**Hsa-let-7c controls the committed differentiation of IGF-1-treated mesenchymal stem cells derived from dental pulps by targeting IGF-1R via the MAPK pathways**

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**Abstract**

The putative tumor suppressor microRNA let-7c is extensively associated with the biological properties of cancer cells. However, the potential involvement of let-7c in the differentiation of mesenchymal stem cells has not been fully explored. In this study, we investigated the influence of hsa-let-7c (let-7c) on the proliferation and differentiation of human dental pulp-derived mesenchymal stem cells (DPMSCs) treated with insulin-like growth factor 1 (IGF-1) via flow cytometry, CCK-8 assays, alizarin red staining, real-time RT-PCR, and western blotting. In general, the proliferative capabilities and cell viability of DPMSCs were not significantly affected by the overexpression or deletion of let-7c. However, overexpression of let-7c significantly inhibited the expression of IGF-1 receptor (IGF-1R) and downregulated the osteo/odontogenic differentiation of DPMSCs, as indicated by decreased levels of several osteo/odontogenic markers (osteocalcin, osterix, runt-related transcription factor 2, dentin sialophosphoprotein, dentin sialoprotein, alkaline phosphatase, type 1 collagen, and dentin matrix protein 1) in IGF-1-treated DPMSCs. Inversely, deletion of let-7c resulted in increased IGF-1R levels and enhanced osteo/odontogenic differentiation. Furthermore, the ERK, JNK, and P38 MAPK pathways were significantly inhibited following the overexpression of let-7c in DPMSCs. Deletion of let-7c promoted the activation of the JNK and P38 MAPK pathways. Our cumulative findings indicate that Let-7c can inhibit the osteo/odontogenic differentiation of IGF-1-treated DPMSCs by targeting IGF-1R via the JNK/P38 MAPK signaling pathways.

**Introduction**

Insulin-like growth factor 1 (IGF-1) is a polypeptide hormone that is mainly synthesized by the liver. It is the most abundant growth factor in the bone matrix and plays an important role in bone maintenance and remodeling. In general, IGF-1 mediates cell behaviors via the IGF-1 receptor (IGF-1R, a transmembrane tyrosine kinase-containing receptor). Following binding of the IGF-1 ligand, IGF-1R can activate downstream signaling pathways that subsequently regulate cell growth, apoptosis, mineralization, differentiation, and osteogenesis, and thus the IGF-1/IGF-1R pathway is known as the IGF-1/IGF-1R axis. Various studies have demonstrated that IGF-1 can promote the proliferation and osteo/odontogenic...
differentiation of mesenchymal stem cells (MSCs) in different tissues. Endogenous and exogenous IGF-1 signaling mediated through IGF-1R plays an important role in the differentiation and morphogenesis of human embryonic stem cells in three-dimensional culture. IGF-1 can significantly improve the proliferative and survival capabilities of neural progenitor-like cells derived from bone marrow mesenchymal stem cells (BMSCs). Moreover, low IGF-1 has been shown to be a risk factor for osteoporosis and bone fractures. IGF-1/IGF-1R signaling has also been shown to be important for both the terminal differentiation of osteoprogenitors from bone MSCs and bone mass acquisition. Thus, the IGF-1/IGF-1R axis plays a critical role in the proliferation and osteogenic differentiation of MSCs. IGF-1 and insulin activate ERK1/2 mitogen-activated protein kinase (MAPK) via the type 1 IGF receptor in mouse embryonic stem cells. Our previous studies have demonstrated that IGF-1 can upregulate the osteo/odontogenic differentiation of MSCs by activating the MAPK signaling pathways. During the process of bone resorption, the release of IGF-1 from the bone matrix can induce the differentiation of MSCs toward an osteoblast lineage by activating mTORC signaling to maintain the bone microarchitecture and mass. Moreover, IGF/IGF-1R can indirectly stimulate the PI3K/Akt pathway in an interactive osteogenic signaling network, which is necessary for both bone development and remodeling. Despite the enormous progress in the mechanistic, pathway-level understanding of IGF-1-mediated differentiation of MSCs, there is still a lack of understanding of the committed differentiation induced by IGF-1 at the microRNA (miRNA) level.

miRNAs are non-coding RNAs that are 21–23 nucleotides in length and posttranscriptionally regulate protein expression by directly binding to the 3’-untranslated regions (3’-UTRs) of target genes. They play important roles in numerous biological processes, including development, apoptosis, longevity, and metabolism. Originally discovered in the nematode Caenorhabditis elegans, let-7 miRNA plays a critical role in regulating cell proliferation and differentiation and also participates in the maintenance of stem cell niches. Let-7c is one member of the let-7 family; it maps to human chromosome 21q11.21 and is known as a putative tumor suppressor in several cancer cell lines. Further, let-7c markedly promotes ectopic bone formation and suppresses adipogenesis by targeting the high-mobility group AT-hook 2 in MSCs derived from human adipose tissues. Differentiation of MSCs is usually under precise regulation by multiple modifiers, including miRNAs and related signaling pathways. To date, little is known about the role of let-7c and its involvement in pathways that are critical for the committed differentiation of IGF-1-treated MSCs.

In the present study, we investigated the potential gene targets of hsa-let-7c by TargetScan, miRDB, and microRNA.org; clarified the interaction between let-7c and IGF-1R as well as the effects of these two genes on the proliferation and differentiation of dental pulp MSCs; and further explored the potential involvement of let-7c in various signaling pathways.

Materials and methods

Cell isolation and culture

Normal human impacted third molars were collected with informed consent from patients (18–25 years of age) in the Oral Surgery Department of Jiangsu Provincial Stomatological Hospital. Dental pulp was carefully isolated from the impacted third molars and primary DPMSs were enzymatically separated, as reported previously. These cells were purified using rabbit anti-STRO-1 antibody (Santa Cruz, Delaware, CA) and sheep anti-rabbit IgG Dynabeads (Dynal Biotech, Oslo, Norway) according to the standard procedure for magnetic-activated cell sorting. Purified DPMScs were seeded at 1 × 10^4 cells/mL into 6 cm culture dishes and subsequently cultured in alpha minimum essential medium (α-MEM, Gibco, Life Technologies, Grand Island, NY, http://www.lifetechnology.com/) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified, 5% CO₂ atmosphere. Cells were subcultured at a 1:3 ratio when they reached 80–90% confluence and the culture medium was replaced every 2 days. DPMScs passaged between three and five times were used in the following experiments.

Cell identification

To investigate the origin of isolated multiclonal cells, cells were harvested and incubated with various combinations of the following fluorochrome-conjugated rabbit anti-human antibodies (CD34-FITC, CD146-APC, CD105-APC, CD73-PE, CD45-PerCP, CD90-PE; Miltenyi, Germany, http://www.miltenyibiotec.com) and rat anti-human antibody STRO-1 (BioLegend, USA, http://www.biolegend.com) for 20 min at room temperature in the dark. The corresponding mouse IgG isotype control antibodies conjugated to FITC, APC, PE, or PerCP were employed as negative controls in each experiment. 0.01 mol/L PBS was used as a negative control for STRO-1 staining. Stained cells were washed twice with 0.01 mol/L PBS and analyzed by flow cytometry (FCM) (FACSCalibur, BD Biosciences, USA).
**Lentivirus transfection**

LV-hsa-let-7c and LV-hsa-let-7c-inhibition lentiviruses were purchased from GENECHEM (Shanghai, China, [http://www.genechem.com.cn/]). The LV-hsa-let-7c (+Let-7c) vector consisted of the pre-hsa-let-7c sequence cloned into a GV159 plasmid. A GV159 plasmid expressing a scrambled RNA was used as a control (+Ctrl). The LV-hsa-let-7c-inhibition (−Let-7c) vector consisted of the reverse complement of the mature hsa-let-7c transcript cloned into a GV159 plasmid. A GV159 plasmid expressing a scrambled RNA was used as a control (−Ctrl). For better observation, all lentiviruses encoded a green fluorescence protein (GFP) gene sequence. DPMSCs at 60–70% confluence were transfected with let-7c lentiviruses for 12 h in 2 mL α-MEM supplemented with 10% FBS and 0.05 mg/mL polybrene (Santa Cruz, USA).

**Cell counting kit-8 assay**

DPMSC proliferation was measured using the commercial cell counting kit (CCK)-8 in accordance with the manufacturer’s protocol. Briefly, cells were seeded into 96-well cell culture cluster plates (Corning, Corning, NY, USA) at an initial density of 3 × 10^4 cells/well in α-MEM containing 10% FBS until 60% confluence, and then serum-starved for 24 h. CCK-8 reagents (Dojindo, Kumamoto, Japan, [http://www.dojindo.eu.com]) were added to a subset of wells at the indicated time points, and the absorbance was quantified using an automated plate reader. Absorbency was measured at a test wavelength of 490 nm and a reference wavelength of 650 nm using a micro-plate reader (Bio-Rad). CCK-8 results were expressed as the mean ± standard deviation (SD) and the experiment was performed three times.

**Flow cytometry**

Cells in each group were harvested with trypsin (Beyotime, Haimen, China) and fixed with 75% ice-cold ethanol at 4 °C for 24 h in the dark. Then, each sample was measured using a FAC-Scan flow cytometer (BD Biosciences, San Jose, CA). Cell cycle fractions (G0/G1, S, and G2/M phases) and cell apoptosis levels were determined by FCM. Three independent experiments were performed for each group.

**Alizarin red staining**

To evaluate the osteogenic differentiation of +Let-7c/−Let-7c DPMSCs, DPMSCs were seeded into 24-well plates (Corning) at a density of 1 × 10^4 cells/well, infected with +Let-7c/−Let-7c lentiviruses and then incubated in four different media (α-MEM, α-MEM+100 ng/mL IGF-1, MM, MM+100 ng/mL IGF-1). MM is the mineralization-inducing medium containing α-MEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (Sigma-Aldrich, American, [http://www.sigmaaldrich.com/]), 50 mg/L ascorbic acid (Sigma-Aldrich, American), 10 mmol/L β-glycerophosphate (Sigma-Aldrich, American), and 10 nmol/L dexamethasone (Sigma-Aldrich, American).

After 2 weeks of mineralization induction, cells were fixed in ice-cold 70% ethanol for 30 min and stained with alizarin red (40 mM, pH = 4.2, Sigma-Aldrich) for 5 min at room temperature. Images were acquired using a scanner and alizarin red was destained with 10% cetylpyridinium chloride (CPC) in 10 mmol/L sodium phosphate for 30 min at 25 °C. Calcium concentrations were determined according to the absorbance reading at 562 nm using a standard calcium curve prepared in the same solution. The final calcium levels in each group were normalized with the total protein concentrations obtained from the duplicate plates.

**Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)**

DPMSCs were cultured in 6 cm dishes until they reached 60–70% confluence. Then, they were transfected with let-7c lentiviruses for 12 h in 2 mL α-MEM supplemented with 10% FBS and 0.05 mg/mL polybrene. After 12 h of transfection, DPMSCs were cultured in 3 mL α-MEM supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 100 ng/mL IGF-1 for 0, 3, and 7 days before they were harvested for RT-PCR. Total cellular RNA was extracted from cells in each group using TRIzol reagent (Invitrogen, Carlsbad, USA, [https://www.thermofisher.com]) according to the manufacturer’s instructions. Isolated RNA precipitates were completely dissolved in diethylpyrocarbonate-treated water (Ambion Inc., Austin, USA) and reverse transcribed using a PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). For the analysis of let-7c over/underexpression, isolated RNA was subjected to reverse transcription with SMART MMLV Reverse Transcriptase (Takara Bio, Otsu, Japan). Real-time RT-PCR was performed using the SYBR® Premix Ex Taq™ kit (TaKara Bio, Otsu, Japan) on an ABI 7300 real-time PCR system. The mRNA expression levels of hsa-let-7c and several osteo/odontogenic markers, including osteocalcin (OCN), osterix (OSX), runt-related transcription factor 2 (RUNX2), dentin sialophosphoprotein (DSP), alkaline phosphatase (ALP), collagen type 1 (COL-1), and dentin matrix protein-1 (DMP-1), were detected using the primer sets listed in Table 1. Real-time RT-PCR reaction conditions were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was employed as the reference housekeeping gene for normalizing mRNA levels of osteo/odontogenic genes. U6 was employed as the reference housekeeping gene for
analysis of hsa-let-7c levels. The results were calculated from three independent experiments. Relative gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method, as previously described\textsuperscript{31,32}. All PCR reactions were performed in triplicate and data are expressed as the mean ± SD. Primers used for the detection of hsa-let-7c are as follows: U6, forward 5′-CTCGCTTCGGCAGCACA-3′ and reverse 5′-AACGCTTCACGAAGACAC-3′, hsa-let-7c, forward 5′-ACACTCCAGCTGGGTGAGGTAGGTTGT-3′ and reverse 5′-TGGTGCTGGAGTCG-3′.

Western blot

Cells used for Western blot analysis were cultured identically to those used for RT-PCR and were harvested at days 0, 3, and 7 for the following experiments. After washing twice with cold PBS, cells were lysed for 15 min on ice in RIPA lysis buffer (Beyotime, China) supplemented with 1 mM phenylmethylsulfonyl fluoride (Beyotime). Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min. Protein concentrations were determined using the Bio-Rad protein assay kit (Pierce, Rockford, IL). 20 μg of each protein sample was used for Western blot analysis according to the previously described protocol. For the analysis of signaling pathways, cytoplasmic and nuclear proteins were extracted with NE-PER\textsuperscript{®} Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA), respectively, at the indicated time points and subsequently analyzed by Western blot.

Statistical analysis

Statistical analysis was performed using SPSS software v17.0. Quantitative results are expressed as the mean ± SD. Data were analyzed by a paired Student’s t-test (two-tailed distribution). P-values less than 0.05 were regarded as statistically significant.

Results

Let-7c inhibits IGF-1R expression in DPMSCs

In culture, DPMSCs exhibited spindle-like and fibroblast-like morphology (Fig. 1a, b). FCM analysis showed that DPMSCs stained positively for several MSC markers, including STRO-1, CD73, CD90, CD105, and CD146, but stained negatively for the hematopoietic markers CD34 and CD45 (Fig. 1c, d).
Fig. 1 Isolation and characterization of DPMSCs. a, b Isolated stem cells derived from human dental pulps displayed spindle-like and fibroblast-like morphology. c Flow cytometric analysis revealed that cultured DPMSCs were immunopositive for CD73 (99.99%), CD90 (99.94%), CD105 (99.83%), and CD146 (77.67%) but immunonegative for CD34 (0.58%) and CD45 (0.86%). Mouse IgG isotype control antibodies conjugated to FITC, PE, APC, or PerCP were used as negative controls. d Flow cytometry revealed that cultured DPMSCs were positive for STRO-1 (20.74%). PBS served as a negative control. Scale bars: 100 μm
Fig. 2 Identification of hsa-let-7c overexpression/knockdown in DPMSCs and effects of hsa-let-7c overexpression/knockdown on IGF-1R. 

(a) The hsa-let-7c binding site within IGF-1R, as predicted by TargetScan. 

(b) Selection of optimal transfection conditions for let-7c lentivirus transfection. MOI: the ratio of the lentiviral concentration to the number of cells, V: lentivirus, P: polybrene, BF: bright field, GFP: green fluorescent protein.

(c) The levels of hsa-let-7c gene expression in +Let-7c and +Ctrl DPMSCs. Values are presented as the mean ± SD, n = 3. *1 < 2^−ΔΔCt < 2, P < 0.01.

(d) The expression of IGF-1R protein in +Let-7c and +Ctrl DPMSCs, as determined by Western blot.

(e) Quantitative analysis of d. **P < 0.01.

(f) The levels of hsa-let-7c gene expression in −Let-7c and −Ctrl DPMSCs. Values are presented as the mean ± SD, n = 3. *1 < 2^−ΔΔCt < 2, P < 0.01.

(g) The expression of IGF-1R protein in −Let-7c and −Ctrl DPMSCs.

(h) Quantitative analysis of g. **P < 0.01.
We then determined that IGF-1R was one of the potential gene targets of hsa-let-7c by TargetScan; the putative binding site is shown in Fig. 2a. Let-7c lentiviruses were subsequently designed according to this binding site. To determine the optimal let-7c lentivirus transfection conditions, let-7c lentiviruses of different concentrations (MOI = 1, 5, 10; MOI is the ratio of the lentiviral concentration to the number of cells) were transfected into DPMSCs in either α-MEM or α-MEM + P (polybrene). After 72 h of transfection, cell fate and transfection efficiency were visualized under a fluorescence microscope. The cell morphology of DPMSCs

Fig. 3 Effects of let-7c on the proliferation of IGF-1-treated DPMSCs. a, b CCK-8 assay revealed no significant differences between the growth curves of +Let-7c DPMSCs and +Ctrl DPMSCs (a) or between those of −Let-7c DPMSCs and −Ctrl DPMSCs (b). Values are presented as the mean ± SD, n = 3. c–f Flow cytometry was used to assess proliferative capabilities. The average proliferation index (PI = S + G2M) in +Ctrl DPMSCs (c), +Let-7c DPMSCs (d), −Ctrl DPMSCs (e), and −Let-7c DPMSCs (f) was 11.59, 10.34, 10.15, and 12.30%, respectively. g–j Flow cytometry was used to assess rates of cell apoptosis. The average rate of cell apoptosis in +Ctrl DPMSCs (g), +Let-7c DPMSCs (h), −Ctrl DPMSCs (i), and −Let-7c DPMSCs (j) was 6.40, 7.89, 6.66, and 8.64%, respectively.
transfected with *let-7c* lentiviruses at MOI = 1 and 5 was normal; however, those transfected at MOI = 10 were dysmorphic and had high rates of cell death (Fig. 2b). Moreover, DPMSCs transfected with *let-7c* lentiviruses at MOI = 1 and 10 exhibited fewer GFP-positive cells than those transfected at MOI = 5 (Fig. 2b). When the MOI was equal to 5, DPMSCs expressed more GFP when transfected in α-MEM + P as compared to α-MEM only. Under MOI = 5 and α-MEM + P conditions, DPMSCs presented with the greatest GFP intensity and normal cell morphology. Therefore, this condition was used for subsequent experiments.

To detect whether *let-7c* levels are up/downregulated in DPMSCs, *let-7c* expression levels were measured by real-time RT-PCR 6 h following the transfection of DPMSCs with *let-7c* lentiviruses. The results showed that expression of the *let-7c* gene was significantly upregulated in +*Let-7c* DPMSCs (Fig. 2c), while IGF-1R protein expression was significantly decreased as compared with the +Ctrl group (Fig. 2d, e). Inversely, DPMSCs in the −*Let-7c* group displayed downregulated *let-7c* gene expression (Fig. 2f) and increased IGF-1R protein expression as compared with the −Ctrl group (Fig. 2g, h). These data revealed the significant inverse correlation between *hsa-let-7c* and IGF-1R.

**Effects of *let-7c* on DPMSC proliferation**

The cell counting kit-8 assay was used to investigate the proliferation of +*Let-7c*/−*Let-7c* DPMSCs after treatment with 100 ng/mL IGF-1 for 0, 1, 3, 5, 7, 9, 11, and 13
consecutive days. The results demonstrated that the proliferation of DPMSCs in the +Let-7c group was not significantly different from those in the +Ctrl group (Fig. 3a). Similarly, the –Let-7c group presented almost the same proliferation rate as the –Ctrl group between days 0 and 13 (Fig. 3b). FCM revealed that the proliferation index (PI = S% + G2M%) in the +Let-7c group (11.59%, Fig. 3d) was very similar to that of the +Ctrl group (10.34%, Fig. 3c). There was also no significant difference in the proliferation indices of the –Let-7c group (10.15%, Fig. 3f) and the –Ctrl group (12.3%, Fig. 3e). Furthermore, FCM demonstrated that the average cell apoptosis rate of DPMSCs in the +Ctrl, +Let-7c, –Ctrl, and –Let-7c groups were 7.89% (Fig. 3g), 6.40% (Fig. 3h), 8.64% (Fig. 3i), and 6.66% (Fig. 3j), respectively. These data demonstrated that let-7c had no obvious effects on the proliferative capacity and cell viability of DPMSCs.

**Let-7c regulates IGF-1-mediated osteo/odontogenic differentiation of DPMSCs**

To detect the effects of let-7c on the osteo/odontogenic differentiation of DPMSCs, real-time RT-PCR and Western blot were conducted on DPMSCs infected with +Let-7c/–Let-7c lentiviruses and treated with 100 ng/mL IGF-1. RT-PCR showed that the expression of osteo/odontogenic genes (OCN, OSX, DSPP, RUNX2, ALP, and COL-1) was remarkably decreased in +Let-7c DPMSCs as compared with the +Ctrl group (Fig. 4a). In particular, the expression levels of OCN, OSX, DSPP, and ALP continued to decline between days 3 and 7 (Fig. 4a). The expression
levels of RUNX2 and COL-1 were significantly declining at day 3 and day 7, respectively (Fig. 4a). Western blot showed that the expression of osteo/odontogenic proteins (OCN, OSX, DSPP, RUNX2, ALP, COL-1, and DMP1) was significantly downregulated from day 3 to day 7, while there was no significant decrease in the expression of these proteins at day 0 in the +Let-7c group (Fig. 4c, e).

After treatment with 100 ng/mL IGF-1, the expression levels of osteo/odontogenic genes (OCN, OSX, DSPP, RUNX2, ALP, and COL-1) in the −Let-7c group were obviously upregulated compared to the −Ctrl group (Fig. 4b). In particular, the expression levels of OCN, OSX, and RUNX2 were significantly increased at day 7 in the −Let-7c group as compared to the −Ctrl group (Fig. 4b). The expression levels of DSPP, ALP, and COL-1 increased from day 3 to day 7 (Fig. 4b). Western blot showed that the expression of osteo/odontogenic proteins (OCN, OSX, DSPP, RUNX2, ALP, COL-1, and DMP1) was upregulated from day 3 to day 7, while there was no significant decrease in the expression of these proteins at day 0 in the −Let-7c group (Fig. 4d, f).

Alizarin red staining revealed that fewer mineralized nodules were detected in the MM/MM +IGF-1 −Let-7c groups at day 14, as compared with the +Ctrl groups (Fig. 5a, b). There was no significant difference between the +Let-7c groups and the +Ctrl groups cultured in either α-MEM or α-MEM+IGF-1 media (Fig. 5a, b). CPC assays also revealed that calcium concentrations in the MM/MM +IGF-1 −Let-7c groups were significantly higher than those in the −Ctrl groups (Fig. 5d).

The above findings demonstrated that let-7c plays an important role in the osteo/odontogenic differentiation of DPMSGs.

**Effects of let-7c on MAPK pathways in DPMSGs**

To determine the potential involvement of MAPK signaling pathways in +Let-7c/−Let-7c-treated DPMSGs, MAPK-related proteins were detected in DPMSGs following infection with +Let-7c/−Let-7c lentiviruses. Total ERK and p38 protein levels significantly decreased in the +Let-7c group as compared with the +Ctrl group (Fig. 6a). The levels of phosphorylated ERK, JNK, and p38 were significantly downregulated in the +Let-7c group in comparison with the +Ctrl group (Fig. 6a). The p-JNK/JNK and p-p38/p38 intensity ratios were markedly decreased in the +Let-7c group as compared with those in the +Ctrl group (Fig. 6b). In the −Let-7c group, the levels of total ERK and p38 as well as phosphorylated ERK, JNK, and p38 were significantly increased compared with the −
hsa-let-7c inhibits the odonto/osteogenic differentiation of IGF-1-treated DPMSCs by targeting IGF-1R via the JNK/P38 MAPK pathways (Fig. 7). Of IGF-1-treated DPMSCs by targeting IGF-1R via the JNK/P38 MAPK pathways (Fig. 7).

**Discussion**

MiRNAs act as posttranscriptional regulators of gene expression by targeting the 3′-UTR regions of target mRNAs. In this study, IGF-1R was predicted to be a target of hsa-let-7c by TargetScan and thus we chose to investigate its effects on the biologic activities of IGF-1-treated DPMSCs. In brief, let-7c inversely regulated the expression of IGF-1R protein and suppressed the JNK/P38 MAPK signaling pathways. Furthermore, let-7c had the capacity to inhibit the osteo/odontogenic differentiation of IGF-1-treated DPMSCs. Since MAPK signaling is downstream of IGF-1/IGF-1R, we proposed that hsa-let-7c inhibits the odonto/osteogenic differentiation of IGF-1-treated DPMSCs by targeting IGF-1R via the JNK/P38 MAPK pathways (Fig. 7).

In this study, knockdown of let-7c increased the protein expression levels of IGF-1R, while overexpression of let-7c reduced IGF-1R expression, suggesting that IGF-1R is suppressed by let-7c in DPMSCs. Previous studies have shown that IGF-1 and IGF-1R are both targets of the let-7 family and that let-7 is closely associated with the IGF-1/IGF-1R axis. Let-7 can bring about the upregulation of IGF-1 and IGF-1R, indicating that let-7 may contribute to the upstream regulation of the IGF-1/IGF-1R axis. In the presence of IGF-1, expression of let-7 family members usually decreases while BMSCs undergo committed differentiation. Decreases in let-7 may allow for the upregulation of target genes such as IGF-1R, thus modulating the proliferation or differentiation of MSCs. In short, IGF-1R is a target of let-7c, and the latter can suppress the osteo/odontogetic ability of IGF-1-treated DPMSCs by inhibiting the expression of IGF-1R.

The p-JNK/JNK and p-p38/p38 intensity ratios distinctly decreased following let-7c overexpression and increased following let-7c knockdown, indicating that the JNK and P38 MAPK pathways are blocked by let-7c in IGF-1-treated DPMSCs. The MAPK and PI3K signaling pathways have been reported to be downstream mechanisms regulated by the IGF-1/IGF-1R axis. siRNA-mediated knockdown of IGF-1 can diminish the activation of ERK1/2 and PI3K. At the same time, either specific inhibitors or knockdown of ERK1/2 and PI3K can effectively abrogate IGF-1/IGF-1R-mediated differentiation. Therefore, the ERK MAPK and PI3K signaling pathways seem to be the downstream mechanisms effecting IGF-1-induced differentiation. Furthermore, P38 MAPK is a downstream target of IGF-1R and IGF-1R may control stem cell quiescence via p38 MAPK signaling. Interestingly, inhibitors of IGF-1R can decrease DPMSC colony-forming units, while inhibitors of p38 can overcome the suppressive effects of IGF-1R inhibition. These findings imply that IGF-1R and p38 MAPK can interact with each other to balance the quiescence and differentiation of DPMSCs. Since IGF-1R is a target gene of let-7c and is downregulated by let-7c, it seems that decreases in JNK and P38 MAPK signaling are associated with let-7c-mediated suppression of IGF-1R. Furthermore, MAP4K4 is the upstream regulator of the ERK, JNK, and p38 MAPK signaling pathways. MAP4K4 contains a let-7 target site in its 3′-UTR, indicating that let-7 family can regulate MAPK pathways by altering MAP4K4 expression levels. Hsa-let-7c-5p can facilitate human enterovirus 71 replication by inhibiting MAP4K4 expression; this mechanism might explain the virus’s ability to subvert the JNK pathway. Previous studies have demonstrated that ERK1/2 MAPK negatively regulates let-7 by inducing LIN28 expression through Myc transcription. Meanwhile, others have demonstrated that Ras, an upstream activator of Raf/MAPK, is inhibited by let-7 family miRNAs. Thus, it seems that let-7 and MAPK signaling pathways may function within a positive feedback loop. However, we found no direct evidence that MAPK signaling pathways can be regulated by let-7c itself. The speculation that JNK and P38 MAPK signaling can be directly regulated by let-7c still requires more extensive study. Therefore, it is reasonable to propose that the overexpression of let-7c may indirectly downregulate the expression of IGF-1R.
the JNK and P38 MAPK signaling pathways by targeting IGF-1R.

ALP, OCN, OSX, RUNX2, COL-1, DSPP/DSP, and DMP-1 are usually recognized as the functional markers of odonto/osteoblast activity, bone/tooth regeneration, and odonto/osteogenesis. Specifically, ALP is an important marker involved in the early stages of osteogenic differentiation and has been widely used to evaluate the osteogenic potential of cells in vitro. OCN is a late-stage marker of osteogenic differentiation and mainly appears in mature bone tissues. The transcription factor RUNX2 serves as an early transcriptional regulator of osteogenic differentiation and its downstream target OSX is involved in both early and late stages of osteogenic differentiation. COL-1 is the most abundant protein in bone and is the main component of the bone matrix. Higher COL-1 expression may reflect matrix mineralization in mineralized bones. DSPP and DSP are odontoblast-specific markers, which are highly expressed in dentin and predentin structures and play critical roles in dentinogenesis. DMP-1 is an extracellular matrix protein mainly expressed in odontoblasts and is essential for dentin mineralization. DSP may act as a downstream effector molecule of DMP1 during dentinogenesis. At days 3 and 7 following the overexpression of let-7c in 100 ng/mL IGF-1-treated DPMSCs, the mineralization capacity and expression of osteo/odontogenic markers (ALP, OCN, OSX, RUNX2, COL-1, and DMP1) were distinctly downregulated. Inversely, the mineralization capacity and expression of osteo/odontogenic markers were clearly upregulated at days 3 and 7 in IGF-1-treated let-7c DPMSCs. These results revealed that let-7c could suppress the IGF-1-induced osteo/odontogenic differentiation of DPMSCs. Together, it is reasonable to suggest that let-7c suppresses the IGF-1-induced osteo/odontogenic differentiation of DPMSCs by inhibiting the expression of IGF-1R.

The IGF-1/IGF-1R axis directly participates in the regulation of gene expression and osteoblast differentiation, a critical step in the maintenance of bone homeostasis. IGF-1R possesses the ability to translocate to the cell nucleus and elicit genomic activities triggered by transcription factors. Nuclear IGF-1R has functional activities similar to transcription factors, providing an additional layer of biological regulation at a genome-wide level.

By stimulating the action of IGF-1R, IGF-1 acts as an important mitogenic factor to promote cell proliferation and differentiation. Our previous studies have demonstrated that exogenous IGF-1 can modify cellular morphology and ultrastructure as well as promote the proliferation and differentiation of DPMSCs, hSCAPs, and hPDLSCs. Exogenous IGF-1 can enhance the proliferative and osteo/odontogenic abilities of DPMSCs by activating the ERK and p38 MAPK signaling pathways. In addition, IGF-1 can induce the osteogenic differentiation of hPDLSCs via MAPK signaling. In this study, let-7c significantly downregulated both JNK/P38 MAPK signaling and the expression of the upstream regulator IGF-1R and inhibited the committed differentiation of IGF-1-treated DPMSCs. In short, let-7c expression depressed IGF-1R expression, which subsequently downregulated the JNK/P38 MAPK signaling pathway and finally led to the downregulation of osteo/odontogenic differentiation in DPMSCs.

In this study, overexpression of miRNA let-7c led to the downregulation of IGF-1R and the downstream JNK/P38 MAPK signaling pathways, subsequently resulting in the weakened osteo/odontogenic differentiation of IGF-1-treated DPMSCs. Inversely, deletion of let-7c caused upregulation of IGF-1R and its downstream JNK/P38 MAPK signaling, resulting in the enhanced osteo/odontogenic differentiation of IGF-1-treated DPMSCs. Thus, it is speculated that a let-7c/IGF-1/IGF-1R/MAPK axis may exist to effect let-7c-mediated changes in IGF-1-treated DPMSCs. Hsa-let-7c inhibited the osteo/odontogenic differentiation of IGF-1-treated DPMSCs by suppressing the expression of IGF-1R on the membrane. This decrease in IGF-1R expression then brought about the inhibition of the cytoplasmic JNK/P38 MAPK signaling pathways. Decreases in the amount of phosphorylated JNK/P38 in the nucleus subsequently resulted in the downregulation of downstream genes associated with the committed differentiation of DPMSCs (Fig. 7). Taken together, the modification of osteo/odontogenic differentiation of IGF-1-treated DPMSCs by let-7c was mediated by IGF-1/IGF-1R and the downstream JNK/P38 MAPK signaling pathways. Further study is required to explore the potential applications of this axis in tooth engineering as well as bone reconstruction.

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Authors’ contributions
GX.L. and SM: project conception and design, data collection, and/or assembly, data analysis and interpretation, manuscript drafting. Y.L.: RT-PCR, cell culture, Y.Y.: manuscript drafting, technical support. Y.K.Z.: Western blot analysis, alizarin red staining. Y.L.: FCM, statistical support. L.J.: CCK-8 assay, cell identification. Z.W.: technical support, lentivirus ordering. J.Y.: study design, technical support, and final approval of manuscript.

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

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