the cytoplasmic polyadenylation element. Their RNA-binding domains recognize mRNAs with specific secondary structures containing U-rich loops and interact with single-stranded uridines as well as double-stranded stems present in the 3′-UTR of the target mRNA (38, 39). Recent studies showed Tob1 interaction with cytoplasmic CPEB2–4, which negatively regulate the expression of a target by tethering to specific mRNA and mediating recruitment of the deadenylase Cnot7, leading to specific mRNA decay (38). Furthermore, a previous study showed that CPEB3 binds to the 3′-UTR of AMPA receptor (*GluR2*) mRNA and negatively regulates its expression, becoming the only known target of CPEB3 at present (39). The *Osx* 3′-UTR contains secondary structures with a stem-loop structure similar to those bound by CPEB2–4 (supplemental Fig. S4) (39). Our RNA pulldown analysis showed that these sequences are directly bound by CPEB proteins and Tob2. Thus, in view of our results, we hypothesize that *Osx* mRNA could be bound by Tob2 and CPEB2–4 and, as a consequence, specifically degraded. Moreover, *in silico* analysis showed that *CPEB2–4* are also putative targets of miR-322 and miR-503, whereas they do not target *CPEB1*. This suggests that, in addition to Tob2, miR-322 could also target *CPEB* genes to coordinately regulate *Osx* expression. Future work is necessary to discern which CPEB-like proteins are involved in the interaction between Tob2 and stem-loop structures in the 3′-UTR of *Osx* and other osteogenic genes.

In summary, we have demonstrated that down-regulation of miR-322 in response to BMP-2 acts as a negative regulator of Osterix expression. We also identified Tob2 accumulation as an important step in fine-tuning the expression of *Osx* and other osteogenic transcription factors during osteoblast differentiation. This novel mechanism highlights the possibility of exploring new therapeutic approaches targeting Tob proteins to treat bone diseases.

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