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Long non-coding RNA IGFBP7-AS1 promotes odontogenic differentiation of stem cells from human exfoliated deciduous teeth through autophagy: An *in vitro* study

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ABSTRACT

Objective: In the present study, we aimed to investigate whether long non-coding RNA (lncRNA) insulin-like growth factor binding protein 7-antisense 1 (IGFBP7-AS1) regulates the odonto-differentiation of stem cells from human exfoliated deciduous teeth (SHED) and its underlying mechanism.

Design: Real-time polymerase chain reaction (PCR) and correlation analysis were used to determine the expression of IGFBP7-AS1 during odontogenesis. Alkaline phosphate staining, alizarin red S staining, and real-time PCR *in vitro* were performed to investigate the effects of IGFBP7-AS1 during odontogenesis. Western blot and immunostaining (with or without chloroquine treatment) were applied to detect the expression of the autophagy-related markers, microtubule-associated proteins 1A/1B light chain 3B (LC3B) and p62. The autophagy inhibitor 3-methyladenine was used to further clarify the effect of autophagy in odonto-differentiation as promoted by IGFBP7-AS1.

Results: The expression of lncRNA IGFBP7-AS1 is significantly upregulated during odonto-differentiation of SHED and promotes odontogenesis of SHED *in vitro*. IGFBP7-AS1 promotes autophagy during odontogenesis.

Conclusions: IGFBP7-AS1 elicits odontogenic differentiation of SHED through autophagy. Furthermore, IGFBP7-AS1 shows promise as a gene target in the regeneration of dental hard tissue and dental–pulp complex.

1. Introduction

Pulpitis, dental traumatology, or dysplasia inevitably lead to the loss of pulp tissue, which plays a key role in dentin formation (Huang et al., 2009). Pulp regeneration is a promising strategy. Seed cells, growth factors, and scaffold are the three basic elements in tissue regeneration engineering (Nakashima & Akamine, 2005). How to directly induce odonto-differentiation of seed cells to odontoblasts is a key issue in this field.

Long non-coding RNAs (lncRNAs), the lengths of which are over 200 bps, have been reported to participate in numerous biological process (Bhan et al., 2017; Bridges et al., 2021) and to play a crucial role in the odontogenic differentiation of mesenchymal stem cells (Liu et al., 2020). For instance, LncRNA H19 have been shown to promote the odontogenic differentiation of dental pulp stem cells (Zeng et al., 2018) and stem cells from apical papilla (D. Li et al., 2019). Conversely, lncRNA DANCR inhibits the odontogenesis of dental pulp stem cells through the Wnt/β-catenin signaling pathway or sponging miR-216a (Chen et al., 2016, 2020). Furthermore, in our previous RNA sequencing research, we revealed that the expression of lncRNA insulin-like growth factor binding protein 7-antisense 1 (IGFBP7-AS1) is upregulated during the odontogenic differentiation of stem cells from human exfoliated teeth (SHED). Moreover, IGFBP7-AS1 is reported to be significantly associated with overall survival in patients with glioblastoma (D. Li et al., 2019). Accordingly, we wonder whether IGFBP7-AS1 regulated the odontogenesis of SHED and the underlying mechanism involved.

Autophagy is a highly conserved catabolic process that is essential for maintaining homeostasis (Mizushima & Komatsu, 2011). There are four main stages of the autophagy process: the formation of phagophores; the subsequent formation of autophagosomes; the combination

Abbreviations: lncRNA, long non-coding RNA; IGFBP7-AS1, insulin-like growth factor binding protein 7-antisense 1; SHED, stem cells from human exfoliated deciduous teeth; LC3B, microtubule-associated proteins 1 A/1B light chain 3B; siRNA, small-interfering RNA; ARS, alizarin red S; DMP1, dentin matrix protein 1; PCR, polymerase chain reaction..

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of autophagosomes and lysosomes; and the formation of autolysosomes (Parzych & Klionsky, 2014). Autophagy regulates cell growth, survival, differentiation, and homeostasis in mammals (Glick et al., 2010), and recent studies have indicated that odontogenesis is related to autophagy (Cho et al., 2021; Lei et al., 2020). For instance, Kim et al. reported that the dental-treatment material mineral trioxide aggregate elicits odontoblastic differentiation in dental pulp stem cells though the activation of autophagy (Kim et al., 2021), while Cho et al. reported that an autophagy-related protein plays a crucial role in the odontogenesis of human dental pulp stem cells by regulating autophagic flux (Cho et al., 2021). In addition, lncRNA has been shown to regulate cellular and pathology processes through autophagy (Levine & Kroemer, 2019). Nevertheless, the relationship between IGFBP7-AS1 and autophagy has not been investigated to date.

We hypothesized that IGFBP7-AS1 promotes the odontogenic differentiation of SHED and does so by regulating autophagy at early stage. Accordingly, in the present study, the odontogenic effect of IGFBP7-AS1 was investigated, and the signaling pathway regarding autophagy was detected under overexpression and knockdown of IGFBP7-AS1 conditions. Autophagy inhibitor was applied to further explore the role of autophagy in odonto-differentiation of SHED.

2. Materials and methods

2.1. Cell culture and odontogenic differentiation

SHED were kindly provided by ORAL STEM CELL BANK operated by Beijing Tason Biotech Co. Ltd. (http://www.kqgxb.com) from children (aged from five to seven) and cultured as previously described. Our experiments were performed with approval from the Ethics Committee of the Peking University School and Hospital of Stomatology, Beijing, China (Approval Number: PKUSSIRB-201732003). SHED at stages Passages 3rd− 6th were used for subsequent experiments. To induce odontogenic differentiation, SHED were exposed to mineralized medium comprising 0.01 mM dexamethasone disodium phosphate, 0.1 mM L-ascorbic acid phosphate, and 1.8 mM monobasic potassium phosphate (Sigma-Aldrich, USA) after reaching 70%− 80% confluence. The mineralized medium was changed every two days.

2.2. RNA oligoribonucleotides and cell transfection

RNA oligoribonucleotides (*e.g.*, small interfering RNAs [siRNAs] targeting lncRNA IGFBP7-AS1, and siRNA control) were purchased from GenePharma (Shanghai, China). SHED were cultured in 12-well plates prior to transfection. After reaching 60% confluence, the cells were transfected with siRNAs using Lipofectamine 3000 according to the manufacture's protocol (Invitrogen, Carlsbad, CA, USA).

2.3. Lentivirus infection

EF-1 aF/GFP and puromycin lentiviruses were created by GenePharma (Shanghai, China) to induce lncRNA-IGFBP7-AS1 overexpression respectively. SHEDs were transfected with lentiviruses at a multiplicity of infection of 20 to increase the level of lncRNA IGFBP7-AS1. Polybrene (5 mg/mL) was used in the lentivirus medium to improve the infection efficiency. In addition, the medium was changed after 8 h. After three days, the infection efficiency was determined using an inverted fluorescence microscope (Olympus, Japan) and verified using real-time polymerase chain reaction (PCR).

2.4. Alkaline phosphatase staining

Alkaline phosphate staining was performed on day 7 using an alkaline phosphate staining kit according to the manufacturer's protocol (CWbiotech, Beijing, China). Briefly, phosphate-buffered saline was used to rinse the cultured cells and the cell layers were fixed in 4% paraformaldehyde for 30 min. Then, they were washed with dH_2O and incubated in alkaline solution for 10 min at room temperature.

2.5. Alizarin Red S (ARS) staining

SHED were rinsed with phosphate-buffered saline on day 14 for three times and fixed in 4% paraformaldehyde for 15 min before staining with 0.1% ARS (pH 4.0–4.6) for 20 min. Finally, the reaction was terminated with dH_2O.

2.6. Real-time PCR

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, and 1 μg of total RNA was converted to cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan). Real-time PCR was performed as previously described(Wang et al., 2019). Each genetic analysis was performed in triplicate, and the primers that were used are listed in Table 1.

2.7. Western blot

A protein lysis buffer containing a phosphatase inhibitor (Applygen Technologies Inc., Beijing, China) was used to harvested cells on day 1 or day 3. The cell suspensions were centrifuged for 30 min at 4 °C and 12,000 ×*g*. BCA protein assays (CWBIO, Beijing, China) were used to determine the protein concentration, and each lane was loaded with equal aliquots of the total protein (20 μg). The sample lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked in 5% bovine serum albumin for 2 h, and probed with the following antibodies at 4 °C overnight: autophagy-related markers microtubule-associated proteins 1A/1B light chain 3B (LC3B) (1:1000, Proteintech, Beijing, China), p62 (1:10000, Proteintech, Beijing, China), and β-actin (1:10000; Cell Signaling Technology, Beverly, MA, USA). The membranes were incubated at room temperature for 1 h with horseradish peroxidase -conjugated anti-rabbit immunoglobulin. Protein expression was detected using a western blot enhanced chemiluminescence blotting kit (SOLIBRO, Beijing, China).

2.8. Immunofluorescence staining

SHED and IGFBP7-AS1-overexpressed SHED were seeded in 12-well plates and exposed to mineralized medium with or without chloroquine for 3 days, then prepared as previously reported (Wang et al., 2019). Thereafter, the cells were incubated with LC3B antibody (1:200) overnight at 4 °C and then with an anti-rabbit secondary antibody (1:1000; Proteintech, China) for 1 h at room temperature. Nuclei were counterstained with 4′,6-diami-dino-2-phenylindole (DAPI, SOLIBRO, Beijing, China). Images were captured using an LSM 5 EXCITER confocal imaging system (Carl Zeiss, Oberkochen, Germany).

2.9. Statistical analysis

SPSS21.0 statistical software (IBM Corp, Armonk, NY, USA) was used to perform all statistical calculations. Comparisons between two groups were performed using independent two-tailed Student's t-tests, and comparisons between more than two groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Correlation analysis was performed by calculating Pearson's correlation coefficient. All data are expressed as mean ± standard deviation (SD) of the results from three experiments per group, and $p < 0.05$ was considered to be statistically significant.

Table 1
Sets of primers used for real-time PCR in this study.

Gene name		5′– 3′	Size (bp)	GenBank accession
GAPDH	F	CCGTCTTGAGAAACCTGCCA	139	NM_001115114.1
	R	GGATGAACGGCAATCCCCAT		
Alkaline phosphate	F	CTCCATACCTGGGATTTCCGC	299	NM_000478.6
	R	GGCCCCAGTTTGTCCTTCTT		
Dentin sialoprotein	F	GGAATGGCTCTAAGTGGGCA	284	NM_014208.3
	R	CTCATTGTGACCTGCATCGC		
DMP1	F	GAGTGGCTTCATTGGGCATAG	260	NM_004407.4
	R	GACTCACTGCTCTCCAAGGG		
IGFBP7-AS1	F	GGTTGGGTTCATGTGCTAC	121	NR_034081.1
	R	AGAATTGCTTCCTGCTAATCT		

3. Results

3.1. Expression of lncRNA IGFBP7-AS1 is significantly upregulated during odontogenesis of SHED

We first induced SHED to odontogenic differentiation for 7 and 14 days with mineralized medium, and alkaline phosphate staining and ARS staining were conducted on days 0, 7, and 14. As shown in Fig. 1 A, alkaline phosphate and ARS staining, which reflect matrix mineralization, are increased upon the odontogenic differentiation of SHED. The mRNA levels of the odontogenesis-related markers: alkaline phosphate, dentin sialoprotein, and dentin matrix protein 1 (DMP1), are also significantly increased during this process (Fig. 1B).

Next, the expression of IGFBP7-AS1 during odontogenic differentiation of SHED was determined on days 7 and 14 (Fig. 1B). The results show that the expression of IGFBP7-AS1 level increases on days 7 and 14 and there are positive correlations between the levels of IGFBP7-AS1 and those of odontogenic markers (Fig. 1 C), indicating that IGFBP7-AS1 may be involved in the odontogenic differentiation of SHED.

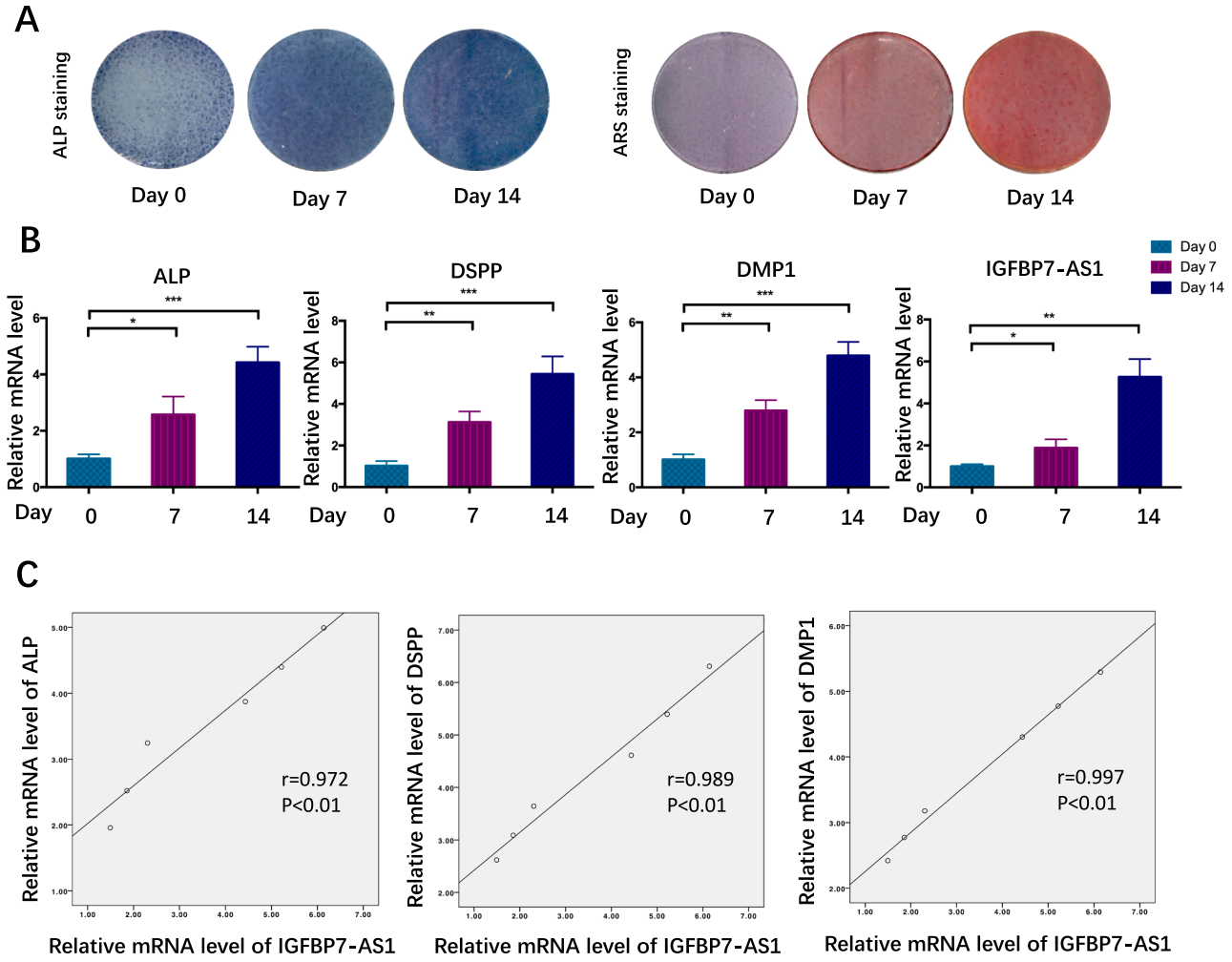

Fig. 1. Odontogenic differentiation of stem cells from human exfoliated deciduous teeth (SHED) on days 0, 7, and 14 when cultured with mineralized medium. (A) ALP staining and ARS staining of SHED on days 0, 7, and 14. ALP, alkaline phosphatase; ARS, alizarin Red S (ALP, alkaline phosphate). (B) Expression levels of the odontogenic markers ALP, DSPP, DMP1, and lncRNA IGFBP7-AS1 are significantly increased on days 7 and 14 during differentiation. Data are all presented as mean ± standard deviation (error bars) for three individual experiments. (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, compared to days 0; DSPP, dentin sialoprotein). (C) Correlation analyses between the levels of IGFBP7-AS1 and odontogenic markers.

3.2. IGFBP7-AS1 promotes odontogenesis of SHED in vitro

In order to determine the effect of IGFBP7-AS1 during the odontogenesis of SHED, we used siRNA and lentivirus-targeting IGFBP7-AS1 to downregulate and upregulate the expression of IGFBP7-AS1 in SHED. The modified SHED were cultured in mineralized medium for 7 and 14 days to determine their odontogenic ability.

After 7 days of culturing, the results show that knockdown of IGFBP7-AS1 decreases alkaline phosphate staining when comparing to negative control siRNA transfection group and overexpression of IGFBP7-AS1 increases alkaline phosphate staining when comparing to negative control group. After 14 days, ARS staining presents similar results to those of alkaline phosphate staining (Fig. 2 A, C). The expression of odontogenic markers at the mRNA level was also determined after 14 days of culturing. The results show that the mRNA levels of alkaline phosphate, dentin sialoprotein, and DMP1 are reduced in IGFBP7-AS1-downregulated group and enhanced in the IGFBP7-AS1-overexpression group (Fig. 2B, D). These results demonstrate that IGFBP7-AS1 promotes odontogenesis of SHED *in vitro*.

3.3. IGFBP7-AS1 upregulates autophagy during odontogenesis

We also determined the activation of autophagy by IGFBP7-AS1 in SHED during odontogenesis. LC3B is a protein that is expressed during autophagy. As shown by immunostaining analysis under confocal microscopy, the expression of LC3B on day3 (red fluorescence) is increased

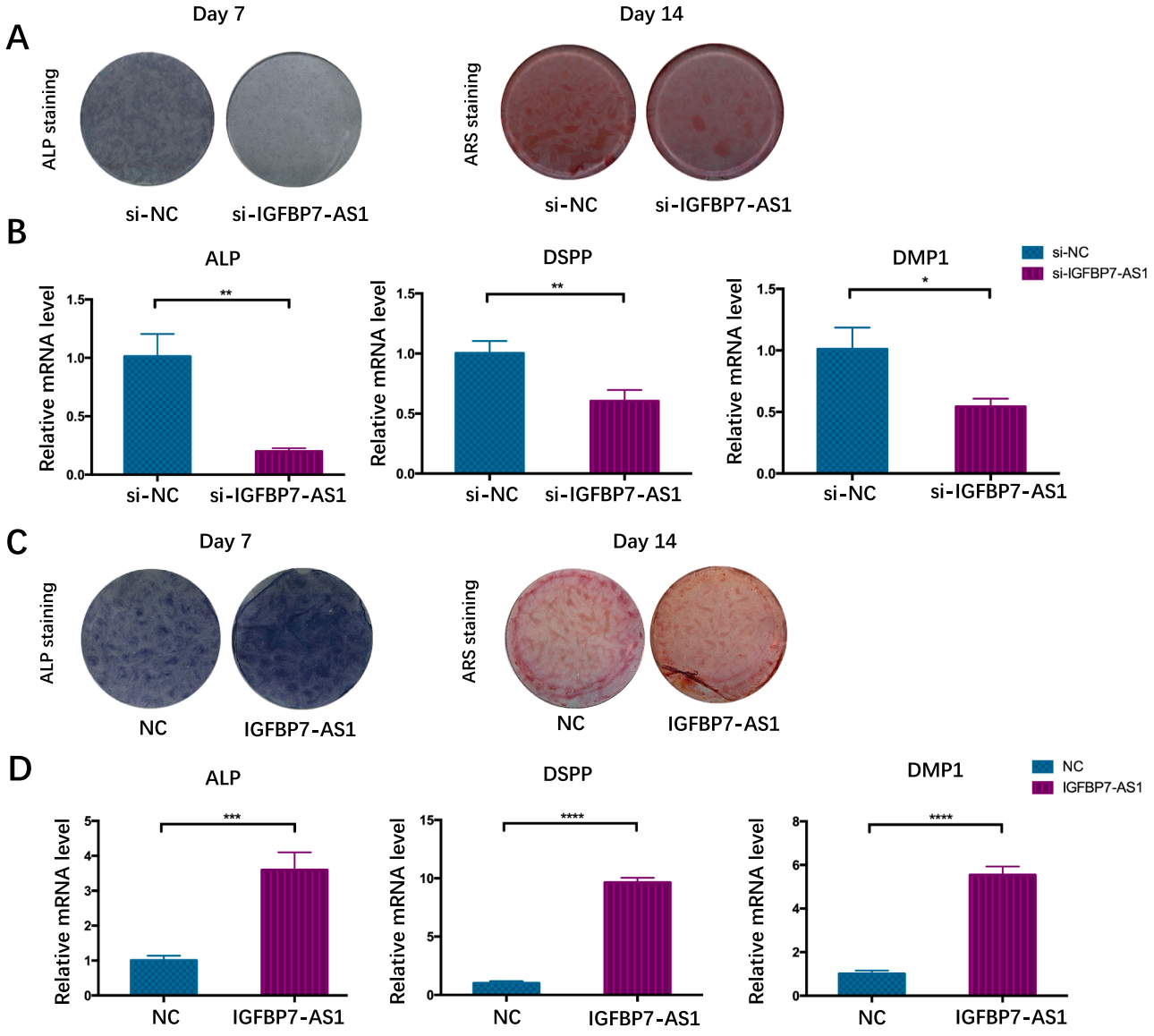

Fig. 2. IGFBP7-AS1 promotes the odontogenic differentiation of SHED *in vitro*. (A) ALP staining of SHED on day 7 after odontogenic differentiation and ARS staining of SHED on day 14 after odontogenic differentiation in the si-NC and si-IGFBP7-AS1groups. Both of the groups were cultured with mineralized medium. si-NC, negative control siRNA transfection group; si-IGFBP7-AS1, IGFBP7-AS1 downregulated group (ALP, alkaline phosphate). (B) Real-time PCR detection of the odontogenic differentiation related markers ALP, DSPP, and DMP1 in the si-NC and si-IGFBP7-AS1 groups. Both of the groups were cultured with mineralized medium. Data are all presented as mean ± standard deviation (error bars) for three individual experiments, (* $p < 0.05$, ** $p < 0.01$, compared to si-NC group; DSPP, dentin sialoprotein). (C) ALP staining of SHED on day 7 after odontogenic differentiation and ARS staining of SHED on day 14 after odontogenic differentiation in the NC and IGFBP7-AS1 groups. Both of the groups were cultured with mineralized medium. NC, negative control group; IGFBP7-AS1, IGFBP7-AS1 overexpressed group. (D) Real-time PCR detection of the odontogenic differentiation related markers ALP, DSPP, and DMP1 in the NC and IGFBP7-AS1 groups. Both of the groups were cultured with mineralized medium. Data are all presented as mean ± standard deviation (error bars) for three individual experiments, (*** $p < 0.001$, **** $p < 0.0001$, compared to NC group;).

in the IGFBP7-AS1-overexpressed group than negative group (Fig. 3 A). Furthermore, the relative levels of p62, which is an autophagy substrate, were also determined by western blot analysis. The results show that the p62 level is decreased in the IGFBP7-AS1 overexpressed group (Fig. 3B), which indicates enhanced autophagic activity.

The most representative and important event in the autophagic process is the conversion of LC3B-I to LC3B-II. Here, the LC3B-II to LC3B-I ratio reflects autophagic activity. However, autophagy is a dynamic process that, accordingly, is also termed autophagic flux. During this process, lysosomes combine with autophagosomes, which leads to the degradation of LC3B-II. In order to measure the actual LC3B-II to LC3B-I ratio, we used chloroquine, an inhibitor that blocks the combination of lysosomes and autophagosomes during autophagy, preventing degradation of LC3B-II. The LC3B-II/LC3B-I ratio determined by western blot is higher for the IGFBP7-AS1 overexpressed group than for the negative control group on both Day 1 and Day 3 with or without chloroquine (Fig. 3 C). The expressions of LC3B with or without chloroquine were also confirmed by immunostaining on day 3 (Fig. 3D).

3.4. Inhibited autophagy suppresses odontogenesis in IGFBP7-AS1-overexpressed SHED

To further investigate the effect of autophagy in IGFBP7-AS1 overexpressed SHED during odontogenesis, the commonly used autophagy inhibitor 3-methyladenine was added to prevent autophagy. The odontogenic differentiation in IGFBP7-AS1 overexpressed SHED was then assessed. The immunostaining on day 3 in Fig. 4 A shows that 3-methyladenine prevents the formation of LC3B induced by the overexpression of IGFBP7-AS1, which confirms the inhibition of autophagy. Furthermore, alkaline phosphate and ARS staining are decreased upon adding 3-methyladenine to IGFBP7-AS1 overexpressed SHED (Fig. 4B). 3-methyladenine also decreases the relative mRNA levels of the odontogenic differentiation indicators alkaline phosphate, dentin sialoprotein, and DMP1 as induced by the overexpression of IGFBP7-AS1 (Fig. 4 C). These results further support the conclusion that IGFBP7-AS1 promotes odontogenesis by inducing autophagy (Fig. 5).

4. Discussion

The formation of dentin is crucial for the regeneration of the pulp–dentin complex (Kim, 2017). To achieve this goal, the directional differentiation of seed cells into odontoblast-like cells is the most critical step (Bleicher et al., 2001; Zhang et al., 2020). LncRNA, which in the past has been considered to constitute genetic “noise” during transcription (Ponting et al., 2009), may, in fact, be a target for regulating the regeneration of dentin (Fang, Zhang, Chen, & Wu, 2019). Previous studies have demonstrated that certain classical lncRNAs such as lncRNA H19 and lncRNA DANCR regulate the odontogenesis of dental mesenchymal stem cells through different mechanisms (Chen et al., 2016; 2020; Z. Li et al. 2019; Zeng et al., 2018). SHED are a kind of dental mesenchymal stem cells that can also be used as seed cells in the regeneration of dentin (Ching et al., 2017; Zhai et al., 2019). In this study, we have identified an lncRNA that may participate in the odontogenesis of SHED. LncRNA IGFBP7-AS1 is an antisense lncRNA located on chromosome four in humans that is related to the survival of cancer patients (D. Li et al., 2019; Liu et al., 2021). We found that the expression of IGFBP7-AS1 at the gene level is upregulated during SHED odontogenesis. Furthermore, its expression shows positive correlation with odontogenic markers such as alkaline phosphate, dentin sialoprotein, and DMP1. This suggests that IGFBP7-AS1 may play a role in the odontogenesis of SHED.

To verify this hypothesis, we upregulated and downregulated the expression of IGFBP7-AS1 in SHED and determined the effects of these changes on odontogenesis. The odontogenic differentiation of IGFBP7-AS1 modified SHED was compared with that of normally treated SHED in terms of the expression of special markers of odontogenic differentiation. Alkaline phosphate and ARS staining, which reflect matrix mineralization at early stage and late stage of hard tissue formation, were also observed. Improved odoogenic differentiation of SHED in the IGFBP7-AS1 overexpressed group was confirmed by alkaline phosphate staining, and mineralized matrix deposition, as evaluated by ARS staining, as well as the upregulation of the odontogenesis related markers alkaline phosphate, dentin sialoprotein, and DMP1. Conversely, the opposite effects were observed for the IGFBP7-AS1 downregulated group. The data provide evidence for the hypothesis that IGFBP7-AS1 plays a key role in the promotion of odontogenesis in SHED.

Autophagy is a highly conserved process in eukaryotes that degrades intracellular misfolded proteins and damaged organelles to maintain the balance of proteins and stability of the intracellular environment (Bagherniya et al., 2018; Johansen & Lamark, 2011; Sciarretta et al., 2018). It begins with the expansion of free bilayer membranes to form cup-shaped structures called phagophores; the second stage is the formation of autophagosomes; and then, in the third stage, lysosomes fuse with autophagosomes to form autolysosomes; finally, the autophagia contents are exposed to lysosomal enzymes and degraded into small molecules (Lamark et al., 2017; Tanida et al., 2004). Autophagy participates in the renewal, pluripotency, and differentiation of human adult stem cells (Chang, 2020; Salemi et al., 2012). It also participates in the odontogenesis of dental-pulp stem cells. Previous studies have shown that autophagy degrades the cellular substance in mature odontoblasts of human teeth, recycling energy so as to ensure the vitality of odontoblasts (Park et al., 2021).

LC3B and p62 are common markers of autophagy. The expression of LC3B represents the activation of autophagy and was detected by immunostaining in this study. P62, also called sequestosome 1, is an autophagy-specific substrate that and is degraded during the autophagic process. The downregulation of p62 reflects the activation of autophagy. In this study, the expression of p62 at the protein level was observed to be lower in the IGFBP7-AS1 overexpressed group than in the negative control group, which indicates that autophagy is more active in the IGFBP7-AS1 overexpressed group. However, autophagy is a continuous dynamic process with the formation and degradation of autophagosomes. Therefore, the determination of autophagic flux may be a better way to quantify autophagy (Yoshii & Mizushima, 2017). There are two forms of LC3B, LC3B-I, which is 17–19 kd, and LC3B-II, which is 14–16 kd, that are produced post-translationally in cells. LC3B-I is cytosolic, whereas LC3B-II is membrane-bound. LC3B-I could convert into LCB-II during autophagy. The amount of LC3B-II is correlated with the extent of autophagosome formation (Tanida et al., 2004; 2008). In addition, the ratio of LC3B-II to LC3B-I can be used to detect autophagic flux. LC3B-II is degraded by lysosomes, so the fact that the expression of LC3B-II was upregulated (Mizushima & Yoshimori, 2007) not only means that the formation of autophagosomes was promoted, it may also mean that lysosome activity was decreased. Lysosomal activity must be taken into account when detecting the expression of LC3B-II. Chloroquine is a common inhibitor that inhibits the activity of lysosomes (Mauthe et al., 2018). Herein, we used chloroquine to inhibit the degradation of autophagosomes by lysosomes and detected the ratio of LC3B-II to LC3B-I at the protein level and expression of LC3B to verify that IGFBP7-AS1 promoted autophagic flux during odontogenesis. The expression of LC3B-II was upregulated significantly compared with the group without chloroquine. The ratio of LC3B-II to LC3B-I was higher in IGFBP7-AS1 overexpressed group which represents the increase of autophagic flux. The expressions of LC3B were also confirmed by immunostaining. Thus, from the results above, we concluded that autophagy is activated during the odontogenesis of IGFBP7-AS1 overexpressed SHED.

To confirm whether IGFBP7-AS1 promotes the odontogenesis of SHED through autophagy, we used 3-methyladenine to inhibit the formation of autophagosomes (Miller et al., 2020) and again determined odontogenic differentiation. Through immunostaining, we observed that the expression of LC3B is reduced by using 3-methyladenine which

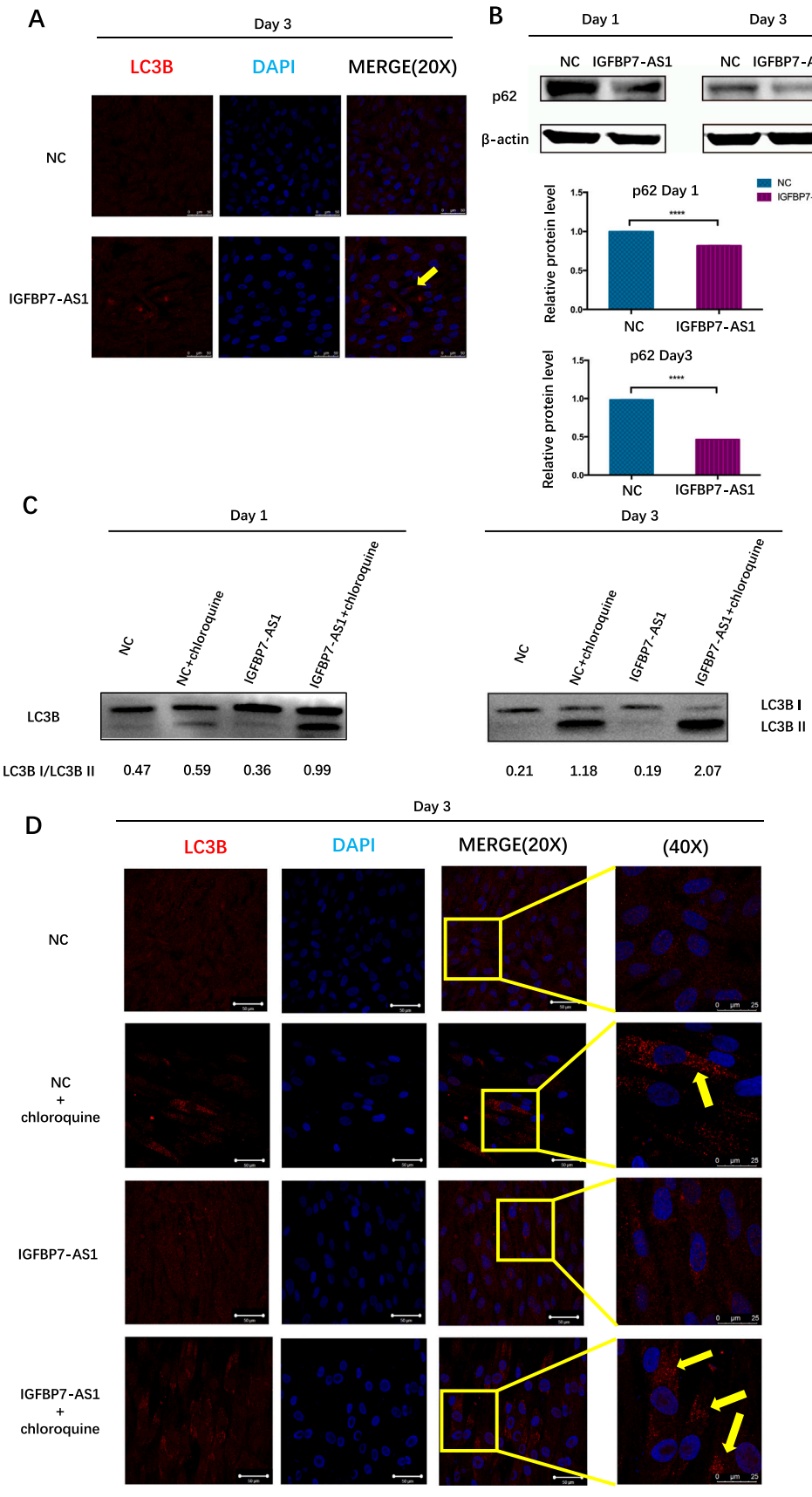

Fig. 3. IGFBP7-AS1 regulates the odontogenic differentiation of SHED through autophagy (A) Immunofluorescence staining on day 3 of LC3B under confocal microscopy. The red fluorescence represents LC3B and the blue fluorescence represents the nucleus. LC3B was strongly expressed in IGFBP7-AS1 group (stressed by the yellow arrow). Bars = 50 μm (20X). (B) Western blot detection of the expression of p62 in the NC and IGFBP7-AS1 groups on Day 1and Day 3. (****$p < 0.0001$, compared to NC group) (C) Western blot detection of the expression of LC3B-I and LC3B-II in the NC and IGFBP7-AS1 groups with or without chloroquine on day 1 and day 3. The ratios of LC3B-II to LC3B-I are also shown. (D) Immunostaining of LC3B with or without chloroquine on day 3 under confocal microscopy. LC3B was strongly expressed after treating with chloroquine (stressed by the yellow arrow), especially in IGFBP7-AS1 group. Bars = 50 μm (20X). Bars = 25 μm (40X). Both of the groups were cultured with mineralized medium. NC, negative control group; IGFBP7-AS1, IGFBP7-AS1 overexpressed group.

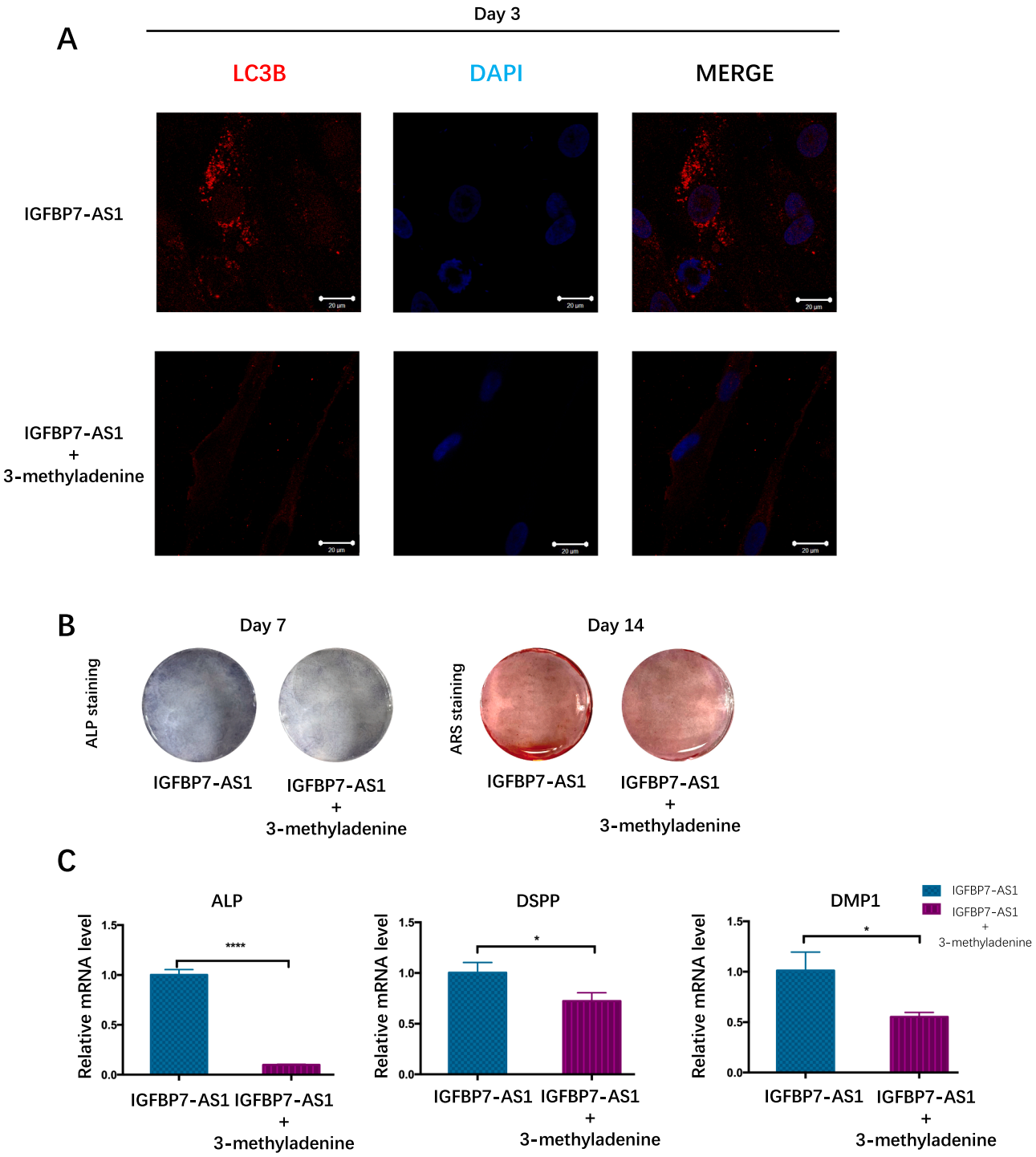

Fig. 4. 3-methyladenine suppresses odontogenesis in IGFBP7-AS1-overexpressed SHED (A) Immunostaining of LC3B on day 3 in IGFBP7-AS1 overexpressed SHED with or without 3-methyladenine. Bars = 20 μm. (B) ALP staining of IGFBP7-AS1 overexpressed SHED on day 7 and ARS staining of IGFBP7-AS1 overexpressed SHED on day 14 with or without 3-methyladenine (ALP, alkaline phosphate). (C) Real-time PCR detection of the odontogenic differentiation related markers ALP, DSPP, and DMP1. Data are all presented as mean ± standard deviation (error bars) for three individual experiments, (*$p < 0.05$, ****p < 0.0001, compared to IGFBP7-AS1 group; DSPP, dentin sialoprotein). IGFBP7-AS1, IGFBP7-AS1 overexpressed group cultured with mineralized medium.

confirms the inhibition of autophagy. For odontogenesis, alkaline phosphate activity and mineralized matrix deposition were significantly decreased by using 3-methyladenine. Furthermore, the expression of the odontogenic markers alkaline phosphate, dentin sialoprotein, and DMP1 were also decreased at the RNA level. 3-methyladenine suppressed the odontogenesis-promoting effects of IGFBP7-AS1 in SHED, illustrating that IGFBP7-AS1 plays a role in autophagy to promote odontogenesis (Fig. 5). This finding is consistent with those of previous studies. Consequently, a rational mechanism is that IGFBP7-AS1 promotes autophagy, increasing the degradation of misfolded proteins, which provides energy to SHED during odontogenesis to maintain cell viability and differentiation capacity.

In conclusion, IGFBP7-AS1 elicits odontogenic differentiation of SHED by increasing autophagic activity at early stage. Thus, IGFBP7-AS1 will be explored as a gene target to promote the regeneration of dental hard tissue and dental–pulp complex in future research.

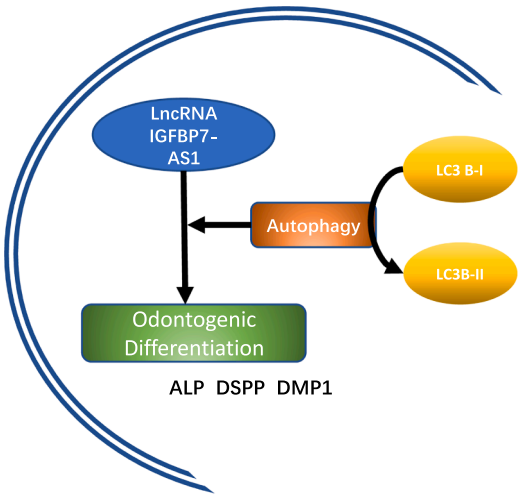

Fig. 5. Schematic showing the role of IGFBP7-AS1 in autophagy to promote the odontogenesis of SHED (ALP, alkaline phosphate; DSPP, dentin sialoprotein).

Furthermore, our results demonstrate the role of autophagy in odontogenic differentiation. Overall, our study provides a deeper understanding of the odontogenic process in dental mesenchymal stem cells.

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CRediT authorship contribution statement

Dan Wang: Conceptualization, Methodology, Investigation, Resources, Writing – original draft. **Ningxin Zhu:** Conceptualization, Methodology, Writing – original draft. **Fei Xie:** Conceptualization, Resources, Writing – original draft. **Man Qin:** Conceptualization, Visualization, Writing – review & editing. **Yuanyuan Wang:** Conceptualization, Writing – review & editing, Funding acquisition. All of the authors approved of the version to be submitted.

Conflict of interests

The authors declare that there is no conflict of interests.

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