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Cbfα1 hinders autophagy by DSPP upregulation in odontoblast differentiation

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ABSTRACT

Odontoblasts forms dentin as the main hard tissue of the tooth. Autophagy is a highly conserved homeostasis involved in odontoblast differentiation. DSPP (Dentin sialophosphoprotein) and Cbfα1 (Core binding factor α1) are typical factors in odontoblasts of the dentin-pulp complex. However, the relationship between Cbfα1 and DSPP as well as the role of Cbfα1 involved in the autophagy during odontoblast differentiation are poorly understood. In this study, we found the stimulation with FGF2 in MDPC-23 cells resulted in the suppression of autophagy and promotion of cell differentiation. We demonstrated that both mRNA and protein levels of Cbfα1 and DSPP were elevated upon FGF2 stimulation coupled with the inhibition of autophagy in MDPC-23 cells. Further results revealed that overdose of Cbfα1 increased the expression of DSPP, and also suppressed autophagy in MDPC-23 cells. Using luciferase reporter assay and EMSA, we illustrated that Cbfα1 upregulated DSPP expression through binding to the specific sites 5′-TACCTCA (-3950 bp to -3944 bp) and 5′-ACCACA (-3106 bp to -3101 bp) in *DSPP* promoter. Furthermore, ChIP assay results revealed that FGF2 treatment enhanced this binding of Cbfα1 to *DSPP* promoter. Signaling pathway inhibitor screening further revealed Cbfα1 suppressed autophagy involved in Wnt I signaling. Moreover, knockdown of Cbfα1 using siRNA silencing approach reversed the FGF2-mediated suppression of autophagy in MDPC-23 cells. Thus, these findings illustrate a novel regulatory mechanism by which Cbfα1 transcriptionally enhances DSPP expression, and also provide an insight into the inhibitory action of Cbfα1 in autophagy thereby facilitating odontoblast differentiation upon FGF2 stimulation.

1. Introduction

Tooth is a special mineralized organ composed of enamel, dentin, cementum and pulp. Dentin is the main hard tissue of the tooth and is formed by odontoblasts (Kawashima and Okiji, 2016; Zhang and Chen, 2018). During dentin formation, instructive epithelial–mesenchymal interactions lead to the cytodifferentiation of ectomesenchymal cells into odontoblasts that produce dentin extracellular matrix (ECM) (Guo et al., 2014; Wang et al., 2017). Odontoblasts are terminally differentiated cells that are subject to a long-lived secretory condition, which are the first cell line usually damaged by such as bacterial infection from dental caries (Zhang and Chen, 2018). In this process, the progenitors in the pulp can migrate and differentiate into odontoblast-like cells forming reparative dentin (Ricucci et al., 2018). Therefore, the behavior of odontoblasts in the dentin-pulp complex is crucial for preserving the healthy pulp.

Autophagy is a highly conserved homeostasis mechanism of living cells under various stress conditions, which was also involved in odontoblast development and aging (Cinque et al., 2015; Li et al., 2016; Zhang and Chen, 2018). The dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are dentin-specific proteins which are responsible for the homeostasis during odontoblast differentiation and hydroxyapatite crystal multiplication (Nurrohman et al., 2016). The disruption of mineralization homeostasis might be a crucial reason for DSPP (Dentin sialophosphoprotein) mutations resulting in dentin disorders (Jia et al., 2015). In DSPP positive odontoblasts, fibroblast growth factor 2 (FGF2) enhances odontoblast differentiation (Vidovic-Zdrilic et al., 2018). Moreover, DSPP is essential for normal development of the dental-craniofacial complex associated with the change in Cbfα1 (Core binding factor α1) expression (Chen et al., 2016). The

Abbreviations: Cbfα1, Core binding factor α1; DSPP, Dentin sialophosphoprotein; FGF2, Fibroblast growth factor 2; EMSA, Electrophoretic mobility shift assay; ChIP, Chromatin immunoprecipitation; PCR, Polymerase chain reaction

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expression of the osteoblast/odontoblast-associated genes Cbfα1 and DSPP was associated with tooth development, and also found to be significantly decreased in the abnormal differentiation of dental pulp cells in cleidocranial dysplasia (Chen et al., 2009; Yan et al., 2015). However, the relationship between Cbfα1 and DSPP involved in the homeostasis during odontoblast differentiation remains unclear.

Cbfα1, also called Runt-related transcription factor 2 (RUNX2), is essential for osteoblastic differentiation and maturation as well as for odontoblasts cytodifferentiation and endochondral ossification (Miyauchi et al., 2017). It acts as a scaffold for nucleic acids and regulatory factor binding to the core site, 5′-PYGPYGGT-3′, of a number of enhancers and promoters, including murine leukemia virus, polyomavirus enhancer, T-cell receptor enhancers, osteocalcin, osteopontin, bone sialoprotein, type I collagen, LCK, IL-3 and GM-CSF promoters (Tarkkonen et al., 2017; Wu et al., 2017). In osteoblasts, Cbfα1 supports transcription activation and synergizes with SPEN/MINT to enhance FGFR2-mediated activation of the osteocalcin FGF-responsive element (OCFRE) (An et al., 2015; Yoon et al., 2014). Cbfα1 is temporally and spatially regulated during dental development (Feng et al., 2016). Nevertheless, the role of Cbfα1 in FGF2-enhanced odontoblast differentiation is still unknown.

In this study, we demonstrated that both of the Cbfα1 and DSPP expression were elevated during the FGF2-mediated suppression of autophagy in MDPC-23 cells. Further results revealed that overdose of Cbfα1 increased the expression of DSPP, and also suppressed autophagy in MDPC-23 cells. Furthermore, we illustrated that Cbfα1 upregulated DSPP expression through binding to *DSPP* promoter. Exactly, Cbfα1 interacted with the specific sites 5′-TACCTCA (-3950 bp to -3944 bp) and 5′-ACCACA (-3106 bp to -3101 bp) in *DSPP* promoter. Based on signaling pathway inhibitor screening, Cbfα1 suppressed autophagy involved in Wnt I signaling. Moreover, Cbfα1 played a positive role in the FGF2-mediated suppression of autophagy in MDPC-23 cells. Therefore, these findings elucidate a novel regulatory mechanism by which Cbfα1 enhances DSPP expression, and also provide a new clue of the positive role of Cbfα1 odontoblast differentiation with the suppression of autophagy.

2. Results

2.1. Elevated expressions of Cbfα1 and DSPP during the FGF2-mediated suppression of autophagy in MDPC-23 cells

The fibroblast growth factor (FGF) family plays essential and important roles in primary and reparative dentinogenesis and continuous exposure of pulp cells to FGF2 inhibited odontoblast differentiation but increased number of DSPP positive odontoblasts (Sagomonyants et al., 2017; Vidovic-Zdrilic et al., 2018). Firstly, we demonstrated the expression of proteins responsible for odontoblast differentiation after FGF2 stimulation in MDPC-23 cells, a type of odontoblast-like cells, including SOX2 (SRY-box transcription factor 2), PPAR-γ (peroxisome proliferator activated receptor gamma), and OSX (Osterix) (Gopinathan et al., 2013; Lee et al., 2013). We revealed that SOX2 and OSX protein expression increased after FGF2 stimulation within 24 h (Fig. 1A and 1B), indicating that FGF2 induced odontoblast differentiation. However, we also noticed that PPAR-γ protein expression decreased after FGF2 stimulation (Fig. 1A and 1B). To investigate the changes of Cbfα1 and DSPP in FGF2-mediated odontoblast differentiation, MDPC-23 cells were treated with FGF2 for different periods. We found that FGF2 increased Cbfα1 and DSPP expression in a time-dependent manner in both RNA level (Fig. 1A) and protein level (Fig. 1C-1E). Since FGF signaling acts as a crucial regulator of autophagy in chondrocytes (Cinque et al., 2015), we also assessed the effect of FGF2-mediated autophagy in odontoblast. According to LC3-II and p62 protein levels, FGF2 stimulation obviously repressed autophagy in MDPC-23 cells from 0 to 24 h (Fig. 1D and 1E). As a typical autophagy inhibitor, Bafilomycin A1 was used as a positive control (Iwashita et al., 2018). We also observed the similar results (Fig. 1C-1E). Furthermore, the images from TEM results exhibited a significant decrease of the number autophagy bodies in MDPC-23 cells treated with FGF2 at different times, which was in accordance with the results from Bafilomycin A1 treatment (Fig. 2A). In the parallel experiments, the images from IF results exhibited a significant decrease of MAP1LC3B (autophagy activation) (Fig. 2B), while increase of LAMP2 (autophagy inhibition) (Fig. 2C) in MDPC-23 cells treated with FGF2 at different times, as the same as Bafilomycin A1 treatment. Taken together, the expressions of Cbfα1 and DSPP were prominently elevated in the inhibition of FGF2 on autophagy, suggesting a role of Cbfα1 in autophagy in MDPC-23 cells.

2.2. Cbfα1 suppresses autophagy through upregulating DSPP in MDPC-23 cells

Considering DSPP is associated with the Cbfα1 in normal development of the dental-craniofacial complex (Chen et al., 2016), we evaluated the regulatory role of Cbfα1 on DSPP. Overexpression of Cbfα1 typically enhanced DSPP expression in a dose-dependent manner both in RNA level (Fig. 3A) and protein level (Fig. 3B and 3C). Furthermore, overdose of Cbfα1 dramatically inhibited autophagy in MDPC-23 cells from LC3-II protein level using western blot analysis (Fig. 3B and 3D). Meanwhile, TEM results revealed that overexpression of Cbfα1 resulted in the decrease of the number autophagy bodies (Fig. 3E). In parallel, the images from IF results exhibited a significant decrease of MAP1LC3B (Fig. 3F), while increase of LAMP2 (Fig. 3G) in MDPC-23 cells transfected with Cbfα1-expressing plasmid. Therefore, the data demonstrated that Cbfα1 enhanced DSPP expression and suppressed autophagy in MDPC-23 cells.

2.3. Cbfα1 transcriptionally promotes DSPP expression

Since Cbfα1 enhanced DSPP expression during FGF2-mediated suppression of autophagy in MDPC-23 cells, we further explore the exact regulatory manner of Cbfα1 on DSPP expression. Although DSPP contains an IRES element responsible for the translation (Zhang et al., 2014), we explored the transcriptional regulation on DSPP expression. Using bioinformatic approach, the truncations of *DSPP* promoter from -4496 bp to 54 bp were generated (Fig. 4A). Then, MDPC-23 cells were transfected with pcDNA3.0 or pcDNA3.0-Cbfα1 with truncations of *DSPP* promoter of pGL3-Enhancer luciferase reporters. Cbfα1 significantly enhanced luciferase activity of the deletion from -4496 bp to -3499 bp and -3519 bp to -2515 (Fig. 4B), suggesting that Cbfα1 potentially bound to these two regions in *DSPP* promoter.

Cbfα1 acts as a scaffold for nucleic acids and regulatory factor binding to the core site 5′-PYGPYGGT (Tarkkonen et al., 2017). To confirm the specific binding sites of Cbfα1 in *DSPP* promoter, EMSA was employed in Cbfα1-expressing MDPC-23 cells. The labeled positive oligo DNA bound with nuclear extract as expected (Fig. 4C, lane 3), but such interaction was disrupted by unlabeled positive oligo DNA (Fig. 4C, lane 1), not by unlabeled negative oligo DNA (Fig. 4C, lane 2). Similarly, two sections of unlabeled oligo DNA in *DSPP* promoter (WT1: 5′-TACCTCA and WT2: 5′-ACCACA) obviously disrupted the DNA-protein complex of labeled positive oligo DNA and nuclear extract (Fig. 4C, lane 4–6). These data indicated that the two core sites of *DSPP* promoter 5′-TACCTCA and 5′-ACCACA might be responsible for Cbfα1-mediated *DSPP* transcription. In addition, in the presence of anti-Cbfα1 antibody, labeled oligo DNA containing 5′-TACCTCA (WT1) and 5′-ACCACA (WT2) generated super-shift bands, respectively (Fig. 4D, lane 2 and 6). However, the super-shift bands were not observed incubated with labeled oligo DNA containing mutant 5′-TAggTCA (MT1) and 5′-AggACA (MT2) (Fig. 4D, lane 4 and 8), even in the presence of anti-Cbfα1 antibody. These results demonstrated that Cbfα1 specially bound to 5′-TACCTCA and 5′-ACCACA in *DSPP* promoter to regulate *DSPP* expression.

Because FGF2 induced DSPP expression, we also assess the

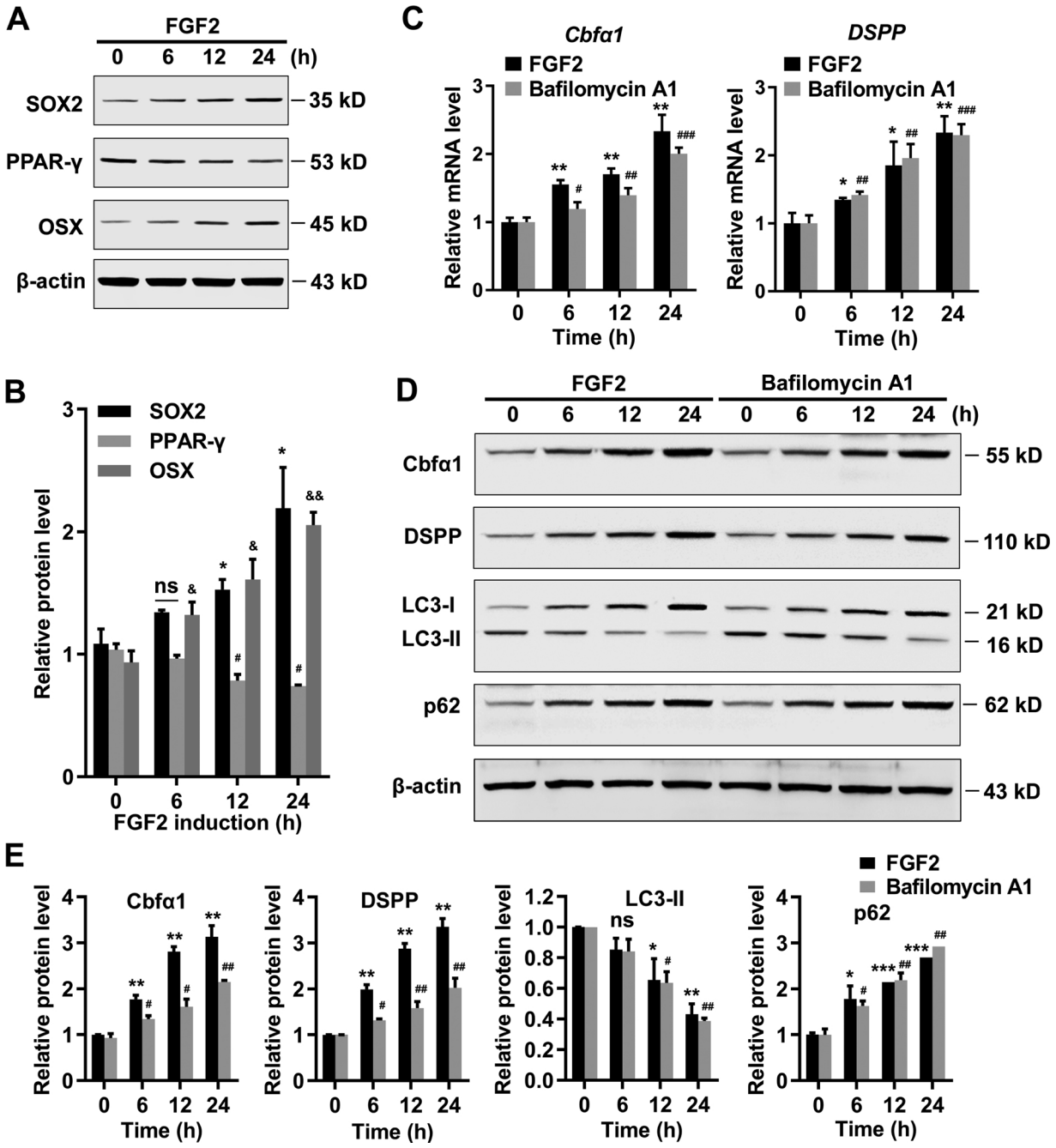

Fig. 1. The expressions of Cbfα1 and DSPP in FGF2-mediated autophagy in MDPC-23 cells.
(**A**) Western blot analysis of osteoblast differentiation associated protein (SOX2, PPAR-γ, and OSX) levels in MDPC-23 cells treated with FGF2 (2 nM) at different time points. (**B**) Quantitative expression of each protein was analyzed by Image J software. The expression level is relative to internal control, β-actin. Data are shown as mean ± SD. ns, not-significant. *, $p < 0.05$, stands for SOX2 expression. $^{\#}$, $p < 0.05$, stands for PPAR-γ expression. $^{\&}$, $p < 0.05$; $^{\&\&}$, $p < 0.01$, stands for OSX expression. (**C**) Quantitative PCR analysis of *Cbfα1* and *DSPP* mRNA levels after FGF2 (2 nM) or Bafilomycin A1 (100 nM) treated in MDPC-23 cells at different time points. Data are shown as mean ± SD. ns, not-significant. *, $p < 0.05$; **, $p < 0.01$, stands for FGF2 treatment. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$; $^{\#\#\#}$, $p < 0.001$, stands for Bafilomycin A1 treatment. (**D**) Western blot analysis of protein levels in MDPC-23 cells treated with FGF2 (2 nM) or Bafilomycin A1 (100 nM) at different time points. (**E**) Quantitative expression of each protein was analyzed by Image J software. The expression level is relative to controls. Data are shown as mean ± SD. ns, not-significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, stands for FGF2 treatment. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$, stands for Bafilomycin A1 treatment.

transcriptional regulation of Cbfα1 on DSPP expression in FGF2 stimulation. Using ChIP assay, we found anti-Cbfα1 antibody specifically immunoprecipitated the complex of Cbfα1 protein and DNA fragment containing 5′-TACCTCA and 5′-ACCACA in *DSPP* promoter (Fig. 4E), and such interactions were enhanced upon FGF2 stimulation (Fig. 3E). Altogether, these data displayed that Cbfα1 transcriptionally promotes DSPP expression in FGF2-mediated autophagy.

2.4. Cbfα1 suppresses autophagy through Wnt signaling

Multiply signaling pathways have been implicated in the modulation of autophagy, such as Hedgehog, Wnt I, Notch I and Insulin I signaling (Ayala et al., 2018; Choi et al., 2018; Sagomonyants and Mina, 2014; Wang et al., 2013). To investigate the specific signaling pathway involved in Cbfα-suppressed autophagy, signaling pathway inhibitor screening was performed. In Cbfα1-expressing cells, according

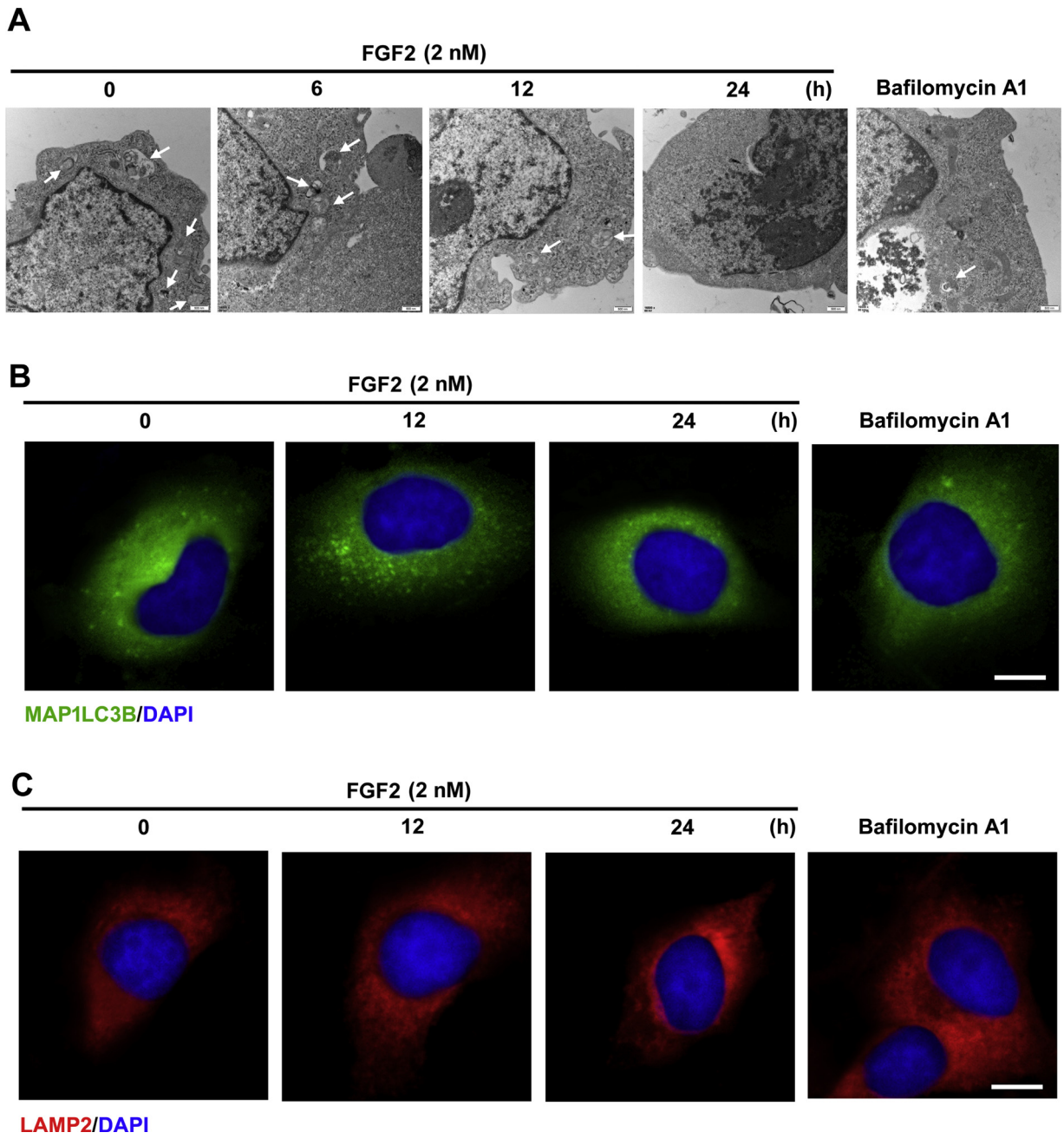

Fig. 2. The autophagy bodies in FGF2-mediated autophagy in MDPC-23 cells.
(**A**) Representative TEM images from MDPC-23 cells treated with FGF2 (2 nM) at different time points or Bafilomycin A1 (100 nM) for 24 h. The autophagy bodies were indicated by white arrows. Bar = 500 nm. (**B** and **C**) The immunofluorescence images from MDPC-23 cells treated with FGF2 (2 nM) at different time points or Bafilomycin A1 (100 nM) for 24 h, followed by staining with DAPI (blue) and MAP1LC3B (green) (B), or LAMP2 (red) (C), respectively. Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

to LC3-II and p62 protein levels, autophagy was dramatically inhibited, which was as similar as the Bafilomycin A1 treatment (Fig. 5A and 5B), whereas this inhibition was significantly impaired when Wnt I signaling repressed (Fig. 5A and 5B). The data suggested that Cbfα1 suppressed autophagy through Wnt signaling.

2.5. Cbfα1 represses FGF2-mediated autophagy in MDPC-23 cells

To further explore whether Cbfα1 is directly related to FGF2-mediated autophagy, we conducted siRNA silencing to knockdown Cbfα1 expression. Upon FGF2 stimulation, both RNA (Fig. 6A) and protein (Fig. 6B and 6C) levels of DSPP were increased, but these increases were reduced after Cbfα1 knocked-down (Fig. 5A-5C), indicating Cbfα1 mediated FGF2-induced DSPP expression. Accordingly, autophagy was dramatically inhibited in FGF2 stimulation, while this inhibition was clearly reversed after Cbfα1 knocked-down (Fig. 6D). Thus, these results illustrated that Cbfα1 facilitated the suppression of autophagy by FGF2 in MDPC-23 cells.

3. Discussion

Dentin forms the main part of tooth and is consisted by odontoblasts. During dentin formation, odontoblasts derived from the progenitors in the pulp produce dentin extracellular matrix (ECM) (Wang et al., 2017). The dentin-specific proteins sialoprotein (DSP) and dentin phosphoprotein (DPP) are essential for the homeostasis during

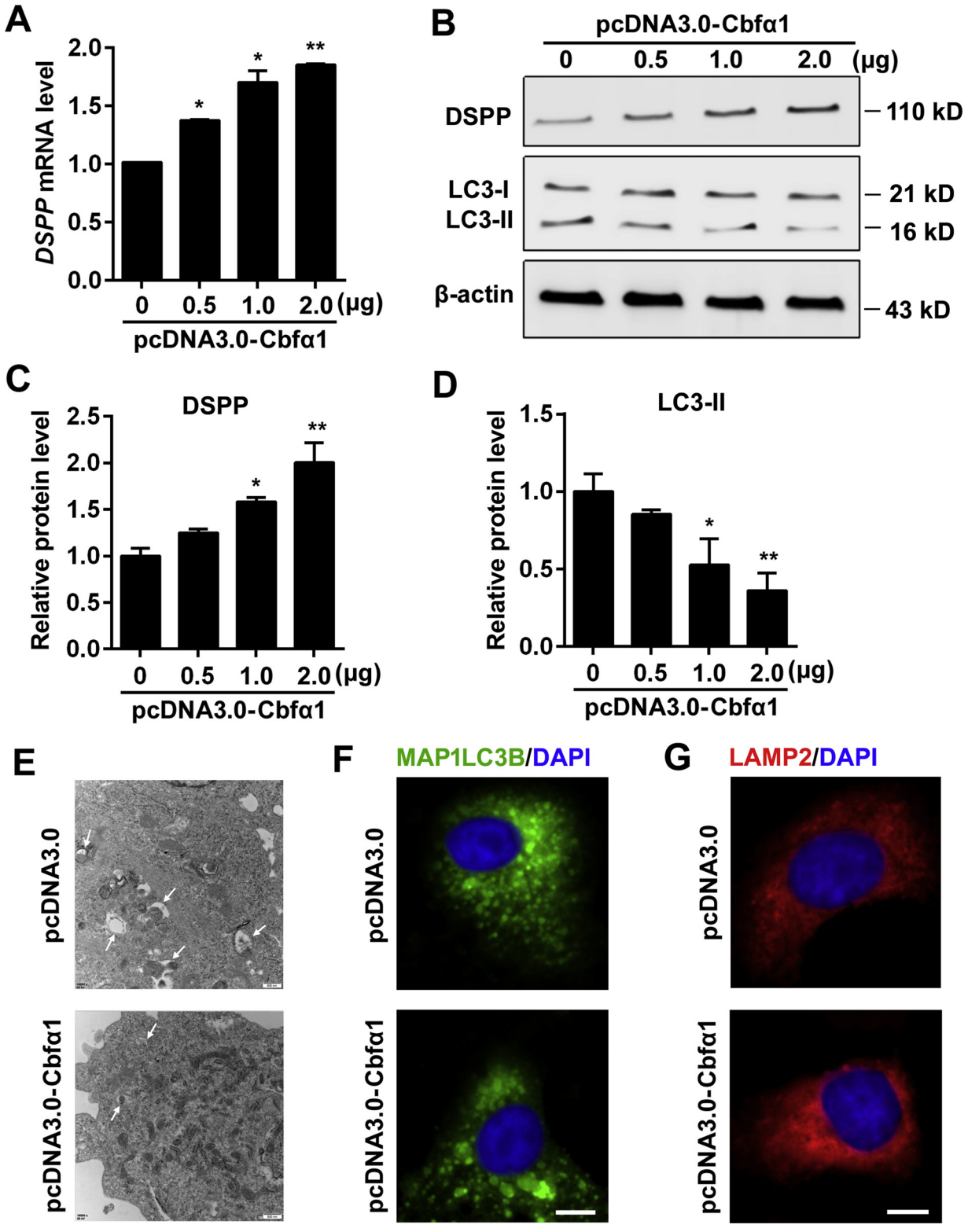

Fig. 3. The suppression role of Cbfα1 on autophagy through upregulating DSPP in MDPC-23 cells.
(**A** and **B**) MDPC-23 cells were transfected with pcDNA3.0-Cbfα1 plasmids at different doses for 48 h. Quantitative PCR analysis of *DSPP* mRNA level (A) and Western blot analysis of protein levels (B) were performed. (**C** and **D**) Quantitative expression of each protein was analyzed by Image J software. The expression level is relative to controls. Data are shown as mean ± SD; n = 3. *, $p < 0.05$; **, $p < 0.01$. (**E**) Representative TEM images from MDPC-23 cells after transfected with pcDNA3.0 or pcDNA3.0-Cbfα1. The autophagy bodies were indicated by white arrows. Bar = 500 nm. (**F** and **G**) The immunofluorescence images from MDPC-23 cells after transfected with pcDNA3.0 or pcDNA3.0-Cbfα1, followed by staining with DAPI (blue) and MAP1LC3B (green) (F), or LAMP2 (red) (G), respectively. Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

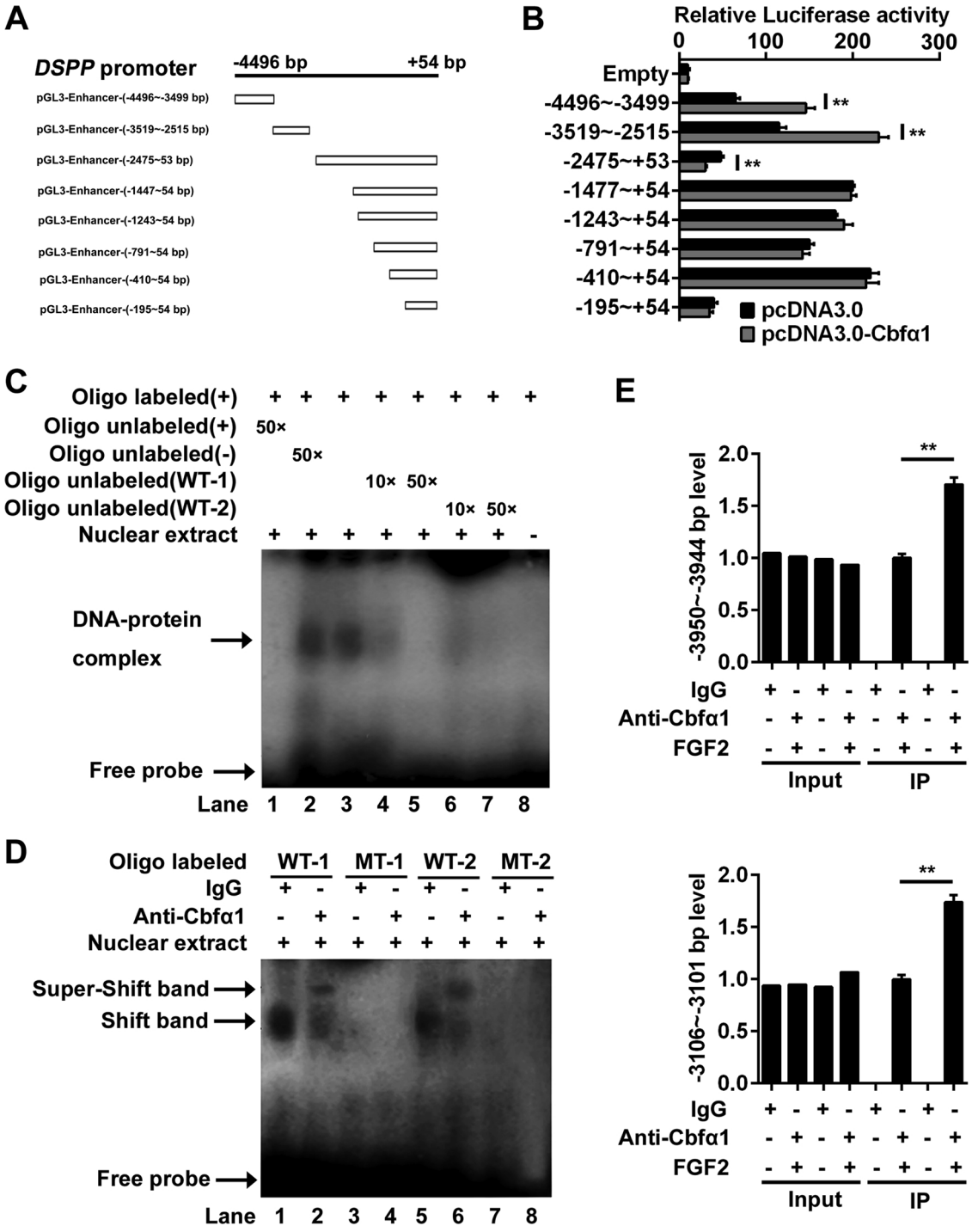

Fig. 4. Transcriptional regulation of Cbfα1 on DSPP expression.
(**A**) The truncations of *DSPP* promoter from -4496 bp to 54 bp. (**B**) MDPC-23 cells were transfected with pcDNA3.0 or pcDNA3.0-Cbfα1 with truncations of *DSPP* promoter of pGL3-Enhancer luciferase reporters. (**C** and **D**) MDPC-23 cells were transfected with pcDNA3.0-Cbfα1 for 48 h. Nuclear extracts were prepared and incubated with oligo DNA (C) or anti-Cbfα1 antibody (D). The samples were subjected to electrophoretic mobility shift assay (EMSA). (**E**) Nuclear extracts were prepared from MDPC-23 cells treated or untreated with FGF2 (2 nM) for 12 h, and then immunoprecipitated (IP) with anti-Cbfα1 antibody. The precipitates were performed with qPCR with specific primers targeted at TACCTCA (-3950 bp to -3944 bp) and ACCACA (-3106 bp to -3101 bp) in *DSPP* promoter. The expression level is relative to controls. Data are shown as mean ± SD; n = 3. **, $p < 0.01$.

odontoblast differentiation (Rathinam et al., 2015). Previous studies uncover that the expression of the osteoblast/odontoblast-associated genes Cbfα1 and DSPP was found to be significantly decreased in the abnormal differentiation of dental pulp cells in cleidocranial dysplasia (Yan et al., 2015). Autophagy is a highly conserved homeostasis which was involved in odontoblast development and aging (Li et al., 2018; Zhang and Chen, 2018). However, the relationship between Cbfα1 and DSPP involved in the autophagy during odontoblast differentiation remains unclear.

FGF signaling prevents the terminal differentiation of odontoblasts through inhibiting autophagy (Sagomonyants et al., 2017). We first assess the expressions of Cbfα1 and DSPP during the FGF2-mediated

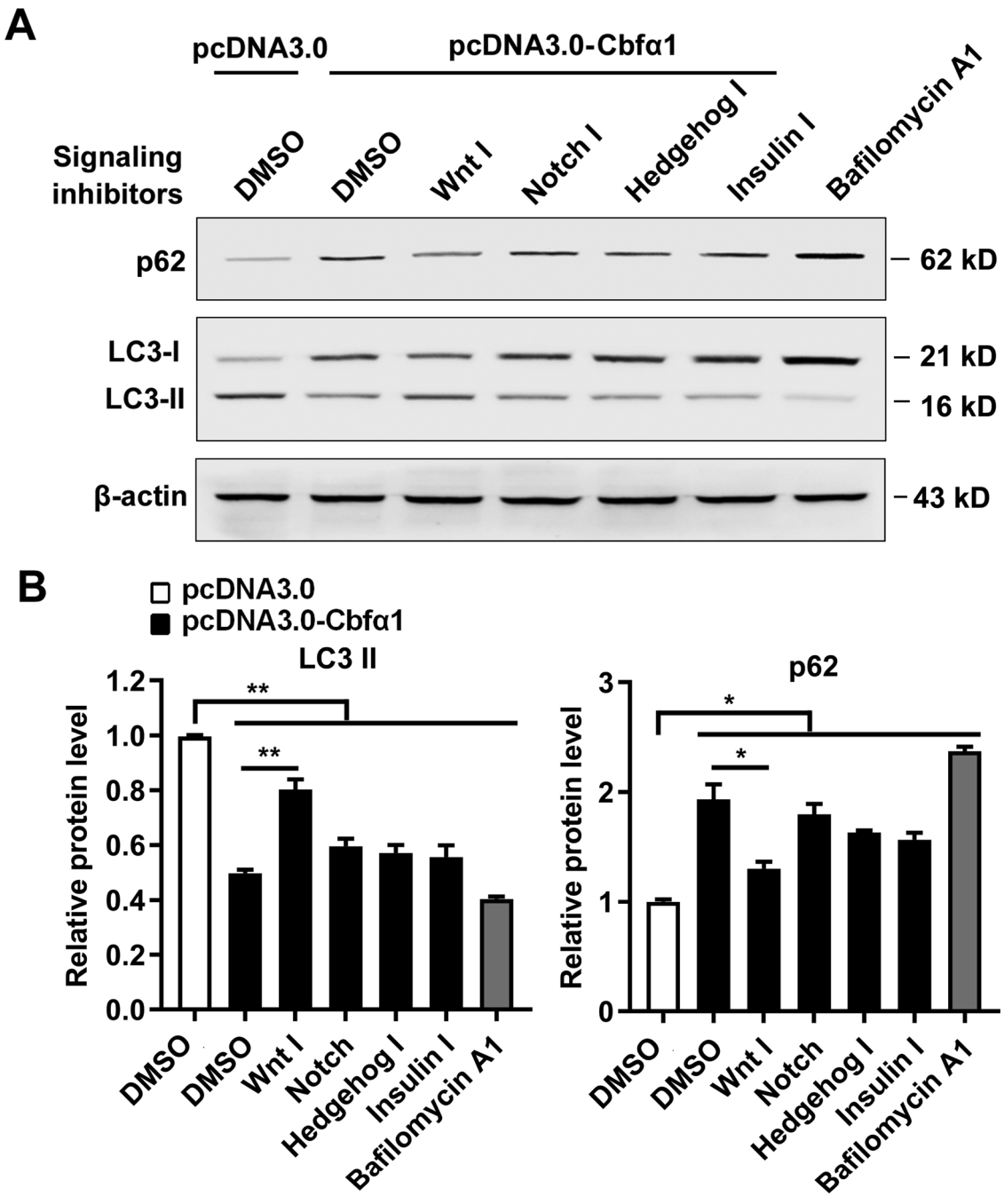

Fig. 5. Cbfα1 repressed autophagy involved in Wnt I signaling.
(**A** and **B**) MDPC-23 cells were transfected with 2 μg pcDNA3.0 or pcDNA3.0-Cbfα1 for 24 h and treated with indicated signaling pathway inhibitors, including autophagy inhibitor Bafilomycin A1, for an additional 24 h. (**A**) Western blot analysis of protein levels was performed (**B**) Quantitative expression of each protein was analyzed by Image J software. The expression level is relative to controls. Data are shown as mean ± SD; n = 3. *, $p < 0.05$; **, $p < 0.01$.

suppression of autophagy in MDPC-23 cells. Upon FGF2 stimulation, both of Cbfα1 and DSPP RNA and protein levels are elevated in a time-dependent manner. In addition, we also observed a great inhibition of autophagy after FGF2 stimulation. FGF2 promotes odontoblast differentiation via increasing DSPP positive odontoblasts (Sagomonyants et al., 2015). Nevertheless, the exact regulatory mechanism of Cbfα1 on DSPP in FGF2-mediated autophagy remains further exploration. Then, the roles of Cbfα1 on DSPP regulation and autophagy were evaluated. In Cbfα1-expressing cells, we found the DSPP RNA and protein levels were increased, while autophagy was obviously reduced.

To explore the exact regulatory mechanism of DSPP by Cbfα1, we firstly employed luciferase report assay to locate the Cbfα1 binding region in DSPP promoter. Then, we conducted EMSA to confirmed specific Cbfα1 binding sites located in 5′-TACCTCA (-3950 bp to -3944 bp) and 5′-ACCACA (-3106 bp to -3101 bp) in *DSPP* promoter. Finally, we continued to perform ChIP assay to reveal that Cbfα1 transcriptionally regulated DSPP expression in FGF2 stimulation. As an odontoblast-specific transcription factor, Cbfα1 is critical for odontoblast differentiation through upregulation of dentin matrix protein 1 (DMP1) expression (Pang et al., 2007). In this study, we identified another Cbfα1 regulating gene DSPP, which further established the function of Cbfα1 in odontoblast differentiation. Meanwhile, we also made clear that Cbfα1 repressed autophagy in MDPC-23 cells.

Autophagy, as a vital degradation and recycling system, has an

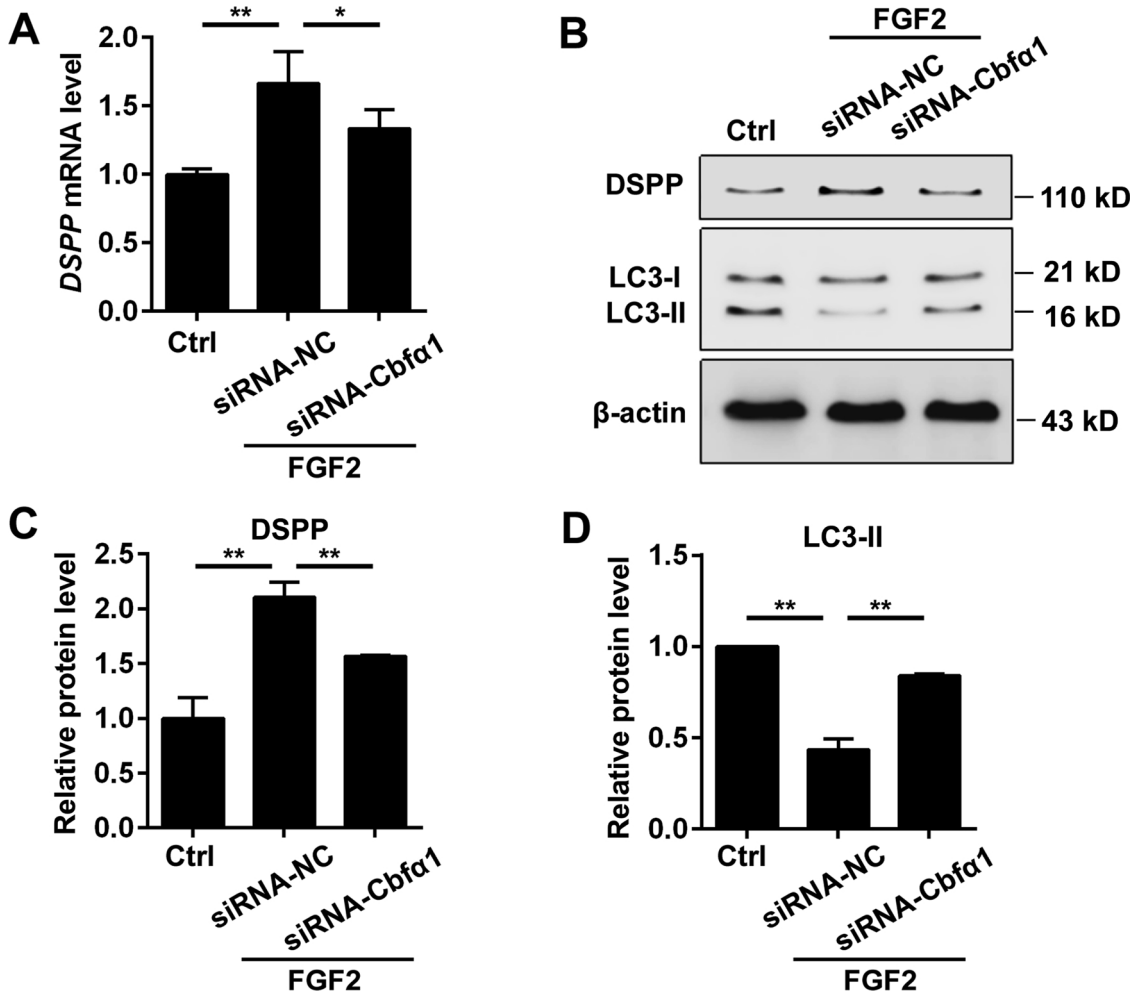

Fig. 6. Cbfα1 facilitated the suppression of FGF2-mediated autophagy in MDPC-23 cells.
(**A** and **B**) MDPC-23 cells were transfected with siRNA control (siRNA-NC) or siRNA targeting Cbfα1 (siRNA-Cbfα1) for 36 h, and treated with FGF2 for an additional 24 h. Quantitative PCR analysis of *DSPP* mRNA level (A) and Western blot analysis of protein levels (B) were performed. (**C** and **D**) Quantitative expression of each protein was analyzed by Image J software. The expression level is relative to controls. Data are shown as mean ± SD; n = 3. *, $p < 0.05$; **, $p < 0.01$.

important protective role in regulating the differentiation of odontoblast, as well as several tooth-related diseases and aging (Couve et al., 2013). During developing and adult teeth, autophagy persists at a low level in the differentiation of odontoblasts and tooth pulp cells, suggesting excessive autophagy may trigger cell death in odontoblasts differentiation (Zhuang et al., 2016). In inflammatory environments, autophagy might act as a double-edged sword in odontoblasts differentiation (Zhang and Chen, 2018). It might protect odontoblasts and enable them to survive at early stages, whereas accelerate cell apoptosis at later stages. Autophagy inhibits odontoblast differentiation by suppressing NF-κB activation (Pei et al., 2016). Hence, we supposed that Cbfα1 increased DSPP expression to repress autophagy, resulting in odontoblast differentiation.

Multiply signaling pathways have been implicated in the modulation of autophagy, including Hedgehog, Wnt I, Notch I as well as Insulin I signaling pathways (Ayala et al., 2018; Choi et al., 2018; Guo et al., 2018; Rahman et al., 2018; Wang et al., 2013). Since Cbfα1 evidently repressed autophagy, we further investigated the specific signaling pathway involved in Cbfα1-suppressed autophagy. Based on signaling pathway inhibitor screening, we revealed that Cbfα1 suppressed autophagy through Wnt signaling. In osteoblasts, Cbfα1 silencing represses the autophagy-related proteins LC3 and RANKL, thereby potentially causing the tooth eruption disorder (Qin and Cai, 2018). To evaluate the role of Cbfα1 in the suppression of autophagy by FGF2 in MDPC-23 cells, Cbfα1 silencing with siRNA was performed and Cbfα1 knocked-down restored the inhibition of autophagy in FGF2 stimulation. Of note, the augment of DSPP enhances odontoblast differentiation by promoting Wnt signaling pathway (Wang et al., 2018). To the end of the study, it could be explained that FGF2-induced Cbfα1 transcriptionally promoted DSPP expression, and the augment of DSPP activated Wnt signaling pathway, which resulted in the repression of autophagy, leading to the odontoblast differentiation. However, it needs more investigations for that whether Cbfα1 suppressed autophagy dependent on DSPP.

In sum, we have demonstrated that upon FGF2 stimulation, both mRNA and protein levels of Cbfα1 and DSPP were elevated coupling with autophagy obviously suppressed. Moreover, Cbfα1 transcriptionally promoted DSPP expression through binding to 5′-TACC TCA (-3950 bp to -3944 bp) and 5′-ACCACA (-3106 bp to -3101 bp) in *DSPP* promoter. In addition, Cbfα1 inhibited autophagy in MDPC-23 cells involved in Wnt signaling. Taken together, these findings elucidate a novel regulatory mechanism by which Cbfα1 enhances DSPP expression, and also provide a new clue of the inhibitory role of Cbfα1 in autophagy during odontoblast differentiation.

4. Methods

4.1. Cell culture

Mouse odontoblast-like MDPC-23 cells were originated from Dr.

Jacques E. Nor (University of Michigan School of Dentistry, MI, USA) as a kind gift (Botero et al., 2003). MDPC-23 cells were cultured in α-MEM medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Gibco, Thermo Fisher Scientific, USA), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate and 50 μg/ml glutamine at 37 °C in a humidified incubator with 5% CO_2.

4.2. Plasmids and reagents

Mouse genome DNA was extracted from an adult Balb/c mouse. The DNA fragments of *DSPP* promoter were amplified using PCR with specific primers as previously described (Guo et al., 2004). The PCR products were ligated into pGL3-enhancer vector (Promega, Madison, WI). The pcDNA3.0 and pcDNA3.0-Cbfα1 plasmids were kindly provided by Dr Yu Qing (The Fourth Military Medical University, Xi'an, China) (Li et al., 2011).

Primary mouse antibody against Cbfα1 (Cat: sc-101145), SOX2 (Cat: sc-365964), and OSX (Cat: sc-393060) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-DSPP (Cat: ab216892) and anti-PPAR-γ (Cat: ab178860) primary antibodies were obtained from Abcam (Cambridge, United Kingdom). Rabbit anti-LC3-I/LC3-II (Cat: #4108), anti-p62 (Cat: #8025) and anti-LAMP2 (Cat: #49,067) primary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-MAP1LC3B rabbit polyclonal antibody (Cat: D163557-0025) was purchased from Sangon Biotech (Shanghai, China).

All of the inhibitors against Hedgehog (Sonidegib), Wnt I (IWP-2), Notch I (IMR-1) and Insulin I (PD168393) signaling pathways, as well as autophagy inhibitor Bafilomycin A1, were obtained from Selleckchem (Houston, TX, USA). Recombinant Mouse Fibroblast Growth Factor Basic (also called FGF2) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

4.3. Quantitative RT-PCR based analysis of mRNA expression

Total RNA samples were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Quantitative real-time reverse transcription PCR (RT-PCR) analysis was performed using an Applied Biosystems 7900 Fast Real-Time PCR System (Foster City, Qiagen, CA). Each 20 μl reaction contained 0.5 μM of each PCR primer, 10 μl of SYBR Green Real-time PCR Master Mix-Plus (ABI Ambion Invitrogen), 1 μl of diluted template, and RNase-free water, were added into 96-well plates. And β-actin was used as an internal control for target genes. The qPCR primers used as followed:

β-actin sense: 5′- GCCCTGAGGCTCTCTTCCA;
β-actin anti-sense: 5′- GCGGATGTCGACGTCACA.
Cbfα1 sense: 5′-AATTAACGCCAGTCGGAGCA;
Cbfα1 anti-sense: 5′-CACTTCTCGGTCTGACGACG.
DSPP sense: 5′- GGCCAATCTCATGGGGGAAA;
DSPP anti-sense: 5′- GAGCTTTTGGTTGTCCTGCG.

4.4. Western blot analysis

Cell lysates were prepared with RIPA lysis buffer (50 mmol/l Tris − HCl pH 7.4, 150 mmol/l NaCl, 1% sodium deoxycholate, 0.5 mol/l EDTA, supplemented by 10% proteinase inhibitors cocktail). Protein concentration was determined with a BCA protein assay reagent kit (Bio-rad). 40–80 μg proteins for each sample were boiled and then separated by SDS-PAGE. Separated proteins in the gel were transferred onto a nitrocellulose membrane (Amersham, Piscataway, NY). The membrane was blocked with 10% skim milk in PBST buffer for 30–60 min at room temperature and incubated with the primary antibodies overnight at 4 °C. The membrane was then incubated with anti-rabbit IgG conjugated-HRP for 45 min. The image of immunoblot was finally digitalized with bioluminescent imaging system (Bio-rad). The relative expression of each protein was analyzed using Image J software.

4.5. Cell transfection

MDPC-23 cells were seeded (1×10^6 cells/well) in 6-well plates at 50–80% confluent density. For each well of cells to be transfected, 2 μg of DNA and 4 μl of Lipofectamine™ 2000 (Invitrogen) were diluted in 100 μl of Opti-MEM I Reduced Serum Media without serum, respectively. The dilutions were mixed gently and incubated at room temperature for 15 min, until the complexes were added directly to each well. The cells were incubated at 37 °C in a CO_2 incubator for 36–48 h post-transfection before assaying for gene expression.

4.6. Luciferase reporter gene assay

MDPC-23 cells were seeded (1×10^5 cells/well) in 24-well plates. According to the manufacturer recommended protocol, 100 ng of *DSPP* promoter luciferase reporter and 300 ng Cbfα1-expressing plasmid were transfected in each well. All transfections were performed using Lipofectamine™ 2000 in Opti-MEM I Reduced Serum Medium (100 μl/well) for 24 h. The luciferase activities from each well were measured using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instruction.

4.7. Electrophoretic mobility shift assay (EMSA)

The purified protein and the crude cell extract are typically incubated with a ^{32}P isotopically labeled DNA probe (Sunbiotech, Beijing, China), and the complex and unbound probe are separated on non-denaturing polypropylene gel electrophoresis. In competition experiments, DNA fragments and oligonucleotide fragments (specific) containing protein-binding sequences, and other unrelated fragments (non-specific) are used to determine the specificity of DNA-binding proteins. The sequences of oligo DNA are shown as follows and the core binding sites are indicated as underlined letters:

Oligo positive DNA, sense: 5′- GTCACCAACCACAGCATCCTTTG; anti-sense: 5′-CAAAGGATGCTGTGGTTGGTGAC; Oligo negative DNA, sense: 5′- GTCACCAAgaACAGCATCCTTTG; anti-sense: 5′- CAAAGG TGCTGTtcTTGGTAC.

Oligo DNA WT1, sense: 5′-TTTAATTTACCTCAGCAGCATTT; anti-sense: 5′-AAATGCTGCTGAGGTAAATTAAA.

Oligo DNA MT1, sense: 5′-TTTAATTTAggTCAGCAGCATTT; anti-sense: 5′- AAATGCTGCTGAccTAAATTAAA.

Oligo DNA WT2, sense: 5′- TTTAGTTCACCACAAATTACATT; anti-sense: 5′- AATGTAATTTGTGGTGAACTAAA.

Oligo DNA MT2, sense: 5′- TTTAGTTCAggACAAATTACATT; anti-sense: 5′- AATGTAATTTGTccTGAACTAAA.

5. Transmission electron microscope (TEM)

MDPC-23 cells treated with different concentrations of FGF2 (2 μM) were harvested by mild trypsinization (0.25% trypsin in PBS containing 0.05% EDTA) and collected by centrifugation at 800 rpm for 5 min in fresh tubes. The samples were immediately fixed with 2.5% glutaraldehyde in culture medium (2 h at room temperature), centrifuged at 2000 rpm for 10 min and washed several times with PBS. Then, samples were post-fixed in 1% OsO_4 for 2 h at room temperature and washed in water. The cell pellets were pre-embedded in 2% agar, dehydrated with increasing concentrations of acetone (30, 50, 70, 90 and 100%, respectively). Finally, the pellets were embedded in EPON resin and polymerized at 60 °C for 48 h. Ultrathin sections were obtained with ultramicrotome Rechter, then placed on nickel grids and stained with uranyl acetate and lead citrate. Sections were observed under a Zeiss EM 900 transmission electron microscope operating at 80 kV and computerized through Epson Perfection 4990 Photo scanner.

5.1. Chromatin immune precipitation (ChIP)

The chromatin immunoprecipitation (ChIP) assay kit was purchased through Upstate Biotechnology (Lake Placid, NY, USA). MDPC-23 cells were treated with formaldehyde to cross-link protein and DNA to the *DSPP* promoter, and sonication was used to shear DNA. Chromatin was immunoprecipitated using antibodies against Cbfα1 or IgG isotype control. The quantification of immunoprecipitated DNA was measured by qRT-PCR with specific primers. All data were normalized to the percentage of input. The specific primers used detect DNA regions in *DSPP* promoter from TACCTCA (-3950 bp to -3944 bp), sense: 5′-GTT GTCCCTGGGTCTGTT; anti-sense: 5′- TTCCCAATGGGCAAACTA; and ACCACA (-3106 bp to -3101 bp), sense: 5′- TAAAGATAAGTTTCGGG AGA; anti-sense: 5′- ACAAATGCTGCTGAGGTA.

6. Statistics

All data were shown as means ± standard deviation (SD) unless stated otherwise. Statistical testing was performed using Prism 5 software (GraphPad Software Inc.) with the statistical tests (Student's *t*-test) indicated in the figure legends. A p-value < 0.05 was considered to statistical significance.

Author contributions

Ting Guo planned and performed experiments, analyzed data, and wrote the paper. Zhi Li performed experiments and analyzed data. Bingyao Liu and Wei Chen performed experiments and contributed reagents or other essential material. Ting Guo and Gang Cao planned experiments and critically revised the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest.

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