GSK3 β Rephosphorylation Rescues ALPL Deficiency Induced-impairment of Odontoblastic Differentiation of DPSCs

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**GSK3 β rephosphorylation rescues ALPL deficiency induced-impairment of
odontoblastic differentiation of DPSCs**

Running title: ALPL regulates DPSCs differentiation via canonical Wnt pathway

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1 **Abstract**

2 **Background:** Premature exfoliation of deciduous teeth is a common manifestation in childhood
3 patients with Hypophosphatasia (HPP), which is an autosomal inherited disease caused by ALPL
4 mutations. Dysplasia of cementum, dentin and alveolar bone have been proposed to be the main
5 reasons for the exfoliation of teeth, while the extraordinarily complex intracellular mechanisms
6 remain elusive. Dental pulp stem cells (DPSCs) have been demonstrated to successfully
7 regenerate functional pulp-dentin like tissue. Dental pulp cells derived from HPP patients impaired
8 mineralization, however insight into the deeper mechanism is still unclear.

9 **Methods:** The effects of ALPL on odontoblastic differentiation of DPSCs from HPP patient were
10 assessed by Alizarin Red staining, immunofluorescent staining, Western blot and RT-PCR, and
11 micro-CT assays.

12 **Results :** Here, we found DPSCs from HPP patient exhibited low ALP activity and impaired
13 odontoblastic differentiation. Meanwhile, we found that loss of function of ALPL reduced
14 phosphorylation of GSK3 β in DPSCs. While GSK3 β rephosphorylation improved odontoblastic
15 differentiation of HPP DPSCs with LiCl treatment. Finally, we demonstrated systemic LiCl
16 injection ameliorated tooth associated defects in ALPL^{+/-} mice by enhanced phosphorylation of
17 GSK3 β in teeth.

18 **Conclusions:** Our study indicates that ALPL regulates odontoblastic differentiation of DPSCs, and
19 provides useful information for understanding how ALPL deficiency leaded to tooth dysplasia and,
20 ultimately, may inform efforts at improvement tooth defects in HPP patients.

21

22 **Keywords:** Hypophosphatasia, tooth defects, odontoblastic differentiation, ALPL, DPSCs, GSK3 β

1 **Background**

2 Hypophosphatasia (HPP) is an areinherited disease characterized with insufficient mineralization
3 of teeth and skeletal system caused by gene mutations of liver/bone/kidney-type alkaline
4 phosphatase (ALPL), which encoded tissue-nonspecific alkaline phosphatase (TNAP) and leading
5 to reduce activity of the enzyme [1-3]. The clinical manifestations of patients with HPP are broad
6 range from life-threatening in infantile or death in utero to mild forms in childhood or adult with
7 only dental or skeletal symptoms. Accumulating clinical trials suggest that asfotase alfa
8 (Strensiq™), a first-in-class drug was permitted to use for therapy the patients with HPP, treatment
9 can significantly improve outcome for the life-threaten patients with HPP [4-6]. However, several
10 problems remain to be worked out, despite asfotase alfa exhibited a significant improvement on
11 bone mineralization. Hypersensitive and ectopic calcification in injection sites, pneumonia, and
12 even severe chronic hepatitis or failed treatment have been reported [7-9], and the longer-time
13 effects of asfotase alfa therapy in these HPP patients are still unknow.

14 Premature exfoliation of deciduous teeth is the most common manifestation in childhood patients
15 with HPP, severely affect life quality of the patients. Asfotase alfa on improvement tooth defects
16 in HPP patients was few reported. In addition, the expensive cost should also be considered,
17 especially in economically under-developed areas. Thus, cheaper and more effective strategies
18 need to be developed based on discovering molecular mechanism for treatment patients with HPP,
19 especially in the mild forms of childhood- or odonto-HPP. Dysplasia or aplasia of cementum,
20 dentin and alveolar bone were frequently observed in patients with HPP [10-12] and have been
21 proposed to be the main reasons for the exfoliation of teeth, however the complex cellular
22 mechanisms remain elusive.

1 Dental pulp stem cells (DPSCs) have been identified to be a population of capable of multilineage
2 differentiation into a variety of cell types, including neurons, adipocytes, osteoblasts and
3 odontoblasts [13]. Strong evidences have demonstrated that DPSCs with tooth slice or root
4 canals-scaffold could regenerate pulp-dentin like complex tissues when transplanted into
5 subcutaneous of immunocompromised mice [14, 15]. Notably, our previous work showed that
6 implanted autologous DPSCs aggregates into necrotic immature permanent incisors of pediatric
7 patients successfully regenerated functional dental pulp with odontoblast layer [16]. These
8 evidences suggest that DPSCs display excellent regenerative ability, which is important for tooth
9 development and regeneration. Despite some studies have showed that dental pulp cells derived
10 from HPP patients reduced ALP activity and actually impaired mineralization [17, 18], however
11 insight into the deeper mechanism remains unclear.

12 In this study, tooth defects were observed both in HPP patients and $ALPL^{+/-}$ mice. In addition,
13 DPSCs from HPP patient (HPP DPSCs) displayed low ALP activity and impaired lineage
14 differentiation into odontoblasts. Subsequently, we found that loss function of ALPL inhibited
15 activation of canonical Wnt pathway. While reactivating of canonical Wnt pathway promoted
16 odontoblastic differentiation of HPP DPSCs with LiCl treatment. Finally, we demonstrated
17 systemic LiCl injection ameliorated insufficient mineralization of tooth and mandible bone, and
18 also improved exposure of collagen fiber network on the surface of dentinal tubules in $ALPL^{+/-}$
19 mice. This work provides useful information for understanding tooth associated disorders caused
20 by loss of function of ALPL and, ultimately, may inform efforts at improvement tooth defects in
21 HPP patients.

22 **Materials and Methods**

1 **Animals**

2 Female ALPL^{+/-} mice (B6.129S7-Akp2tm1Sor/J, pure C57BL/6J genetic background) were
3 purchased from Jackson labs (Bar Harbor, USA) and maintained in specific pathogen-free
4 conditions at 24°C with a 12 h light/dark cycle. All of the procedures that involved animals were
5 approved by the Animal Ethics Committee of the Xi'an Jiaotong University. Primers used for
6 genotyping include: mutant-5'CCGTGCATCTGCCAGTTTGAGGGGA3', wild
7 type-5'CTGGCACAAAAGAGTTGGTAAGGCAG3',
8 common-5'GATCGGAACGTCAATTAACGTCAAT3'.

9 **Isolation and culture of DPSCs**

10 Tooth samples were collected from healthy teeth extracted for orthodontic reasons and one patient
11 with HPP (Dental Clinic of the Fourth Military Medical University, Xi'an, China). To isolate
12 dental pulp stem cells (DPSCs), dental pulp were enzymatically digested with type I collagenase
13 (0.66 mg/ml; Sigma, USA) for 30min, single-cell deposits were suspended and cultured in a-MEM
14 (Gibco, USA) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen, USA), 100 U/ml
15 penicillin and 100 mg/ml streptomycin (Gibco) at 37 °C of 5% CO₂.

16 **Alkaline phosphatase activity assay**

17 To analyze alkaline phosphatase activity, the total proteins of DPSCs from normal and HPP patient
18 were isolated and examined using an Alkaline phosphatase assay kit from NJCBIO company
19 (NJCBIO, A059-2-2, China) according to the manufacturer's instructions. ALP activity was
20 corrected with total proteins and ultimately were present as U/g.

21 **Flow cytometry analysis**

22 For identification of DPSCs phenotype, 3 × 10E5 DPSCs (passage 3) were incubated with PE or

1 FITC conjugated monoclonal antibodies against human CD29, CD44, CD105, CD34, CD45
2 (eBioscience, CA) and Stro-1 (R&D Systems, MN) (sFig. 1), and flow cytometric analysis were
3 performed using a Beckman CytoFLEX S flow cytometer (Beckman Coulter, USA).

4 To analyze cell cycle, DPSCs cultured with 0.1 % a-MEM medium for 12 hours and then replaced
5 and cultured with 10 % a-MEM medium for 24 hours. Subsequently, the cells were collected, and
6 cell cycle tests were performed with a Cell Cycle and Apoptosis Analysis Kit (Yeasen, 40301ES60,
7 China) according to the manufacture's instruction. For apoptosis assays, the cells were collected,
8 and cell apoptosis tests were performed with a Annexin V-FITC/PI Apoptosis Detection Kit
9 (Yeasen, 40302ES60, China) according to the manufacture's instruction.

10 **DPSCs induced odontoblastic differentiation**

11 DPSCs were incubated in odontoblastic medium (100 nM dexamethasone, 50 mg/ml ascorbic acid,
12 and 5 mM β -glycerophosphate) for 3 weeks. To assess odontoblastic differentiation, the
13 expression of DSPP and DMP1 were detected by real-time polymerase chain reaction (RT-PCR)
14 and western blotting after induced in odontoblastic medium 1 week. To analyze the mineralized
15 nodes, the cells were stained with 1% Alizarin Red (Sigma, USA) after induced in odontoblastic
16 medium 3 weeks.

17 **Scan Electron Microscopy (SEM) assay**

18 The human teeth, or molars from wild type or ALPL^{+/-} mice were fixed in 2.5% glutaraldehyde,
19 washed with 10% sodium hypochlorite in ultrasonic cleaning instrument for 30min. Then samples
20 were dried, coated with gold and were imaged with a scanning electron microscope (SEM,
21 S-3400N) at a voltage of 5.0 kV. All measurements were made using the Cell Sense morphometric
22 program (Olympus).

Real time PCR (RT-PCR) analysis

Total DPSCs RNAs were isolated and extracted by RNAiso plus (TaKaRa, Japan) according to the manufacture's instruction. Then, the mRNA was reversely transcribed into cDNA by PrimescriptTM RT master mix (TaKaRa, RR036A, Japan). Real-time PCR was performed with TB Green[®] Premix Ex TaqTM GC (TaKaRa, RR071A, Japan), and detected by CFX96 Trademark Real-time PCR detection system (Bio-rad, USA). The primers used in Real time PCR were listed in the supplementary table 1.

Western blotting analysis

Total proteins from DPSCs were harvested with RIPA lysis buffer (Beyotime, China). The proteins were separated in sodium dodecyl sulfonate-polyacrylamide gels (SDS-PAGE), transferred to PVDF membranes (Millipore, USA), blocked in TBST containing 5% BSA, and then incubated in first antibodies with DSPP (Santa Cruz Biotechnology, sc-73632, 1:500), DMP1 (Santa Cruz Biotechnology, sc-6551, 1:500), total GSK3 β (Cell signaling technology, 9832, 1:1000), p-GSK3 β (Cell signaling technology, 9323, 1:1000), β -catenin (Cell signaling technology, 8480s, 1:1000), active β -catenin (Millipore, 05-665, 1:1000), p-AMPK (Cell signaling technology, 2535, 1:1000), p-ERK (Cell signaling technology, 9101s, 1:1000), Gli1 (Novus Biological, NBP1-78259) and GAPDH (Cwbiochem, CW0100, 1:4000), respectively. Next day, the membranes incubated in secondary antibodies coupled to peroxidase (Cwbiochem, China). Finally, the signals were detected by an enhanced chemiluminescence kit (ECL, Cwbiochem, China).

Immunofluorescent staining

For immunofluorescent staining, single mandibular hemi-sections were fixed in 4% paraformaldehyde overnight, decalcified with 10% EDTA (pH 7.0), and embedded in Optimal

1 Cutting Temperature (OCT, Leica), and sectioned into 10 μ m sections. Then, the sections were
2 washed with PBS, incubated with 0.03% triton-100 for 10 minutes at room temperature, and
3 blocked with PBS containing 1% BSA for 30 minutes at room temperature. Then, the sections
4 were incubated overnight with primary antibodies to p-GSK3 β (1:100, Cell signaling technology,
5 9323), and active β -catenin (1:100, Millipore, 05-665) at 4°C respectively, and subsequently
6 incubated with fluorescent secondary antibodies (1:200) for 2 hours at room temperature. The
7 positive cells were examined under a laser scanning confocal microscope (Olympus FluoViem FV
8 1000, Tokyo, Japan).

9 **Micro-computed tomography analysis**

10 The micro-CT scans of single mandibular hemi-sections were performed at the molar level using
11 an GE micro-CT system (GE, USA). X-ray source was set at 80 kV, and 80 μ A microfocus.
12 Three-dimensional images were reconstructed and data analysis were performed by GEHC
13 MicroView analysis software. The single mandibular regions and sagittal planes were chosen for
14 comparison.

15 **Lentiviral vector construction and transduction**

16 To upregulate ALPL, ALPL was amplified from cDNA of human SEHD by PCR. Then, the PCR
17 product was digested with the restriction enzymes BamH I and Xho I, and then inserted into the
18 pLenti 6.3 vector (Invitrogen), named pLenti-ALPL. For downregulation ALPL, the shRNA
19 sequences for targeting human ALPL were inserted in the pLko.1 vector (Invitrogen). The
20 lentivirus contained with pLenti-ALPL or ALPL shRNA were produced by co-transfecting 293T
21 cells with the transfer lentiviral vector and two packaging vectors (psPAX2 and pMD2.G). The
22 primers used to construct the lentiviral vectors of ALPL are listed in Supplementary Table 2.

β-catenin siRNA transduction

To silence β-catenin expression in HPP DSPCs, β-catenin siRNA (Ribobio, China) were transfected into HPP DSPCs at a final concentration of 50 nM using riboFECT™ CP (Ribobio, China). NC siRNA was transfected as negative control. The silence efficiency on β-catenin were examined after transduction 48 hours with β-catenin siRNA by RT-PCR and Western blotting.

LiCl treatments in vitro

A total of 1×10^5 or 2×10^5 DPSCs were plated in each well of 12-well or 6-well plates and cultured. After DPSCs reached above 90% confluence, the cells were cultured in odontoblastic differentiation medium containing 10 mM LiCl (Sigma, 213233) or 10 mM NaCl, and the medium was changed three times per week. On days 7 and 21, the cells were harvested and performed with RC-PCR, Western blotting and Alizarin Red staining as described above.

LiCl intraperitoneal injection *in vivo*

A total of 16 4-month-old ALPL^{+/-} mice were randomly divided into NaCl group and LiCl group (n=8 per group). The LiCl group were injected 100 mg/kg LiCl with body weight by intraperitoneal injection every other day for 4 weeks. The control mice received a comparable volume of NaCl. All mice were sacrificed after injection 30 days for teeth analysis by micro-CT, immunofluorescent staining, H&E, and SEM.

Statistics

The data were presented as the mean $\pm$ s.d. All of the experiments were repeated more than three times. Statistical analysis was made using Student's t-test or One-way ANOVA, the $p < 0.05$ were considered significant.

Results

Characteristics of teeth from HPP patients and ALPL^{+/-} mice

HPP patients commonly face premature exfoliation teeth caused by dysplasia or aplasia of root or cementum [2, 19, 20]. We have collected two childhood HPP patients reported in our previous study [21]. Two patients displayed premature exfoliation of primary or permanent teeth. According to corresponding panoramic radiograph results, the height of upper and lower jaws was insufficient, the permanent dentition featured short roots and delayed eruption in HPP1 (Fig. 1a). The alveolar bone height insufficient and short roots were observed in HPP2 (Fig. 1a). Fortunately, one lower cuspid from HPP1 were collected due to severe odontoseisis. The shape of pediatric deciduous cuspid is normal, with no root resorption, and rough root surface (Fig. 1b). Masson staining and H&E staining showed that the cementum layer was apparent thin and uneven surface. In addition, minority root was not covered with cementum, and cementum separated with dentin were observed (Fig. 1b). SEM results further showed that the surface of cementum in HPP1 patient were uneven and rough, and visible dentinal tubules open at root surface, while the surface of cementum is smooth and homogenous in healthy tooth (Fig. 1c). The dentinal tubules in the HPP1 patient were irregular and low mineralized dentin, and were larger lumen with obvious exposure of collagen fiber network than that in the normal deciduous tooth (Fig. 1d).

Previous studies have revealed that ALPL^{-/-} mice suffered severe insufficient mineralization of teeth and skeletal systems [22-24], and skeletal mineralization were significantly impaired in ALPL^{+/-} mice [21, 25]. To investigate tooth defects in HPP, ALPL^{+/-} mice were chosen as animal model, because of ALPL^{-/-} mice die from 14 - 21 days after birth [26]. First, we found the ALP activity in serum of ALPL knockdown mice reduced approximately 60% as compared with that of

1 wild type mice (Fig. 2a). Next, to evaluate the effects of ALPL deficiency on tooth, micro-CT
2 analysis were performed. The results showed that alveolar bone height, mandibular bone mass,
3 and dentin mineral density (DMD) dramatically reduced in ALPL^{+/-} mice compared with that in
4 control group (Fig. 2b). Moreover, ALPL knockdown significantly decreased the alveolar bone
5 mass, and led to cementum partially segregated from dentin (Fig. 2c, d). In addition, dentinal
6 tubules of the molars in ALPL^{+/-} mice exhibited larger lumen with collagen fiber network
7 exposure contrast to the control group (Fig. 2d), which were observed in the HPP1 patient.
8 Collectively, these results suggested ALPL^{+/-} mice exhibited tooth defects similar to HPP
9 associated periodontal disorder.

10 **The influence of ALPL mutation on cell proliferation and apoptosis of DPSCs**

11 Increasing data provided the evidences that DPSCs could be induced to differentiate into
12 odontoblasts, and regenerate a dental pulp-dentin like tissue in vivo [13, 14]. To further assess the
13 role of ALPL in teeth development, DPSCs derived from HPP1 patient were isolated and cultured.
14 First, we found that alkaline phosphatase activity and the protein expression of ALPL in HPP
15 group decreased significantly compared with that of the normal group (Fig. 3a). Then, to evaluate
16 the roles of ALPL on cell proliferation and apoptosis, the tests of colony formation, cell cycle, and
17 cell apoptosis were performed. In contrast to normal DPSCs, the CFU efficiency (Fig. 3b) and the
18 phase of S+G2 in cell cycle (Fig. 3c) obviously reduced in HPP group, but the difference in cell
19 apoptosis was not observed (Fig. 3d).

20 **ALPL regulates the odontoblastic differentiation of DPSCs**

21 Given that impaired mineralization and exposure of collagen fiber network on dentinal tubules
22 were observed both in HPP patient and ALPL^{+/-} mice, we next evaluated the capacity of DPSCs

1 from HPP differentiation into odontoblasts *in vitro*. The results of Alizarin Red staining showed
2 that the potential odontoblastic differentiation capacity of HPP DPSCs significantly impaired (Fig.
3 4a), along with decreased expression of odontoblast associated genes and proteins, DSPP and
4 DMP1 (Fig. 4b). To further validate the role of ALPL in regulatory odontoblastic differentiation,
5 we up- or downregulated the expression of ALPL in HPP DPSCs with plenti-ALPL lentivirus or
6 normal DPSCs with plko.1-ALPL shRNA lentivirus (sFig. 2). The odontoblastic differentiation of
7 normal DPSCs was inhibited by downregulation of ALPL (Fig. 4c, d), mimicking the
8 characteristic of HPP DPSCs. Whereas recovery ALPL expression in HPP DPSCs remarkably
9 promoted odontoblastic differentiation (Fig. 4e, f) as evidenced by Alizarin Red staining, and
10 expressions of odontoblast associated genes and proteins. Therefore, our results suggest that ALPL
11 could be essential for DPSCs maintaining odontoblastic differentiation property.

12 ALPL regulates DPSCs differentiation into odontoblasts by controlling canonical Wnt signaling
13 pathway

14 Our previous study has demonstrated that ALPL deficiency-induced dysregulation of the
15 phosphorylation of intracellular signaling proteins in MSCs [21]. We found that the protein levels
16 of phosphorylation of glycogen synthase kinase 3 β (p-GSK3 β), and active β -catenin in HPP
17 DPSCs significantly decreased compared with that in healthy cells (Fig. 5a). In addition, Gli1 and
18 phosphorylation of AMP-activated protein kinase α (AMPK α) also decreased in HPP DPSCs, but
19 not extracellular signal-regulated kinase1/2 (p-ERK1/2) (sFig. 3). Considering that GSK3 β
20 mediated canonical Wnt signaling pathway is a key signaling pathway for regulating tooth
21 development [27, 28], the expression of p-GSK3 β and active β -catenin in the first molars were
22 examined by immunofluorescent staining. Notably, contrast to wild type group, the expression of

1 p-GSK3 β and active β -catenin significantly decreased especially in dental pulp and alveolar bone
2 in ALPL^{+/-} mice as showed in immunofluorescence staining (Fig. 5b).

3 Then, to evaluate whether ALPL could regulate p-GSK3 β and active β -catenin expression, ALPL
4 were down-regulated in healthy DPSCs or up-regulated in HPP DPSCs, respectively. The western
5 blotting results showed that downregulation of ALPL in normal cells significantly inhibited
6 p-GSK3 β and active β -catenin expression, while ALPL overexpression effectively promoted the
7 expression of p-GSK3 β and active β -catenin in HPP DPSCs (Fig.5 c, d). These results suggested
8 that ALPL deficiency could disrupt canonical Wnt pathway activation and then impair DPSCs
9 differentiation into odontoblasts.

10 To resolve above question, the small molecule LiCl, which is an agonist for high effective
11 activating canonical Wnt signaling pathway by inhibited GSK3 β activity [29, 30], were used to
12 treat HPP DPSCs. As we expected, the expression of phosphorylation of GSK3 β and active
13 β -catenin increased dramatically in HPP DPSCs with LiCl treatment (Fig. 5e). Importantly,
14 contrast to control group, the odontoblastic differentiation was prominently improved in HPP
15 DPSCs after treated with LiCl by enhanced Alizarin Red staining, and the protein expression of
16 DSPP and DMP1 (Fig. 5f, g).

17 To further demonstrate that LiCl rescued the odontoblastic differentiation of HPP DPSCs by
18 activating canonical Wnt pathway, these cells were treated with LiCl meanwhile interfered with
19 β -catenin siRNA to block canonical Wnt pathway activation. The RT-PCR and Western blotting
20 results showed that β -catenin siRNA strong silenced the expression of β -catenin in HPP DPSCs
21 (Fig. 6a). Then, we found that increased the expression of active β -catenin, but not p-GSK3 β , in
22 HPP DPSCs with LiCl treatment were inhibited by β -catenin siRNA (Fig. 6b). In addition, LiCl

1 improved odontoblastic differentiation of HPP DPSCs were almost blocked by silence β -catenin
2 expression according to the results of Alizarin Red staining and Western blotting (Fig. 6c, d).
3 Collectively, these results indicated that reactivated GSK3 β to β -catenin signaling cascade could
4 improve odontoblastic differentiation of HPP DPSCs.

5 **LiCl treatment ameliorates insufficient mineralization of dentin and mandible in ALPL^{+/-}** 6 **mice**

7 Next, to evaluate whether LiCl plays therapeutic benefit on improving insufficient mineralization
8 of molars and mandible in ALPL knockout mice, the mice were administered LiCl every other day
9 for 1 month. First, we observed the protein levels of p-GSK3 β and active β -catenin increased
10 significantly in dental pulp and alveolar bone of ALPL^{+/-} mice after LiCl treatment 1 month,
11 compared with those in NaCl group (Fig. 7a). Notably, micro-CT analysis confirmed that ALPL^{+/-}
12 mice treated with LiCl displayed high alveolar bone height, increased bone mass of mandibular
13 bone and alveolar bone, and DMD compared with those of the NaCl group (Fig. 7b). Further, the
14 H&E staining showed that LiCl injection increased the alveolar bone mass and improved
15 segregation between dentin and cementum in ALPL knockdown mice (Fig. 7c). Meanwhile, the
16 dentinal tubules with the larger lumen and collagen fiber network exposure in the molars of
17 ALPL^{+/-} mice were also ameliorated after LiCl treatment (Fig. 7d). In summary, our results
18 provided the convincing proof for the mechanism that ALPL deficiency inhibited intracellular
19 GSK3 β to β -catenin signaling cascade leading to impairment of odontoblastic differentiation in
20 DPSCs. While LiCl treatment could rescue such impairment through reactivating canonical Wnt
21 pathway (Fig. 8).

22

1 Discussion

2 Tooth associated defects, such short roots with thin dentin, lack of acellular cementum, and
3 alveolar bone loss, are the most common clinical manifestations of patients with HPP [1, 19, 20,
4 31, 32], and these phenotypes were also found in ALPL knockout mice or condition ALPL
5 knockout mice [11, 24, 33]. However, the mechanism of dysplasia or aplasia of teeth caused by
6 loss of function of ALPL remains elusive. In this study, our goals were to evaluate the influence of
7 ALPL mutation on bio-behaviors of DPSCs and reveal the potential mechanism on cellular signal
8 transduction. Here, we provided the evidence that ALPL deficiency inhibited cell proliferation and
9 odontoblastic differentiation of DPSCs from HPP patient. Furthermore, ALPL gene mutation
10 caused GSK3 β maintaining low phosphorylation status, and prevented β -catenin translocation into
11 nucleus and inhibited activation of canonical Wnt signaling pathway, which is one of main
12 signaling pathways for controlling lineage differentiation of MSCs [34, 35]. Notably, LiCl
13 treatment reactivated intracellular GSK3 β to β -catenin signaling cascade in DPSCs or dental pulp,
14 and restored impairment of odontoblastic differentiation, then ameliorated teeth and periodontal
15 related disorders in ALPL^{+/-} mice.

16 Previous studies showed the accumulation of pyrophosphate (PPi), one of substrates of ALPL, in
17 culture medium impaired mineralization of dental pulp cells from HPP patients [17, 36]. However,
18 researchers have not elucidated whether ALPL induces a series of intracellular changes. Here, we
19 provided evidences that ALPL deficiency disrupted GSK3 β to β -catenin signaling cascade and
20 then inhibited expression of odontoblastic associated genes, DMPP and DMP1, in DPSCs from
21 HPP patient. Traditional knowledge indicates that ALPL is a downstream gene of canonical Wnt
22 pathway [37-39], and plays main function on extracellular matrix mineralization of osteoblast,

1 odontoblast and ameloblast. Nevertheless, our results indicated that ALPL might be as a distinct
2 regulator to control cellular signals cascade in MSCs. It may provide useful information for
3 understanding ALPL mutations leading to tooth dysplasia.

4 Currently, enzyme replacement therapy with asfotase alfa were recognized as the only effective
5 clinical treatment option for HPP. Increasing clinical data demonstrated that the most severe forms
6 of HPP (prenatal and infantile) with asfotase alfa treatment significantly increased in bone mineral
7 density and improved in respiratory and motor function [9, 40, 41]. One study recently reported
8 about the dental status of HPP patients with asfotase alfa treatment. In their study, the author
9 showed that enzyme replacement therapy significant decreased the mobility of the primary tooth
10 in some cases [12], but a clinically defined improvement was also observed in some patients
11 despite with long-term asfotase alfa treatment. The efficacy evaluation of the asfotase alfa on
12 dental defects in HPP patients is very limited based on the current clinical data. Thus, prior to
13 asfotase alfa therapy for dentist, the potential risks, effective treatment and also expensive cost
14 should be considered.

15 In the present study, we observed systemic LiCl injections effectively improved mineralization of
16 dentinal tubules, but also increased height and bone mass of alveolar bone, and DMD in ALPL
17 knockdown mice. We could not exclude whether other types of cells in periodontal tissues were
18 also affected by systemic LiCl injections. Indeed, periodontal ligament stem cells derived from
19 tooth and BMSCs from jaw are also important for tooth development and functional maintenance
20 [42]. Despite our previous works demonstrated LiCl treatment improved bone mass loss of femora,
21 it might explain why systemic LiCl injections also increased bone mass of alveolar bone in
22 ALPL^{+/-} mice. It still needs to be evaluated whether ALPL deficiency disrupts GSK3 β / β -catenin

1 cascade, and LiCl exerts therapeutic benefits in these cells. In addition, dephosphorylation of
2 AMPK in HPP DPSCs were also observed in BMSCs from HPP [25], and our previous work
3 demonstrated metformin, an AMPK agonist, effectively ameliorated bone disorder in ALPL
4 knockdown mice [25]. Considering that metformin as one of the most popular and cheapest oral
5 medications is widely used treatment for patients with initial type 2 diabetes mellitus [43, 44].
6 Therefore, it urgently needs to evaluate metformin therapeutic benefits in dental stem cells and
7 tooth defects in ALPL knockdown mice in the following works, ultimately, these works may
8 inform efforts at improvement tooth defects in HPP patients.

9 **Conclusions**

10 Here, our study indicated that ALPL might be as a distinct regulator to control intracellular
11 GSK3 β to β -catenin signals cascade, and subsequently regulate DPSCs differentiation into
12 odontoblast. ALPL deficiency impaired odontoblastic differentiation of DPSCs. While LiCl
13 treatment could rescue such impairment through rephosphorylation of GSK3 β . This work may
14 provide useful information for understanding how ALPL mutations leading to tooth dysplasia and,
15 ultimately, may inform efforts at improvement tooth defects in patients with HPP.

16 **Abbreviation**

17 ALPL: liver/bone/kidney-type alkaline phosphatase; HPP: hypophosphatasia; DPSC: Dental pulp
18 stem cells; t-GSK3 β : total Glycogen synthase kinase 3 beta; DSPP: Dentin sialophosphoprotein;
19 DMP1: Dentin matrix acidic phosphoprotein 1; p-GSK3 β : phosphorylation of Glycogen synthase
20 kinase 3 beta; micro-CT: Micro-computed tomography; RT-PCR: Real time polymerase chain
21 reaction;

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2 work.

3 **Author contributions**

4 Wenjia Liu and Kun Xuan designed the project. Liqiang Zhang and Jiangdong Zhao did the
5 majority of the experiments and collected data. Jiayi Dong did the experiment of microCT and
6 collected data. Yuting Liu participated in the experiments and collected data. Liqiang Zhang
7 drafted the manuscript. All authors contributed to and approved the final version of the
8 manuscript.

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14 **Availability of data and materials**

15 All datasets used and/or analyzed during the current study are available from the corresponding
16 author on reasonable request.

17 **Ethics approval and consent to participate**

18 Mice were used according to federal guidelines and as approved by the Animal Ethical and
19 Welfare Committee of Xi'an Jiaotong University (No. XJTULAC201).

20 **Consent for publication**

21 Not applicable.

22

Competing interests

All the authors declare no conflict of interest.

References

1. Beumer J, Trowbridge HO, Silverman S, Jr., Eisenberg E. Childhood hypophosphatasia and the premature loss of teeth. A clinical and laboratory study of seven cases, *Oral Surg Oral Med Oral Pathol.* 1973;35:631-40.
2. Haliloglu B, Guran T, Atay Z, Abali S, Mornet E, Bereket A, et al. Infantile loss of teeth: odontohypophosphatasia or childhood hypophosphatasia, *Eur J Pediatr.* 2013;172:851-3.
3. Simon S, Resch H, Klaushofer K, Roschger P, Zwerina J, Kocijan R. Hypophosphatasia: From Diagnosis to Treatment, *Curr Rheumatol Rep.* 2018;20:69.
4. Rush ET. Childhood hypophosphatasia: to treat or not to treat, *Orphanet J Rare Dis.* 2018;13:116.
5. Whyte MP. Hypophosphatasia: Enzyme Replacement Therapy Brings New Opportunities and New Challenges, *J Bone Miner Res.* 2017;32:667-75.
6. Whyte MP, Greenberg CR, Salman NJ, Bober MB, McAlister WH, Wenkert D, et al. Enzyme-replacement therapy in life-threatening hypophosphatasia, *N Engl J Med.* 2012;366:904-13.
7. Costain G, Moore AM, Munroe L, Williams A, Zlotnik Shaul R, Rockman-Greenberg C, et al. Enzyme replacement therapy in perinatal hypophosphatasia: Case report of a negative outcome and lessons for clinical practice, *Mol Genet Metab Rep.* 2018;14:22-26.
8. Scott LJ. Asfotase Alfa in Perinatal/Infantile-Onset and Juvenile-Onset Hypophosphatasia: A Guide to Its Use in the USA, *BioDrugs.* 2016;30:41-8.
9. Whyte MP, Simmons JH, Moseley S, Fujita KP, Bishop N, Salman NJ, et al. Asfotase alfa for infants

- 1 and young children with hypophosphatasia: 7 year outcomes of a single-arm, open-label, phase 2
- 2 extension trial, *Lancet Diabetes Endocrinol.* 2019;793-105.
- 3 10. Cheung WS. A mild form of hypophosphatasia as a cause of premature exfoliation of primary teeth:
- 4 report of two cases, *Pediatr Dent.* 1987;949-52.
- 5 11. Foster BL, Kuss P, Yadav MC, Kolli TN, Narisawa S, Lukashova L, et al. Conditional Alpl Ablation
- 6 Phenocopies Dental Defects of Hypophosphatasia, *J Dent Res.* 2017;9681-91.
- 7 12. Kiselnikova L, Vislobokova E, Voinova V. Dental manifestations of hypophosphatasia in children and
- 8 the effects of enzyme replacement therapy on dental status: A series of clinical cases, *Clin Case Rep.*
- 9 2020;8911-18.
- 10 13. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human
- 11 exfoliated deciduous teeth, *Proc Natl Acad Sci U S A.* 2003;1005807-12.
- 12 14. Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, et al. Dental pulp tissue engineering
- 13 with stem cells from exfoliated deciduous teeth, *J Endod.* 2008;34962-9.
- 14 15. Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, et al. Stem/progenitor cell-mediated de
- 15 novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model,
- 16 *Tissue Eng Part A.* 2010;16605-15.
- 17 16. Xuan K, Li B, Guo H, Sun W, Kou X, He X, et al. Deciduous autologous tooth stem cells regenerate
- 18 dental pulp after implantation into injured teeth, *Sci Transl Med.* 2018;10.
- 19 17. Liu H, Li J, Lei H, Zhu T, Gan Y, Ge L. Genetic etiology and dental pulp cell deficiency of
- 20 hypophosphatasia, *J Dent Res.* 2010;891373-7.
- 21 18. Rodrigues TL, Foster BI Fau - Silverio KG, Silverio Kg Fau - Martins L, Martins L Fau - Casati MZ,
- 22 Casati Mz Fau - Sallum EA, Sallum Ea Fau - Somerman MJ, et al. Hypophosphatasia-associated

1 deficiencies in mineralization and gene expression in cultured dental pulp cells obtained from human
2 teeth, *J Endod.* 2012;902-12

3 19. Bruckner RJ, Rickles NH, Porter DR. Hypophosphatasia with premature shedding of teeth and
4 aplasia of cementum, *Oral Surg Oral Med Oral Pathol.* 1962;151351-69.

5 20. Mussolino Ribeiro ZM, Assed S, Borsatto MC, Campos de Freitas A. [Hypophosphatasia--premature
6 loss of deciduous teeth], *Rev Fac Odontol Ribeiro Preto.* 1986;23107-14.

7 21. Liu W, Zhang L, Xuan K, Hu C, Li L, Zhang Y, et al. Alkaline Phosphatase Controls Lineage Switching
8 of Mesenchymal Stem Cells by Regulating the LRP6/GSK3beta Complex in Hypophosphatasia,
9 *Theranostics.* 2018;85575-92.

10 22. Oikawa H, Tomatsu S, Haupt B, Montano AM, Shimada T, Sly WS. Enzyme replacement therapy on
11 hypophosphatasia mouse model, *J Inherit Metab Dis.* 2014;37309-17.

12 23. Millan JL, Narisawa S, Lemire I, Loisel TP, Boileau G, Leonard P, et al. Enzyme replacement therapy
13 for murine hypophosphatasia, *J Bone Miner Res.* 2008;23777-87.

14 24. Gasque KC, Foster BL, Kuss P, Yadav MC, Liu J, Kiffer-Moreira T, et al. Improvement of the skeletal
15 and dental hypophosphatasia phenotype in *Alpl*^{-/-} mice by administration of soluble (non-targeted)
16 chimeric alkaline phosphatase, *Bone.* 2015;72137-47.

17 25. Liu W, Zhang L, Xuan K, Hu C, Liu S, Liao L, et al. *Alpl* prevents bone ageing sensitivity by specifically
18 regulating senescence and differentiation in mesenchymal stem cells, *Bone Res.* 2018;627.

19 26. Waymire KG, Mahuren Jd Fau - Jaje JM, Jaje Jm Fau - Guilarte TR, Guilarte Tr Fau - Coburn SP,
20 Coburn Sp Fau - MacGregor GR, MacGregor GR. Mice lacking tissue non-specific alkaline phosphatase
21 die from seizures due to defective metabolism of vitamin B-6, *Nat Genet.* 1995;1145-51.

22 27. Song Y, Liu X, Feng X, Gu Z, Gu Y, Lian M, et al. NRP1 Accelerates Odontoblast Differentiation of

- 1 Dental Pulp Stem Cells Through Classical Wnt/beta-Catenin Signaling, Cell Reprogram. 2017;19324-30.
- 2 28. Wang C, Song Y, Gu Z, Lian M, Huang D, Lu X, et al. Wedelolactone Enhances Odontoblast
- 3 Differentiation by Promoting Wnt/beta-Catenin Signaling Pathway and Suppressing NF-kappaB
- 4 Signaling Pathway, Cell Reprogram. 2018;20236-44.
- 5 29. Hao HP, Wen LB, Li JR, Wang Y, Ni B, Wang R, et al. LiCl inhibits PRRSV infection by enhancing
- 6 Wnt/beta-catenin pathway and suppressing inflammatory responses, Antiviral Res. 2015;11799-109.
- 7 30. Li C, Zhang Y, Lu Y, Cui Z, Yu M, Zhang S, et al. Evidence of the cross talk between Wnt and Notch
- 8 signaling pathways in non-small-cell lung cancer (NSCLC): Notch3-siRNA weakens the effect of LiCl on
- 9 the cell cycle of NSCLC cell lines, J Cancer Res Clin Oncol. 2011;137771-8.
- 10 31. el-Labban NG, Lee KW, Rule D. Permanent teeth in hypophosphatasia: light and electron
- 11 microscopic study, J Oral Pathol Med. 1991;20352-60.
- 12 32. Hayashi-Sakai S, Numa-Kinjoh N, Sakamoto M, Sakai J, Matsuyama J, Mitomi T, et al.
- 13 Hypophosphatasia: Evaluation of Size and Mineral Density of Exfoliated Teeth, J Clin Pediatr Dent.
- 14 2016;40496-502.
- 15 33. Ikeue R, Nakamura-Takahashi A, Nitahara-Kasahara Y, Watanabe A, Muramatsu T, Sato T, et al.
- 16 Bone-Targeted Alkaline Phosphatase Treatment of Mandibular Bone and Teeth in Lethal
- 17 Hypophosphatasia via an scAAV8 Vector, Mol Ther Methods Clin Dev. 2018;10361-70.
- 18 34. James AW. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic
- 19 Differentiation, Scientifica (Cairo). 2013;2013684736.
- 20 35. Visweswaran M, Pohl S, Arfuso F, Newsholme P, Dilley R, Pervaiz S, et al. Multi-lineage
- 21 differentiation of mesenchymal stem cells - To Wnt, or not Wnt, Int J Biochem Cell Biol.
- 22 2015;68139-47.

- 1 36. Rodrigues TL, Foster BL, Silverio KG, Martins L, Casati MZ, Sallum EA, et al.
2 Hypophosphatasia-associated deficiencies in mineralization and gene expression in cultured dental
3 pulp cells obtained from human teeth, J Endod. 2012;38907-12.
- 4 37. Heo JS, Lee SY, Lee JC. Wnt/beta-catenin signaling enhances osteoblastogenic differentiation from
5 human periodontal ligament fibroblasts, Mol Cells. 2010;30449-54.
- 6 38. Morsczeck C, Reck A, Reichert TE. WNT3A and the induction of the osteogenic differentiation in
7 adipose tissue derived mesenchymal stem cells, Tissue Cell. 2017;49489-94.
- 8 39. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase
9 expression and osteoblast mineralization by a Wnt autocrine loop, J Bone Miner Res. 2003;181842-53.
- 10 40. Hofmann C, Seefried L, Jakob F. Asfotase alfa: enzyme replacement for the treatment of bone
11 disease in hypophosphatasia, Drugs Today (Barc). 2016;52271-85.
- 12 41. Whyte MP, Rockman-Greenberg C, Ozono K, Riese R, Moseley S, Melian A, et al. Asfotase Alfa
13 Treatment Improves Survival for Perinatal and Infantile Hypophosphatasia, J Clin Endocrinol Metab.
14 2016;101334-42.
- 15 42. Dave JR, Tomar GB. Dental Tissue-Derived Mesenchymal Stem Cells: Applications in Tissue
16 Engineering, Crit Rev Biomed Eng. 2018;46429-68.
- 17 43. Inzucchi SE, Lipska KJ, Mayo H, Bailey CJ, McGuire DK. Metformin in patients with type 2 diabetes
18 and kidney disease: a systematic review, JAMA. 2014;3122668-75.
- 19 44. Sanchez-Rangel E, Inzucchi SE. Metformin: clinical use in type 2 diabetes, Diabetologia.
20 2017;601586-93.

Figure Legends

Figure 1. The manifestations of teeth from patients with HPP

A. The panoramic radiograph analysis of full dentition of two patients with HPP. B. The morphology features of pediatric deciduous cusps from health and HPP1 patient, and histology of tooth structures in HPP1 patient including acellular cementum (AC) and dentin (DE). Scale bar = 500 μ m. C and D. The surface structures of the cementum (C) and ultra-structures of dentinal tubules (D) in tooth from health or HPP patient were detected by the SEM.

Figure 2. The morphological and histological characters of teeth from ALPL^{+/-} mice

A. The activity of alkaline phosphatase in serum were examined in WT and ALPL^{+/-} mice. B. The low jaws including molars, alveolar bone height (arrowheads), and bone mass of alveolar bone and mandible bone (arrows), dentin mineral density (DMD) were analyzed by micro-CT in WT and ALPL^{+/-} mice. Scale bar = 2 mm. C. The histology of dentoalveolar structures were analyzed including acellular cementum (AC), periodontal ligament (PDL), dentin (DE), and alveolar bone (AB). Scale bar = 100 μ m. D. The ultra-structures of dentinal tubules in the first molars from WT and ALPL^{+/-} mice were analyzed by SEM, the exposure of collagen network as show by the arrows. The data were presented as the mean $\pm$ s.d. of triplicate samples and analyzed by Student's t-test. ** $p < 0.01$, *** $p < 0.001$.

Figure 3. The influence of ALPL mutation on cell proliferation and apoptosis of DPSCs

A and B. The ALP activities (A) and the ALPL expression on protein were examined in DPSCs from normal and patient with HPP. C. The effective CFU of DPSCs in culture medium were compared between control and HPP groups *in vitro*. D and E. The cell cycle (D) and cell apoptosis (E) of DPSCs from normal and HPP patient were performed by flow cytometry analysis. The data

1 were analyzed by Student's t-test and presented as the mean $\pm$ s.d. of triplicate samples from a
2 representative experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3 Figure 4. ALPL regulates the odontoblastic differentiation of DPSCs

4 A. Alizarin Red staining were performed in DPSCs from health and HPP groups after cultured in
5 odontoblastic medium for 3 weeks. B. The expression of DMP1 and DSPP were examined on
6 gene and protein level in DPSCs from health and HPP groups after cultured in odontoblastic
7 medium for 7 days. To evaluate whether ALPL could regulate DPSCs differentiation into
8 odontoblasts, we up- or down-regulated expression of ALPL in HPP DPSCs with pLenti-ALPL or
9 normal DPSCs with pLko.1-ALPL shRNA lentiviral transduction. C and E. Alizarin Red staining
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11 HPP and HPP with pLenti-ALPL (E) after cultured in odontoblastic medium for 3 weeks. D and F.
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17 Figure 5. ALPL deficiency disrupted canonical Wnt pathway leading to impaired odontoblastic
18 differentiation of HPP DPSCs were restored by LiCl treatment

19 A. Canonical Wnt pathway related key proteins, including total and phosphorylation of GSK3 β ,
20 and total and active β -catenin in DPSCs from health and HPP groups were examined by Western
21 blotting. B. Immunofluorescence staining of p-GSK3 β and active β -catenin were performed in the
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2 pLko.1-ALPL shRNA (C), DPSCs from HPP and HPP with pLenti-ALPL (D), or DPSCs from
3 HPP and HPP treated with LiCl (E). F. Alizarin Red staining were performed in DPSCs from HPP
4 and HPP treated with LiCl after cultured in odontoblastic medium for 3 weeks. G. The protein
5 expression of DMP1 and DSPP were examined in DPSCs from HPP and HPP treated with LiCl
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8 Figure 6. LiCl rescued odontoblastic differentiation of HPP DPSCs were blocked by β -catenin
9 siRNA

10 A and B. The β -catenin silence efficiency was examined by RT-PCR (A) and Western blotting (B)
11 in HPP DPSCs with β -catenin siRNA transfection for 48 hours. C. The expression of t-GSK3 β and
12 p-GSK3 β , and total and active β -catenin were examined by Western blotting in HPP DPSCs from
13 NaCl control, LiCl+Nc siRNA and LiCl+ β -catenin siRNA groups, which were cultured in
14 odontoblastic medium for 7 days. D and E. To evaluate LiCl rescue odontoblastic differentiation
15 of HPP DPSCs by canonical Wnt pathway, the cells were treated with LiCl meanwhile transected
16 with β -catenin siRNA. The odontoblastic differentiation was examined by Alizarin Red staining
17 (D) after in odontoblastic medium for 3 weeks, and Western blotting (E) after in odontoblastic
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20 Figure 7. GSK3 β rephosphorylation ameliorated tooth associated defects in ALPL^{+/-} mice with
21 LiCl treatment

22 A. Immunofluorescence staining of p-GSK3 β and active β -catenin were performed in the first

1 molars of ALPL^{+/-} mice and ALPL^{+/-} mice with LiCl treatment. Scale bar = 100 μ m. B. The
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12 Figure 8. Schematic diagram depicts how ALPL deficiency impairs DPSCs differentiation into
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17 caused by ALPL mutation, disrupted intracellular GSK3 β to β -catenin signaling cascade, then
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19 of odontoblastic differentiation of HPP DPSC through reactivating intracellular GSK3 β to
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21

22 **Supplemental figure legends**

1 sFigure 1. identification of DPSCs phenotype by flow cytometer

2 The makers of DPSCs, including CD29, CD44, CD105, CD34, CD45, and Stro-1, were examined
3 by flow cytometric cytometer in health and patient with HPP.

4 sFigure 2. The silence and overexpression efficiency of ALPL

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9 sFigure 3. The expression of signaling proteins in DPSCs

10 The expression of signaling proteins, including p-ERK, p-AMPK, Gli1, and TGF β , were
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12

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Figures

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