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RESEARCH ARTICLE

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Hypoxia-responsive miRNA-21-5p inhibits Runx2 suppression by targeting SMAD7 in MC3T3-E1 cells

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Abstract

Sustained hypoxia inhibits osteogenesis and osteoblast differentiation by downregulating the expression of runt-related transcription factor 2 (Runx2). MicroRNAs (miRNAs) have been shown to regulate osteogenesis and osteoblast differentiation. In the present study, we profiled miRNAs, with microRNA array and quantitative real-time polymerase chain reaction (RT-PCR) methods, in mouse osteoblast (MC3T3-E1) cells under hypoxia. Then, we investigated regulation by miRNA-21-5p on the expression of Runx2 and other osteoblast differentiation-associated markers via gain-of-function and loss-of-function strategies. We found that expression of miRNA-21-5p, miRNA-210-5p, and other eight miRNAs was upregulated significantly in hypoxia-treated MC3T3-E1 cells. miRNA-21-5p overexpression downregulated the expression of the mRNA and protein of suppressor of mothers against decapentaplegic (*SMAD7*) markedly, the 3′-untranslated region (3′-UTR) of which was highly homologous with the miRNA-21-5p sequence. miRNA-21-5p overexpression upregulated the protein expression of Runx2 in hypoxia-treated MC3T3-E1 cells, although mRNA expression of Runx2 and other osteoblast differentiation-associated molecules (eg, osteocalcin, procollagen type 1 amino-terminal propeptide, P1NP) were not regulated by it; such upregulation was SMAD7-dependent. In conclusion, hypoxia-responsive miRNA-21-5p promoted Runx2 expression (at least in part) by targeting the 3′-UTR and downregulating *SMAD7* expression. Our study suggests a protective role of miRNA-21-5p in promoting osteoblast differentiation under hypoxia.

KEYWORDS

hypoxia, miRNA-21-5p, osteoblast differentiation, Runx2, SMAD7

1 | INTRODUCTION

Sustained hypoxia can induce bone loss under various pathophysiologic conditions, such as ischemia and vascular diseases.[1] Studies have demonstrated inhibition to osteogenesis by hypoxia in human mesenchymal stem cells (MSCs) by downregulation of expression of runt-related transcription factor 2 (Runx2).[2] Direct exposure to

Abbreviations: IGF1, insulin-like growth factor 1; JAKs, Janus activated kinases; MAPKs, p38 mitogen-activated kinases; MCSs, mesenchymal stem cells; miRNAs, microRNA; Runx2, runt-related transcription factor 2; STAT, signal transducer and activator of transcription.

hypoxia alone is sufficient to suppress Runx2 expression in osteoblasts grown in standard tissue culture plates and, thus, leads to suppressed osteoblast differentiation.[3]

Revealing bone-related pathology using the mechanism of osteoblast differentiation is problematic.[4,5] Diverse molecules and signaling pathways have regulatory roles in osteoblast differentiation to various degrees, such as insulin-like growth factor-1,[6] p38 mitogen-activated kinases[7] and Janus-activated kinases/signal transducer and activator of transcription (STAT) pathways.[8,9] However, Runx2 has a pivotal regulatory role in osteoblast differentiation because of posttranslational modifications.[10-12]

In terms of Runx2-mediated regulation of osteoblast differentiation, other molecules and processes have been implicated.[12-14] Runx2 activity is regulated negatively by posttranslational modifications such as acetylation, phosphorylation, sumoylation, and ubiquitination.[11,15] Suppressor of mothers against decapentaplegic (SMAD), STAT1, twist, and hey1 form complexes with Runx2, resulting in inhibition of osteoblast differentiation.[10,15]

Micro-RNAs (miRNAs) are a group of endogenous RNA molecules of size 18 to 25 nucleotides (nts). They have recently been recognized as regulating osteoblast differentiation via inhibition of expression of target genes during differentiation of MSCs to osteoblasts. Accumulating evidence suggests that certain miRNAs can regulate Runx2 expression and osteoblast differentiation, such as miRNA-451,[16] miRNA-455-5p,[17] and miRNA-133a-5p.[18]

We wished to study miRNA regulation of osteoblast differentiation in response to hypoxia, and the association of miRNA expression with Runx2 expression. First, we profiled miRNA expression and measured Runx2 expression in hypoxia-treated mouse embryo osteoblast precursor (MC3T3-E1) cells. Then, we overexpressed the hypoxia-upregulated miRNA-21-5p and investigated its regulation of expression of Runx2 and other osteoblast differentiation-associated molecules. Our study provides a novel understanding of the role of miRNAs in the regulation of Runx2 expression and osteoblast differentiation.

2 | MATERIALS AND METHODS

2.1 | Cell culture and hypoxia treatment

MC3T3-E1 cells (American Type Culture Collection, Rockville, MD) were cultured with α-minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Pittsburgh, PA) and 1% penicillin/streptomycin solution (Invitrogen). Cell culture was undertaken at 37°C with 18% CO_2 and 5% CO_2 in a humid incubator.

For hypoxia culture, a mixture of 5% CO_2, 94% nitrogen and 1% oxygen was supplied to the incubator and monitored. To promote or downregulate cellular expression of miRNA-21-5p, 85%-confluent MC3T3-E1 cells in 12- or 24-well plates were transfected with miRNA-21-5p mimics (30 or 60 nM) or with a miRNA-21-5p inhibitor (20 or 40 nM) (scrambled RNA was taken as the control for miRNA-21-5p mimics or the inhibitor) with INTERFERin in a silent RNA transfection reagent (Polyplus Transfection, San Marcos, CA) according to the kit's manual. Scrambled RNA of equal concentration was taken as a negative control. To overexpress SMAD7, the *SMAD7* coding sequence was cloned into a eukaryotic expression vector: pcDNA3.1(+). The recombinant SMAD7-pcDNA3.1(+) was transfected into MC3T3-E1 cells with lipofectamine 3000 (Invitrogen), and then the expression of the mRNA and protein of SMAD7 was measured.

2.2 | Extraction and microarray analyses of miRNAs

Cellular miRNAs from hypoxia- or normoxia-treated MC3T3-E1 cells were extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), supplemented with RNase Inhibitor (Thermo Fisher Scientific, Rockford, IL), and then stored at − 80°C before use. miRNA samples for microarray analyses had a purity of 1.8 to 2.0 according to the absorbance at 260 nm/280 nm. miRNA samples were labeled with a Flash Tag Biotin HSR RNA Labeling kit (Affymetrix, Santa Clara, CA) and then hybridized with microRNA 4.0 Array (Affymetrix) under manufacturer protocols. Scan Array Express 1.0 (PerkinElmer, Waltham, MA) was utilized for scanning of hybridization signals, which were analyzed with Expression Console (Affymetrix). Each miRNA value was calculated by the $\log_2$ transformation of normalized data. Quantitative real-time polymerase chain reaction (qRT-PCR) was undertaken to quantify deregulated miRNAs. qRT-PCR was done with a mirVana qRT-PCR miRNA Detection Kit (Thermo Fisher Scientific) according to the kit manual. The qRT-PCR value was calculated by the $2^{-\Delta\Delta C_t}$ method[19] using U6 as the internal control.

2.3 | mRNA extraction and qPCR

Total mRNA was extracted from MC3T3-E1 cells with TRIzol Reagent (Life Technologies, Grand Island, NY) and was dissolved in RNase-free water. The mRNA level of SMAD2, SMAD3, SMAD4, SMAD7, osteocalcin, procollagen type 1 amino-terminal propeptide (P1NP), and Runx2 were analyzed by qRT-PCR. The primers for qRT-PCR were designed by Primer premier 5, according to the mRNA sequence of each marker provided by the National Center for Biotechnology Information. qRT-PCR was done with a One-Step SYBR Prime Script

PLUS RT-PCT kit (Takara Bio, Tokyo, Japan) according to manufacturer instructions. The mRNA level of each marker was calculated by the $2^{-\Delta\Delta C_t}$ method[19] using β-actin as the internal control and presented as the mean ± SEM for three experiments carried out independently.

2.4 | Western blot analysis

The protein expression of SMAD7, osteocalcin, P1NP, and Runx2 was examined by Western blot analysis. MC3T3-E1 cells were harvested by scratching and were lysed into ice-cold cell lysis buffer (Bio-Rad Laboratories, Hercules, CA). Cellular proteins were purified in the supernatant from cell debris after centrifugation at 12 000 × *g* for 15 minutes at 4°C. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). PVDF membranes were blocked with 5% skimmed milk powder overnight at 4°C. PVDF membranes were incubated with SMAD7-, osteocalcin-, P1NP- or Runx2-specific rabbit polyclonal antibodies

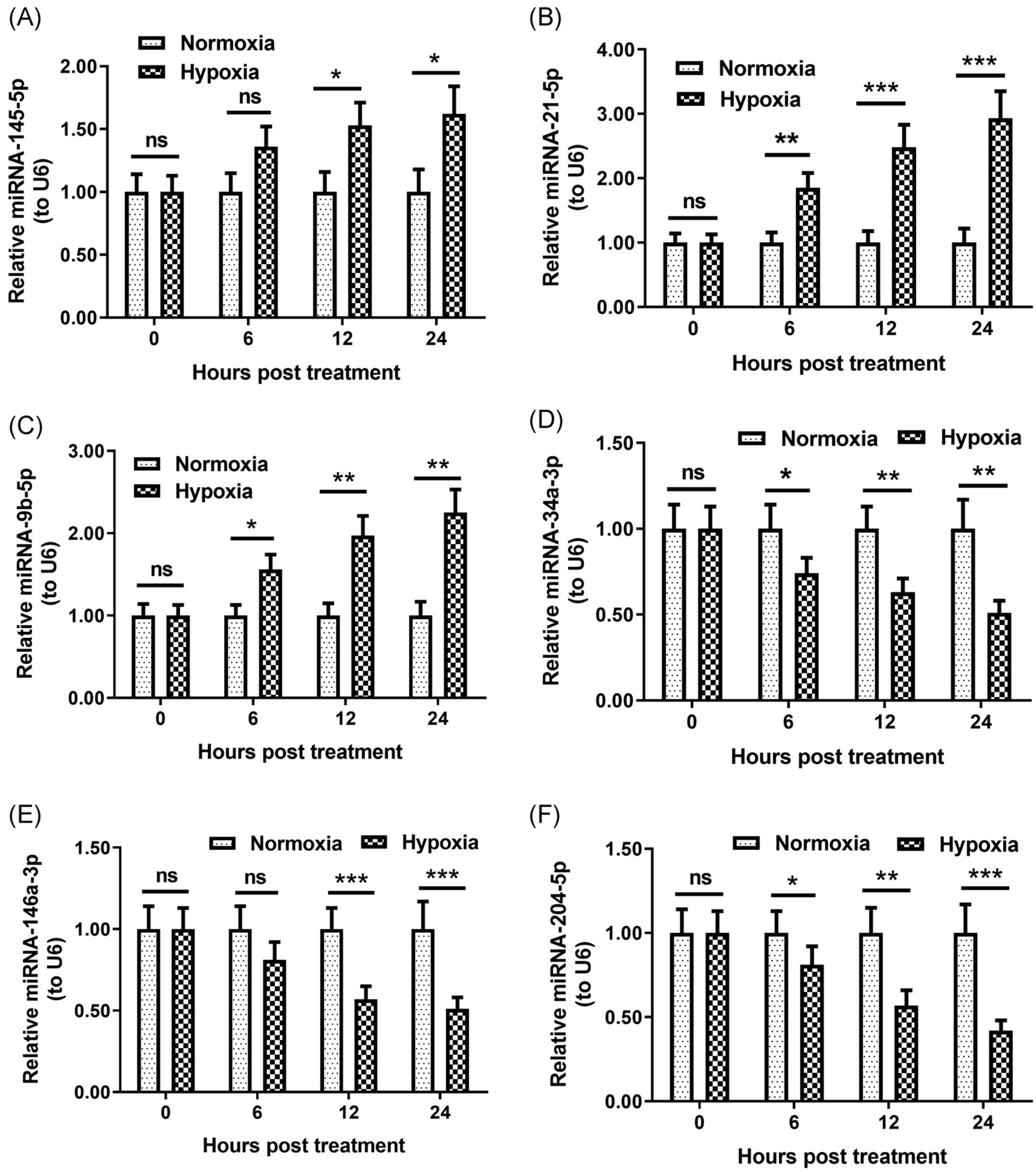

FIGURE 1 Upregulated and downregulated miRNAs in MC3T3-E1 cells under hypoxia. MC3T3-E1 cells were cultured under hypoxia or normoxia for 0, 6, 12, or 24 hours, and cellular miRNAs examined by qPCR. A-F, Relative levels of miR-145-5p (A), miR-21-5p (B), miR-9b-5p (C), miR-34a-3p (D), miR-146a-3p (E), miR-204-5p (F). Each of these miRNAs with >1.5-fold value change in the microarray. miRNA, microRNAs; qPCR, quantitative polymerase chain reaction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant, by the Student *t*-test

(1:1000 dilution; Abcam, Cambridge, UK) in Tris-buffered saline adding Tween 20 (TBST) at 37°C for one h. Subsequently, they were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories) for 30 minutes at 37°C. Finally, PVDF membranes were incubated with electrochemiluminescence solution (Thermo Fisher Scientific) and the specific protein bands were scanned by a Smart Chemi Lamp system (Thermo Fisher Scientific). PVDF membranes were washed three times with TBST before each incubation. The protein level of each marker was quantified according to the band density with β-actin as the loading control. Each value was averaged for three independent results.

2.5 | Luciferase reporting assay

We wished to examine target regulation by miRNA-21-5p on SMAD7 expression. We used the luciferase reporting assay with the reporter recombinant plasmids pLuc-SMAD7 3′-untranslated region (3′-UTR) and pLuc-SMAD7 3′-UTR (mutant) with the wild or mutated 3′-UTR of *SMAD7*. The paired sequences between miRNA-21-5p and 3′-UTR of *SMAD7* were selected after alignment. The sequence of the wild and mutant 3′-UTR of *SMAD7* was synthesized by SangonBio (Shanghai, China) and was inserted into the pCMV-GLuc two vectors (New England Biolabs, Ipswich, MA) just downstream of the luciferase reporter. The recombinant plasmid pLuc-SMAD7 3′-UTR and pLuc-SMAD7 3′-UTR^{mut} and miRNA-21-5p mimics were cotransfected into MC3T3-E1 cells for 24 hours. Relative luciferase activity was assayed with a Dual-Luciferase Assay kit (Promega, Madison, WI) by GloMax (Promega).

2.6 | Statistical analyses

Quantitative results are the mean ± SEM for three or more independent experiments. The Student *t* test analyzed the difference between two groups with Prism (GraphPad, La Jolla, CA). $P < 0.05$ was considered significant.

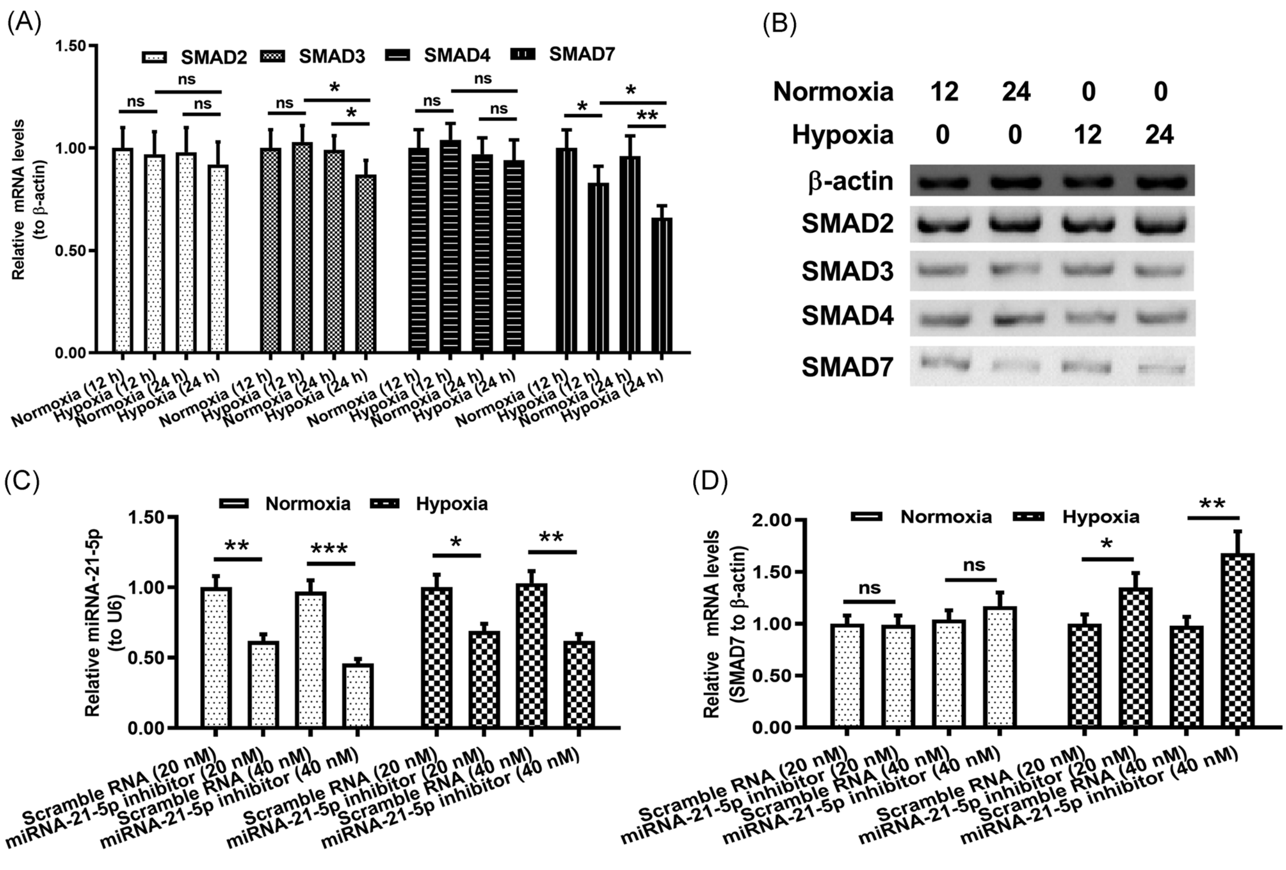

FIGURE 2 Hypoxia-promoted miRNA-21-5p downregulates SMAD7 expression in MC3T3-E1 cells. MC3T3-E1 cells were cultured under normoxia or hypoxia for 12 or 24 hours, and then were collected for the quantification of SMADs. A and B, qPCR for mRNA level (A) and Western blot analysis for protein level of SMAD2, 3, 4, and 7 in the MC3T3-E1 cells under normoxia or hypoxia for 12 or 24 hours; C and D, qPCR for miRNA-21-5p (C) and for SMAD7 (D) in the hypoxia- or normoxia-treated MC3T3-E1 cells after transfection of 20 or 40 nM of scrambled RNA or an miRNA-21-5p inhibitor. miRNA, microRNA; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SMAD, suppressor of mothers against decapentaplegic. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant by the Student *t*-test

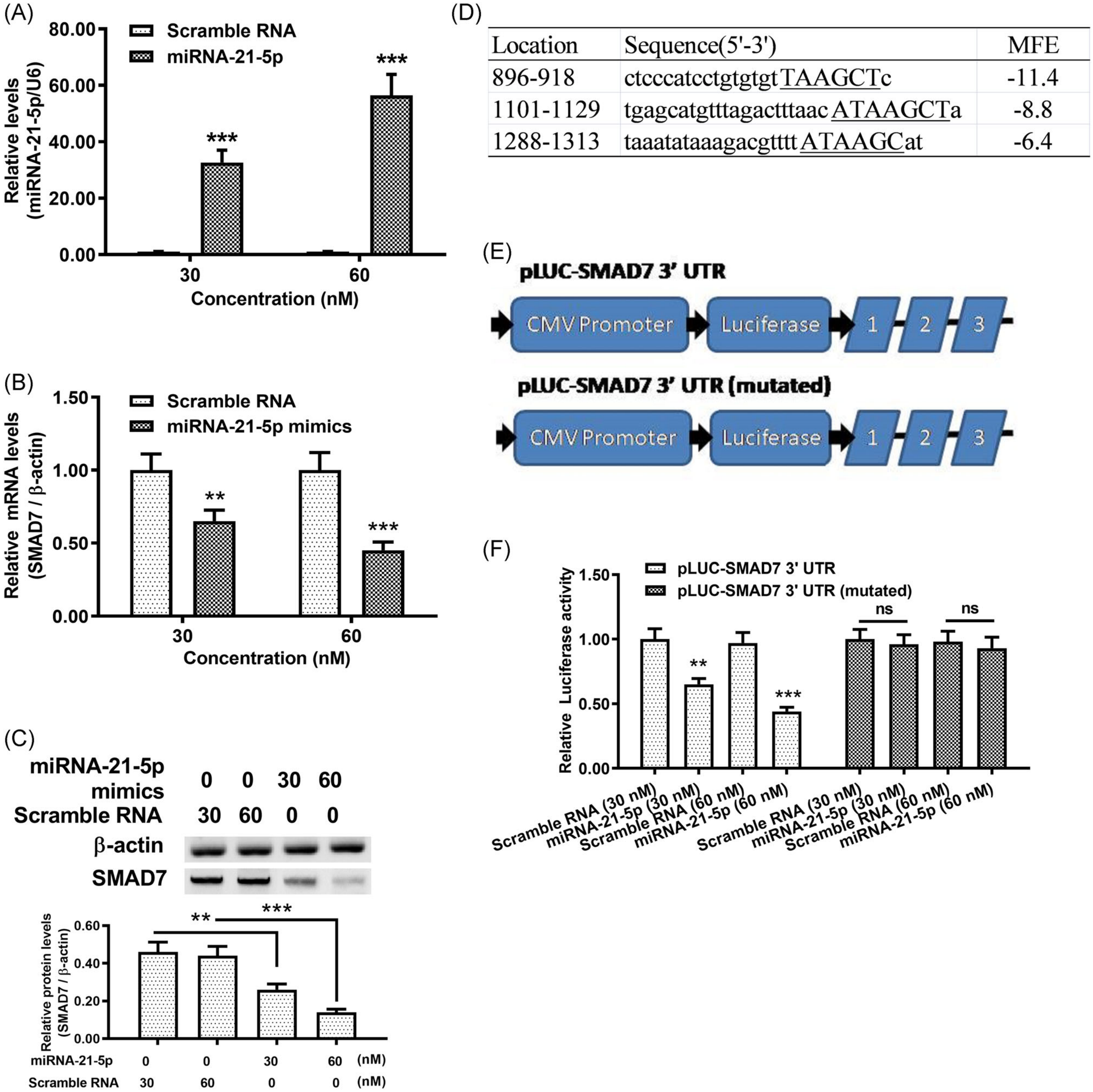

FIGURE 3 miRNA-21-5p targets the 3′-UTR of *SMAD7* and downregulates SMAD7 expression in MC3T3-E1cells. MC3T3-E1 cells were transfected with 30 or 60 nM scrambled RNA or miRNA-21-5p mimics for 24 hours, and then the expression of miRNA-21-5p (A) in MC3T3-E1 cells were quantified with qPCR; the mRNA (B) and protein (C) levels of SMAD7 were quantified in control (scrambled) or miRNA-21-5p-overexpressed MC3T3-E1 cells. D, Alignment of the miRNA-21-5p sequence with the 3′-UTR of *SMAD7*. E, Luciferase reporter plasmid with the normal or mutant 3′-UTR of SMAD7 (schematic). F, Relative luciferase activity in MC3T3-E1 cells after transfection with 30 or 60 nM of scrambled RNA or miRNA-21-5p mimics with normal or mutant reporters. 3′-UTR, 3′-untranslated region; miRNA, microRNA; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SMAD7, suppressor of mothers against decapentaplegic homolog 7 **$P < 0.01$, ***$P < 0.001$ or ns, no significance

3 | RESULTS

3.1 | miRNA profile in MC3T3-E1 cells under hypoxia

miRNA profiling indicated that expression of various miRNAs was upregulated or downregulated in hypoxia-treated MC3T3-E1 cells. There were 725 and 721 valid miRNA signals in hypoxia and normoxia groups, respectively (Figure S1A). These miRNAs ranged in length from 18 to 28 nts, without a significant difference in length between the two groups. We found 663 ± 94 miRNAs in both groups.

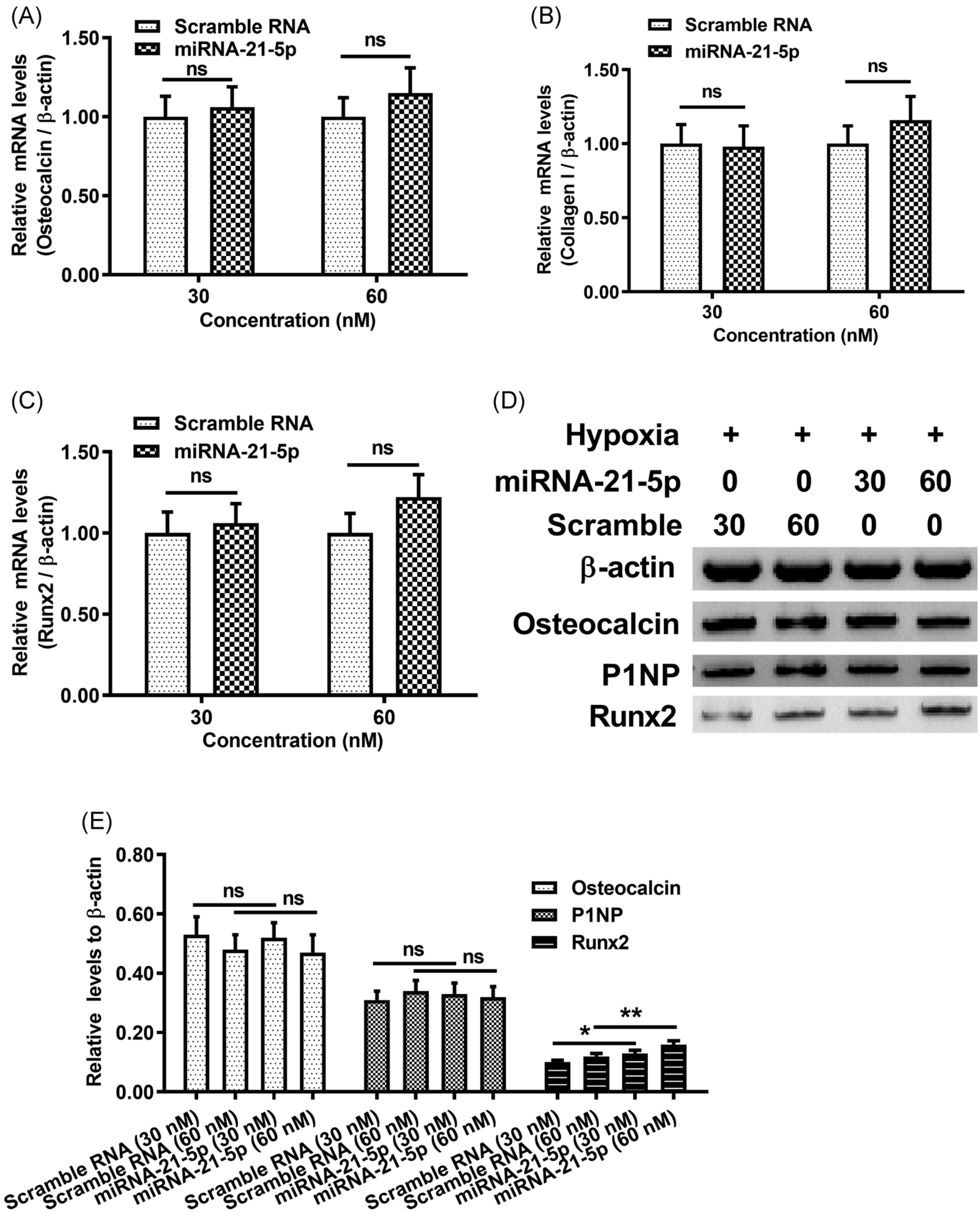

FIGURE 4 Expression of osteoblast differentiation-associated molecules in MC3T3-E1 cells after miR-21-5p overexpression. A–C, Relative mRNA levels of osteocalcin, P1NP, and Runx2 in MC3T3-E1 cells transfected with 30 or 60 nM of miRNA-21-5p mimics or scrambled miRNA for 12 hours; D and E, Western blots (D) for osteocalcin, P1NP, and Runx2 in miRNA-21-5p-overexpressed or control MC3T3-E1 cells (24 hours posttransfection). Each protein is presented as a relative level in comparison with β-actin (E). miRNA, microRNA; P1NP, procollagen type 1 amino-terminal propeptide; Runx2, runt-related transcription factor 2. *$P < 0.05$, **$P < 0.01$; ns, no significance

Among them, there were 10 miRNAs with > 1.5-fold higher, and 7 miRNAs with > 1.5-fold lower signals than those in hypoxia-treated cells (Figure S1B). Quantified signals for the most-regulated 17 miRNAs are indicated in a "heatmap" (Figure S1C) for all four independent results. The heatmap demonstrated that 10 miRNAs (21-5p, 252a-5p, 9b-5p, 122-3p, 7a-5p, 200-5p, 181a-5p, 128a-5p, 210-5p, and 145-5p) were upregulated and 7 miRNAs (30b-3p, 146a-5p, 182-5p, 522-3p, 34a-5p, 204-5p, and 184b-5p) were down-regulated. We also undertook qRT-PCR for significantly regulated (both upregulated) and down-regulated) miRNAs. The significantly up- or down-regulated miRNAs for two or more time points are indicated in Figure 1A-F ($P < 0.05$, 0.01 or 0.001 for 6, 12, or 24 hours posttreatment, respectively).

3.2 | Hypoxia-associated miRNA-21-5p targets SMAD7 in hypoxia-treated MC3T3-E1 cells

SMAD families have been hypothesized to be deregulated in response to hypoxia in multiple cell types.[20-22] We measured the expression of four types of SMADs (2, 3, 4, and 7) in hypoxia- or normoxia-treated MC3T3-E1 cells. Expression of SMAD3 and SMAD7 was downregulated significantly 12 hours and 24 hours after hypoxia ($P < 0.05$ or $P < 0.01$) (Figure 2A). No significant difference was found between hypoxia and normoxia groups for SMAD2 or SMAD4. The downregulation on SMAD3 and SMAD7 was also observed in protein level by Western blot analysis (Figure 2B). miRNA-21-5p has been shown to regulate *SMADs*,[23] particularly *SMAD7*.[23,24] To investigate the role of miRNA-21-5p on downregulation of SMAD7 expression by hypoxia, we blocked upregulation of miRNA-21-5p expression with a miRNA-21-5p-specific inhibitor in hypoxia-treated MC3T3-E1 cells (Figure 2C). Relatively higher SMAD7 expression was found in miRNA-21-5p inhibitor-transfected cells under hypoxia ($P < 0.05$ or $P < 0.01$) (Figure 2D).

Conversely, promotion of miRNA-21-5p expression via miRNA-21-5p mimicked transfection ($P < 0.01$) (Figure 3A) and reduced expression of SMAD7 mRNA ($P < 0.01$ or $P < 0.001$) (Figure 3B) and protein ($P < 0.01$ and $P < 0.001$) markedly (Figure 3C) in MC3T3-E1 cells. Also, according to the sequence alignment of miRNA-21-5p with the 3′-UTR of *SMAD7*, there were three highly homologous matching sites between them (Figure 3D). Next, we constructed luciferase reporter plasmids with these three highly homologous sequences to the 3′-UTR of SMAD7 or with the mutant sequences (Figure 3E). Figure 3F demonstrates targeted inhibition of luciferase activity by miRNA-21-5p mimics (30 or 60 nM) ($P < 0.01$ or $P < 0.001$), whereas significant regulation by the mimics was not observed when the mutated reporter was utilized. Thus, we showed that miRNA-21-5p down-regulated *SMAD7* expression by targeting its 3′-UTR in response to hypoxia in MC3T3-E1 cells.

3.3 | Upregulation of miRNA-21-5p expression inhibits the hypoxia-induced reduction in Runx2 expression in MC3T3-E1 cells

Inhibition of osteoblast differentiation by hypoxia has been documented in MC3T3-E1 cells.[25] To examine a possible regulatory role of miRNA-21-5p in this process, we measured the expression of the osteoblast differentiation-associated markers osteocalcin, P1NP and Runx2 in MC3T3-E1 cells with or without upregulation of miRNA-21-5p expression. qRT-PCR results indicated that mRNA expression of osteocalcin, P1NP and Runx2 was not significantly different between miRNA-21-5p-transfected and scrambled RNA-transfected MC3T3-E1 cells (Figure 4A-C), though mRNA expression of each marker seemed to be high in the

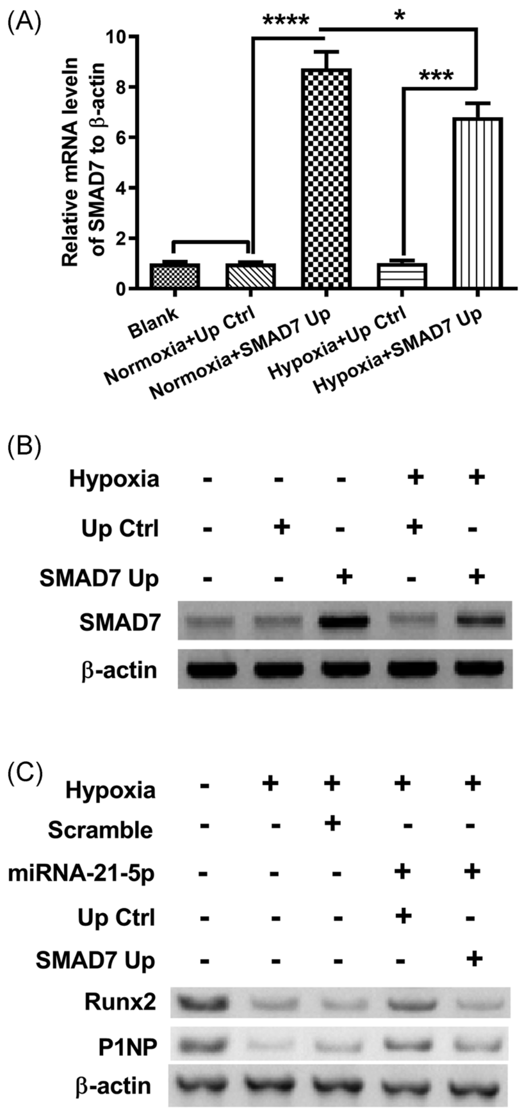

FIGURE 5 Influence of SMAD7 overexpression on miRNA-21-5p-mediated regulation of Runx2 expression in MC3T3-E1 cells. A and B, mRNA (A) and protein (B) expression of SMAD7 in the SMAD7-overexpressed MC3T3-E1 cells under normoxia or hypoxia; C, Western blot analysis for P1NP and Runx2 in the miRNA-21-5p-upregulated MC3T3-E1 cells with or without SMAD7 overexpression (24 hours posttransfection). Up Ctrl: Control for SMAD7 overexpression (SMAD7 UP); miRNA, microRNA; mRNA, messenger RNA; P1NP, procollagen type 1 amino-terminal propeptide; Runx2, runt-related transcription factor 2; SMAD7, suppressor of mothers against decapentaplegic homolog 7. *$P < 0.05$, ***P < 0.001, ****$P < 0.0001$

miRNA-21-5p group. Western blot analysis confirmed no significant regulation by miRNA-21-5p on protein expression of osteocalcin or P1NP (Figure 4D,E). However, Runx2 expression was upregulated markedly in miRNA-21-5p-transfected MC3T3-E1 cells at 30 or 60 nM ($P < 0.05$ and $P < 0.01$) (Figure 4D,E).

Next, we repeated experiments in hypoxia-treated MC3T3-E1 cells with or without SMAD7 overexpression. SMAD7 expression was upregulated significantly by SMAD7 overexpression at mRNA ($P < 0.001$) (Figure 5A) and protein (Figure 5B) levels under normoxia or hypoxia. Next, we measured the expression of Runx2 and P1NP in MC3T3-E1 cells: under normoxia; under hypoxia; with or without upregulation of miRNA-21-5p expression; with or without SMAD7 overexpression. Expression of Runx2 and P1NP was downregulated markedly by hypoxia in MC3T3-E1 cells (Figure 5C). However, upregulation of miRNA-21-5p expression attenuated such downregulation. Conversely, additional SMAD7 overexpression blocked such attenuation.

4 | DISCUSSION

miRNAs (eg, miRNA-21 family) have been shown to regulate osteoblast differentiation. In the present study, we demonstrated marked downregulation and upregulation of miRNA expression in MC3T3-E1 cells under hypoxia using qRT-PCR. miRNA-21-5p and miRNA-9b-5p showed the most significant upregulation of expression in hypoxia-treated MC3T3-E1 cells. miRNA-21-5p has been reported widely to be responsive to hypoxia, especially in hypoxic tumor microenvironments.[26,27] miRNA-21-5p overexpression has also been verified to regulate osteoblast proliferation in vitro.[28] SMAD families have been suggested to be deregulated in response to hypoxia in multiple cell types.[28] In the present study, we found four types of SMADs (2, 3, 4, and 7) to be regulated differently by hypoxia in MC3T3-E1 cells. Expression of SMAD3 and SMAD7 was downregulated significantly in hypoxia-treated MC3T3-E1 cells.

Recently, targeting of Smad7 by miR-21 and its family member, miR-590, has been reported by several reports.[29-31] Given the possible roles of miRNA-21-5p and SMADs in osteoblasts in response to hypoxia, we manipulated the miRNA-21-5p with mimics and an inhibitor of miRNA-21-5 and then investigated its well-recognized target SMAD, SMAD7. Our results confirmed negative target regulation by miRNA-21-5p on SMAD7 expression in MC3T3-E1 cells under normoxia or hypoxia, as indicated by luciferase reporter with three predicted target sites. Interestingly, each of them (without repeats) was markedly less responsive to miR-21-5p. We speculated that miRNA-21-5p might be a protective marker in response to hypoxia in osteoblasts.

To link the hypoxia-responsive miRNA-21-5p with osteoblast differentiation, we measured the expression of the osteoblast differentiation-related markers Runx2, osteocalcin, and P1NP in hypoxia-treated MC3T3-E1 cells by upregulating or downregulating miRNA-21-5p expression. Interestingly, the mRNA expression of these markers was not regulated markedly by hypoxia. Only Runx2 expression was upregulated significantly by miRNA-21-5p overexpression. Moreover, such upregulation was associated with target regulation by miRNA-21-5p on *SMAD* and was blocked by SMAD7 overexpression. However, we could not exclude other Runx2-downregulation pathways under hypoxia. Our study just indicates the regulation on Runx2 by miRNA-21-5p/Smad7 pathway. It is hard to say what degree the endogenous miRNA-21-5p is responsible for the Runx2 protein level under the hypoxia condition.

5 | CONCLUSIONS

miRNA-21-5p can regulate markers of osteoblast differentiation, in association with its target inhibition of SMAD7 expression, in response to hypoxia in osteoblasts. Our study suggests that miRNA-21-5p might be a protective marker for osteoblast differentiation under hypoxia.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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