Periodontal Ligament Stem Cells Modulate Root Resorption of Human Primary Teeth via Runx2 Regulating RANKL/OPG System

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Physiological primary teeth exfoliation is a normal phenomenon during teeth development. However, retained primary teeth can often be observed in the patients with cleidocranial dysplasia (CCD) caused by mutation of Runx2. The potential regulative mechanism is still unknown. In the present study, periodontal ligament stem cells (PDLSCs) were derived from different resorbed stages of primary teeth and permanent teeth from normal patients and primary teeth from CCD patient. The proliferative, osteogenic and osteoclast-inductive capacities of PDLSCs from each group were detected. We demonstrated here that the proliferative ability of PDLSCs was reduced while the osteogenic and the osteoclast-inductive capacity of PDLSCs were enhanced during root resorption. The results also showed that PDLSCs from permanent teeth and CCD patient expressed low level of Runx2 and RANKL while high level of OPG. However, expression of Runx2 and RANKL were increased while expression of OPG was decreased in PDLSCs derived from resorbed teeth. Furthermore, Runx2 regulating the expression of RANKL and OPG and the osteoclast-inductive capacity of PDLSCs were confirmed by gain or loss of function assay. These data suggest that PDLSCs promote osteoclast differentiation via Runx2 upregulating RANKL and downregulating OPG, leading to enhanced root resorption that results in physiological exfoliation of primary teeth.

Introduction

EXFOLIATION OF ORGANS IS A COMMON physiological event in the biosphere, especially for plants and some inferior creatures such as gecko and deer. According to previous reports, the phenomenon of exfoliation of organs is closely related with regeneration [1–6]. As primate mammals, human beings have only one organ exfoliation experience all through our lives, which is the exfoliation of primary teeth. Based on the physiological root resorption, physiological primary teeth exfoliation can guarantee the correct eruption of permanent teeth, which is of great significance for the development of the dentition. Otherwise, malocclusion will develop when primary teeth cannot exfoliate due to abnormal root resorption.

Previous studies indicated that periodontal ligament (PDL) tissue played an important role in regulating osteoclast differentiation, suggesting PDL might be involved in

root resorption [7,8]. Periodontal ligament stem cells (PDLSCs), which were isolated from periodontal tissue, are capable of differentiating into osteoblast, adipocyte, and tooth cementoblast in vitro and regenerating cementum/PDL tissue in vivo [9]. When PDLSCs were transplanted into surgically created defects at the periodontal area, they repaired the defect and integrated into PDL compartment, which suggests PDLSCs maintain the homeostasis of PDL tissue [10]. However, as the stem cells in PDL tissue, whether PDLSCs play an important role in the root resorption is still unknown.

Cleidocranial dysplasia (CCD), which is caused by mutation of Runx2, is a hereditary congenital disorder characterized by the abnormalities of bone development such as clavicle dysplasia, unclosed fontanels, and retained primary teeth due to no root resorption [11–16]. Previous studies reported that Runx2, which is the master osteogenic transcription factor regulating osteogenesis metabolism, also induces

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differentiation of osteoclast through regulation of RANKL and OPG [17–21]. However, the mechanism of Runx2 mutation of CCD patient leading to no root resorption of primary teeth is still not answered yet.

Thus, to explore whether Runx2 in PDLSCs regulates the root resorption of primary teeth in the present study, we isolated PDLSCs from different resorbed stages of primary teeth from normal patients and primary teeth from CCD patient. As the expression of Runx2 was markedly increased in PDLSCs derived from resorbed teeth, we want to investigate the role of Runx2 in the regulation of RANKL/OPG system and osteoclast differentiation both in physiological and pathological conditions.

Materials and Methods

Tooth samples

Primary teeth of children were extracted because of retained primary teeth or malocclusion. According to the degree of root resorption of these primary teeth and convenience for harvesting PDL tissues, we divided the physiological root resorption into three periods: unresorbed (UN group, no resorption can be seen on the root surface), moderate (M group, the lingual surface of the root were resorbed to the middle third while the labial side were unresorbed), and severe (S group, the lingual side were resorbed to the cervical third while the labial were basically complete) period. We also extracted several premolars of adolescents due to orthodontic reasons (permanent, P group). Besides, we collected a clinical case, which was a 10-year-old boy. Based on the clinical, radiographical examinations and genome sequencing results, the boy was diagnosed as a CCD patient [22]. The retained primary incisors were extracted for the eruption of the successors; no resorption can be seen on root surface (CCD group). Each group contained at least three teeth except the CCD group. The PDL tissue of the teeth from the five groups would be obtained for PDLSCs culture. Written informed consent was provided by all participants, and ethical approval had been obtained from the Ethics Committee of the School of Stomatology, the Fourth Military Medical University.

Cell culture

PDLSCs were isolated and cultured as we previously described [10,23–25]. Briefly, the PDL tissues were dissociated with 0.1% collagenase (Sigma) for 15 min at 37°C. The solution was passed through a 75- μ m filter to remove the undissociated tissues, neutralized with α -modified Eagle's medium (α -MEM; Hyclone) containing 10% (v/v) fetal bovine serum (FBS; Hyclone), and centrifuged at 800 rpm for 5 min. Then, the cell pellet was resuspended in α -MEM containing 10% (v/v) FBS with 1% (v/v) penicillin/streptomycin solution and cultured in an incubator at 37°C with 5% carbon dioxide. The culture medium was first changed 7 days later and then changed regularly at 3-day intervals. The cells were passaged with trypsin/EDTA (Sigma) when required. To obtain homogeneous population of PDLSCs, single cell colony was obtained using limiting dilution technique and then expanded. These multiple colony-derived PDLSCs at two to four passages were used in our experiments. Each experiment was repeated at least three times.

Immunophenotype analysis

PDLSCs were stained with stem cell surface markers and analyzed by flow cytometry as described previously [26–29]. Briefly, the second-passage PDLSCs of each group were harvested using trypsin, washed twice with PBS and incubated with FITC-conjugated monoclonal or polyclonal antibodies against STRO-1 (Biolegend), CD29 (eBioscience, Inc.), CD105 (eBioscience, Inc.), CD14 (eBioscience, Inc.), and PE-conjugated antibodies against CD146 (Biolegend), CD31 (eBioscience, Inc.). The cells were analyzed using the Elite ESP flow cytometry (Beckman Coulter).

Cell proliferation assay

For each of the five groups, PDLSCs were seeded into a 96-well culture plate (2×10^3 cells/well) with 100 μ L α -MEM. After 24 h, 10 μ L of WST-1 reagent (Roche Applied Science) was added into each well, incubated for 2 h, and the absorbance was measured against the background (blank) using a microplate reader (Bio-Rad Model 680, Lifescience Research) at 450 nm. The proliferation assay continued for 10 days. Besides, 400 cells of each group were plated in a 90-mm Petri dish and cultured until colony formation were apparently observed under a phase-contrast microscope (Fifty or more cells clustered together were considered a colony). After fixation for 20 min with 5 mL of 4% paraformaldehyde, cells were stained with 1% toluidine blue to assess colony formation efficiency.

Osteogenic differentiation assay

Osteogenic differentiation of PDLSCs was performed according to previous publications [9,23,29]. Briefly, PDLSCs of each group were seeded into a 12-well plate (2×10^5 cells/well) and cultured in osteogenic induction medium. The medium was changed every 2 days. After 21 days of culture with osteogenic supplements, the cells were fixed in 4% paraformaldehyde, washed twice in PBS, and incubated in 0.1% Alizarin red solution (Sigma-Aldrich; cat. number A5533) in Tris-HCl (pH 8.3) at 37°C for 30 min. After washing in PBS, the cells were observed using an inverted microscope and imaged. The nodule area per well were quantitatively measured with an image analysis system Image-Pro Plus 5.0 software.

Total RNA extraction and quantitative reverse transcriptase–polymerase chain reaction

Total RNA was extracted from in vitro culture samples using TRIzol reagent (Invitrogen). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with 1 μ g of RNA using a PrimeScriptTM RT reagent kit (TaKaRa). The primer sequences used in the experiment were listed in Table 1, and related genes were quantified by quantitative RT-PCR (qRT-PCR) using the SYBR Premix Ex Taq II kit (TaKaRa) and the Applied Bio-systems CFX96TM Real-Time sequence detection system (Applied Biosystems).

Protein isolation and western blot analysis

Total proteins were extracted with lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulfate, 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM

TABLE 1. PRIMER SEQUENCES INVOLVED IN THE RESEARCH

Gene	Primer sequences
<i>β-actin</i>	Forward: 5'-TGG CAC CCA GCA CAA TGA A-3' Reverse: 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'
<i>Runx2</i>	Forward: 5'-CAC TGG CGC TGC AAC AAG A-3' Reverse: 5'-CAT TCC GGA GCT CAG CAG AAT AA-3'
<i>ALP</i>	Forward: 5'-CCT TGT AGC CAG GCC CAT TG-3' Reverse: 5'-GGA CCA TTC CCA CGT CTT CAC-3'
<i>Col-1</i>	Forward: 5'-GCA AGG TGT TGT GCG ATG A-3' Reverse: 5'-TGG TCG GTG GGT GAC TCT G-3'
<i>RANKL</i>	Forward: 5'- ATC ACA GCA CAT CAG AGC AGA GA-3' Reverse: 5'- AGG ACA GAC TCA CTT TAT GGG AAC-3'
<i>OPG</i>	Forward: 5'- GGA ACC CCA GAG CGA AAT ACA-3' Reverse: 5'- GGG AAC AGC AAA CCT GAA GAA TG-3'

β-glycerophosphate, and 50 mM sodium fluoride). The protein concentration in the extracted lysates was determined with a protein assay kit (Beyotime) following the manufacturer's recommended protocol. Aliquots of 20–60 μg per sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to the polyvinylidene fluoride membranes (Millipore), and blocked with 5% nonfat milk powder in PBST (PBS with 0.1% Tween), then incubated with following primary antibodies overnight: anti-Runx2 (Santa Cruz Biotechnology, Inc.), anti-RANKL (Abcam), anti-OPG (Santa Cruz Biotechnology, Inc.), and anti-β-actin (Abcam). Then, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Boster). The blots were visualized using an enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer's recommended instructions.

Co-culture with RAW264.7 cells or human peripheral blood mononuclear cells and TRAP staining

RAW264.7 cell line was used as osteoclast progenitors. PDLSCs were seeded into a 24-well culture plate (1×10^5 cells/mL). After 12 h, RAW264.7 cells (1×10^6 cells/mL) were directly loaded on PDLSCs and cultured with α-MEM (containing 30 ng/mL M-CSF). Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood obtained from healthy volunteers, using LSM lymphocyte separation medium (MP Biomedicals). PBMCs (1×10^5 cells/mL) were also co-cultured with PDLSCs, which seeded into a 24-well culture plate (1×10^3 cells/mL), in α-MEM (containing 30 ng/mL M-CSF). The medium was semi-changed every 3 days. After 7 days, the cells were stained for TRAP staining analysis using Acid Phosphatase, leukocyte kit (Sigma-Aldrich; cat. number 387A-1KT).

RNAi

Runx2 siRNA (siRunx2 group) and GFP siRNA oligos (negative control group) were purchased from Santa Cruz Biotechnology, Inc. The control group contained no siRNA. Briefly, PDLSCs were trypsinized, counted, and seeded at 1×10^5 cells/well into six-well culture plates, so that the cells were 40%–45% confluence on the day of transfection. Transfection of siRNA was then conducted with lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. The PDLSCs were incubated with serum-free α-MEM at 37°C with 5% carbon dioxide. After 4–6 h, the medium were replaced with complete α-MEM. The PDLSCs were cultured for another 48 h and 72 h for qRT-PCR and western blot analysis, respectively.

Besides, for the PDLSCs from M group, PDLSCs were seeded at 1×10^5 cells/well into 24-well culture plates. After 4–6 h of siRNA transfection, RAW264.7 cells (1×10^6 cells/mL) were directly loaded on PDLSCs and cultured with α-MEM (containing 30 ng/mL M-CSF). The medium was semi-changed every 3 days. After 7 days, the cells were stained for TRAP staining analysis as described above.

Overexpression of Runx2

Ad-Runx2 GV208 Plasmid (ad-Runx2 group) was constructed and provided by GeneChem Company. The blank plasmid was used as negative control and the control group contained no plasmid. PDLSCs were seeded at 5×10^5 cells/well into six-well culture plates, so that the cells were 90% confluence on the day of transfection. The plasmids were transfected into PDLSCs by lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. qRT-PCR and western blot analysis were conducted to assay the expression of Runx2 as previously described.

On the other hand, for the PDLSCs from UN and CCD group, PDLSCs were seeded at 1×10^5 cells/well into 24-well culture plates. After 4–6 h of plasmids transfection, RAW264.7 cells (1×10^6 cells/mL) were directly loaded on PDLSCs and cultured with α-MEM (containing 30 ng/mL M-CSF). The medium was semi-changed every 3 days. After 7 days, the cells were stained for TRAP staining analysis as described above.

Statistical analysis

All results are presented as mean ($\pm$ SD) from at least three independent experiments and analyzed by a two-tailed unpaired Student's *t*-test using SPSS software. *P*-values less than 0.05 were considered statistically significant.

Results

Isolation and characterization of PDLSCs from different groups

First, we isolated PDLSCs from each group and examined whether inherent properties of PDLSCs were altered in each group. In the present study, the primary teeth were divided into three groups (UN, M, and S group) based on the degree of root resorption to simulate the process of physiological root resorption. Beside of permanent premolars (P group), retained primary incisors from CCD patient (no resorption

on root surface) were added to the research as well to explore the regulative effects of Runx2 on root resorption (CCD group). PDLSCs were derived from each group (Fig. 1A). The WST-1 proliferation curves revealed that the proliferation of PDLSCs from primary teeth was higher compared with that from permanent teeth. Besides, the proliferation of PDLSCs from resorbed primary teeth (M and S group) was lower than that from unresorbed primary teeth (UN group), indicating that the proliferative ability of PDLSCs from primary teeth might be inhibited during root resorption. Interestingly, PDLSCs from CCD group presented a similar proliferative ability with those from UN group indicated by the WST-1 proliferation assessment (Fig. 1B). Besides, the colony formation ability of PDLSCs from CCD group ($18.92\% \pm 1.33\%$) was significantly higher than that from other groups (UN: $4.25\% \pm 0.35\%$; M:

$11.00\% \pm 1.97\%$; S: $13.63\% \pm 1.38\%$; and P: $8.25\% \pm 0.89\%$; Fig. 1C). Flow cytometry assay revealed a typical pattern for MSCs: STRO-1(+), CD146(+), CD29(+), CD105(+), CD31(-), and CD14(-). The overall expression patterns were similar among each group, indicating PDLSCs remained stem cell-like population (Fig. 1D).

Enhanced expression of Runx2 and higher osteogenic capacity of PDLSCs derived from resorbed primary teeth

We then examined both gene and protein expression of Runx2 of PDLSCs derived from each group. Gene expression assay indicated that expression of Runx2 was significantly enhanced in PDLSCs from S group ($P < 0.05$) by qRT-PCR (Fig. 2A). Western blot assay showed that protein expression of

FIG. 1. Isolation and characterization of periodontal ligament stem cells (PDLSCs) from different groups. (A) Physiological root resorption of primary teeth were divided into three periods: unresorbed (UN, no resorption can be seen on the root surface), moderate (M, the lingual surface of the root were absorbed to the middle third while the labial side were unresorbed), and severe (S, the lingual side were absorbed to the cervical third while the labial were basically complete) period. Premolars extracted due to orthodontic reasons were taken as permanent group (P). Unresorbed retained primary incisors from a cleidocranial dysplasia (CCD) patient were taken as CCD group (CCD). PDLSCs were isolated from each group. (B) Proliferation of PDLSCs from each group was determined by WST-1 assay. (C) Colony formation efficiency of PDLSCs from each group. (D) Immunophenotype analysis of PDLSCs from each group was determined by flow cytometry assay. Data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$), ns, no significance.

FIG. 2. Enhanced expression of Runx2 and higher osteogenic capacity of PDLSCs derived from resorbed primary teeth. The expression of Runx2 in PDLSCs from each group was detected by (A) quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and (B) western blot analysis. (C) PDLSCs from each group were cultured in osteogenic medium and osteogenic differentiation was determined by Alizarin red S staining at 21 days. Representative entire plate views of Alizarin red staining in six-well plates for PDLSCs from each group. (D–F) The expression of osteogenesis-related genes Runx2, ALP, and Col-I were determined by qRT-PCR after 14 days of culture with osteogenic supplement. Data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

Runx2 was increased in PDLSCs from M and S group compared with other groups. Protein assay also showed PDLSCs from CCD patient expressed low level of Runx2 (Fig. 2B).

In terms of the enhanced expression of Runx2 in PDLSCs from M and S group, the osteogenic differentiation capacity was analyzed after 21 days of culture with osteogenic supplement. The Alizarin red S staining results revealed a multiple differentiation ability of the isolated cells. Alizarin red S staining showed that PDLSCs from M and S groups formed more mineralized nodules, which indicated stronger osteogenic capacities compared with PDLSCs from the other groups (Fig. 2C). The mRNA expression of osteogenesis-related genes Runx2, ALP, and Col-I in M and S group were significantly higher than the other three groups, which was consistent with Alizarin red S staining result (Fig. 2D–F). Our data suggested that PDLSCs showed higher Runx2 expression and osteogenic capacity during root resorption.

Enhanced induction of osteoclast differentiation by PDLSCs derived from resorbed primary teeth

To examine the function of PDLSCs in root resorption, osteoclast progenitor RAW264.7 cells were co-cultured with PDLSCs to detect the osteoclast-inductive capacity of

PDLSCs from each group. The TRAP staining assay revealed that PDLSCs were able to induce osteoclast differentiation (Fig. 3A–E). Furthermore, the quantitative results demonstrated that the numbers of TRAP (+) cells in M and S co-culture group were more than the other three groups (Fig. 3F, $P < 0.05$). In addition, we also performed co-culture assay, which used human PBMCs with PDLSCs of different resorption stage. TRAP staining showed similar results that the numbers of TRAP (+) cells were increased in M and S co-culture group compared with the other three groups (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd; $P < 0.05$). These data indicated that the osteoclast-inductive capacity of PDLSCs derived from resorbed primary teeth is enhanced.

According to our previous research, dental pulp stem cells derived from resorbed primary teeth showed upregulated RANKL and downregulated OPG expression [30]. To further explore whether PDLSCs regulate osteoclast differentiation through RANKL/OPG system, we examined expression of RANKL and OPG in PDLSCs from each group. The qRT-PCR analysis showed that the mRNA expression of RANKL was increased and expression of OPG was decreased in PDLSCs from M and S group compared with UN and P group (Fig. 3G, H). The western blot assays also revealed a similar protein result (Fig. 3I). PDLSCs from CCD patient

FIG. 3. Enhanced induction of osteoclast differentiation by PDLSCs derived from resorbed primary teeth. PDLSCs from each group were co-cultured with RAW264.7 cells for 7 days to detect their osteoclast-inductive capacities. Osteoclast differentiation was determined by TRAP staining. Representative images showed TRAP staining results of (A) UN co-culture group, (B) M co-culture group, (C) S co-culture group, (D) P co-culture group, and (E) CCD co-culture group. (F) The number of TRAP (+) cells per visual field in each co-culture group was counted and compared. (G–I) Expressions of RANKL and OPG in PDLSCs from each group were examined by qRT-PCR and western blot assays. The white arrow points to the TRAP (+) cells. Data represent the means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

expressed less RANKL and more OPG indicated by both qRT-PCR and western blot assay (Fig. 3G–I). These results were in accordance with the TRAP staining assay after co-culture. Therefore, these data suggested that the expression of RANKL might be positively regulated by Runx2 while OPG might be negatively regulated by Runx2 in PDLSCs during the physiological root resorption process.

Runx2 regulates the expression of RANKL and OPG in PDLSCs

To explore whether Runx2 regulates the expression of RANKL and OPG in PDLSCs, RNAi and overexpression experiments were conducted. Runx2 expression was blocked by siRNA in PDLSCs from M and S group due to their higher expression. Transfection efficiency was $93.14\% \pm 2.36\%$ and $90.36\% \pm 4.63\%$ for M and S group, respectively, which was demonstrated by fluorescence control (Fig. 4A, B). The qRT-PCR and western blot assays demonstrated that the expression of Runx2 was successfully interfered after siRNA was transfected into PDLSCs from M and S group (Fig. 4C–F). Additionally, RANKL was downregulated when Runx2 was knocked down in PDLSCs from M and S group by gene and protein assay (Fig. 4C–F). The qRT-PCR and western blot assays demonstrated that knockdown of Runx2 also lead to increasing the expression of OPG in PDLSCs from these two groups (Fig. 4C–F).

For the PDLSCs from UN, P, and CCD group, Runx2 was overexpressed by transfecting ad-Runx2 GV208 Plasmid. The qRT-PCR and western blot assays indicated that the expression of Runx2 was successfully upregulated in the overexpression experiment (Fig. 5A–F). Furthermore, the expressions of RANKL were accordingly increased when Runx2 was upregulated in PDLSCs from UN, P, and CCD group. On the contrary, the expressions of OPG were inhibited as a result of overexpression of Runx2 in these groups demonstrated by qRT-PCR and western blot assays (Fig. 5A–F). These results indicated that Runx2 upregulate RANKL and downregulate OPG in PDLSCs.

Runx2 regulates PDLSCs induced osteoclast differentiation

According to the data above, the osteoclast-inductive capacity of PDLSCs was enhanced during root resorption, which was consistent with the Runx2 expression. To reveal the regulative effects of Runx2 on the osteoclast-inductive capacity of PDLSCs, PDLSCs from M, UN, and CCD group, with Runx2 interfered and overexpressed respectively, were co-cultured with RAW264.7 cells. The TRAP staining assay revealed that the number of TRAP (+) cells was decreased accordingly when Runx2 was knocked down in PDLSCs from M group (Fig. 6A–C); while the number of TRAP (+) cells was increased as Runx2 of PDLSCs was

FIG. 4. RNAi of Runx2 in PDLSCs from M and S group. siRNA was transfected into PDLSCs from M and S group to knockdown Runx2 expression. (A–B) For M and S group, transfection efficiency was $93.14\% \pm 2.36\%$ and $90.36\% \pm 4.63\%$, respectively, which was demonstrated by fluorescence control. (C–D) The gene expression of Runx2, RANKL, and OPG were examined by qRT-PCR. (E–F) Western blot assay showed expression of Runx2, RANKL, and OPG after knockdown of Runx2. β -Actin was used as the internal control. Data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$), ns, no significance.

overexpressed in UN group (Fig. 6D–F). Significantly, overexpression of Runx2 also increased the number of TRAP (+) cells induced by PDLSCs from CCD group. It can be suggested that during the process of physiological root resorption, PDLSCs promote osteoclast differentiation via Runx2 upregulating RANKL and downregulating OPG, leading to enhanced root resorption, which results in exfoliation of primary teeth.

Discussion

Root resorption of primary teeth is a normal physiological phenomenon during physiological primary teeth exfoliation and teeth development. However, the mechanism of root

resorption is still not clear yet. Once, it was generally accepted that PDL can protect the root of teeth from resorption [31–33]. Nevertheless, according to a recent study, significant root resorption lacuna could be observed on the surface of the root with PDL after subcutaneous transplantation in the mice, while in the control group without PDL, lacuna could seldom be observed [8]. It was suggested that PDL could induce root resorption.

Additionally, previous study reported that PDL cells (PDLCs) derived from deciduous teeth (DPDLCs) could synthesize both RANKL and OPG. And TRAP (+) osteoclast-like cells could be induced when DPDLCs were co-cultured with mouse bone marrow cells [7]. However, as the stem cells derived from PDL and as the progenitors for

FIG. 5. Overexpression of Runx2 in PDLSCs from UN, P, and CCD group. Ad-Runx2 GV208 Plasmids were transfected into PDLSCs from UN, P, and CCD group to overexpress Runx2 in PDLSCs. (A–C) The gene expression of Runx2, RANKL, and OPG were determined by qRT-PCR among control, negative control, and transfection group. (D–F) Western blot assay showed expression of Runx2, RANKL, and OPG among control, negative control, and transfection group. β -Actin was used as the internal control. Data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$), ns, no significance.

PDLSCs, whether PDLSCs regulate the root resorption is still not answered. In the present study, we isolated PDLSCs from teeth with different degree of root resorption. We found that after co-culture with RAW264.7 cells, PDLSCs derived from resorbed primary teeth could induce more osteoclast differentiation and PDLSCs from CCD patient induced less osteoclast formation. We also found that during root resorption, the RANKL expression was increased while OPG expression was decreased in PDLSCs. Our study suggests that PDLSCs play an important role during root resorption.

Runx2 is a key transcription factor during osteogenic development. For CCD patients with mutation of Runx2, addition to abnormalities of bone development, retained primary teeth as a result of none root resorption is the main oral symptom of CCD patients with Runx2 mutation. Previous study indicated that PDLSCs from CCD patients showed a

reduced capacity to induce the differentiation of active osteoclasts [34]. Therefore, the question whether Runx2 expression of PDLSCs is closely related with root resorption comes out. We have demonstrated that the expression level of Runx2 in PDLSCs was increased during root resorption, which was consistent with the enhanced osteoclast-inductive capacity of PDLSCs. However, PDLSCs from CCD patient expressed less Runx2 both in gene and protein level, which was also consistent with the decreased osteoclast differentiation after co-culture with RAW264.7 cells. Furthermore, our result indicated that the osteoclast-inductive capacity of PDLSCs could be reversed via knockdown or overexpression of Runx2. Therefore, it can be concluded that Runx2 in PDLSCs can induce the differentiation of osteoclasts during root resorption. Moreover, overexpression of Runx2 might rescue the oral manifestation of CCD patient caused by Runx2 mutation.

FIG. 6. Runx2 regulates PDLSCs induced osteoclast differentiation. PDLSCs from M, UN, and CCD group, with Runx2 interfered and overexpressed respectively, were co-cultured with RAW264.7 cells to explore the regulative effects of Runx2 on the osteoclast-inductive capacity of PDLSCs. Osteoclast differentiation was determined by TRAP staining. Representative images showed TRAP staining results of (A) M-negative control co-culture group, (B) M-siRunx2 co-culture group, (D) UN-negative control co-culture group, (E) UN-adRunx2 co-culture group, (G) CCD-negative control co-culture group, and (H) CCD-adRunx2 co-culture group. (C, F, I) The number of TRAP (+) cells per visual field in each co-culture group was counted and compared. The white arrow points to the TRAP (+) cells. Data represent the mean $\pm$ SD. * $P < 0.05$ ($n = 3$).

As previously reported, osteoclasts were absent in Runx2^{-/-} mice, due to diminished RANKL expression [19]. Besides, CA120-4 (a Runx2^{-/-} calvaria-derived cell line) expressed OPG strongly but RANKL barely. However, adenoviral introduction of Runx2 into CA120-4 cells was able to induce RANKL expression while inhibit OPG expression to induce osteoclast differentiation [19]. These findings indicated that Runx2 promotes osteoclast differentiation by inducing RANKL and inhibiting OPG. After knockdown of Runx2 in PDLSCs from M and S group and overexpression of Runx2 in PDLSC from UN, P, and CCD group, we confirmed that Runx2 upregulated RANKL and downregulated OPG expression in PDLSCs.

Though our study didn't check the mechanism of Runx2 regulating RANKL and OPG expression in PDLSCs during root resorption, recent studies demonstrated that the 5'-flanking region of RANKL gene contained Runx2-specific binding site [20]. Chip and EMS assays showed that the expression of RANKL in vascular smooth muscle cells can be upregulated by Runx2 via direct binding to the specific site in vascular calcification [35]. Moreover, the binding site was found to be highly conserved among mouse, rat, and human species by bioinformation analysis [35]. Therefore,

we believe that during root resorption, Runx2 was able to bind with the same site to increase the expression of RANKL in PDLSCs. Additionally, previous study demonstrated that 12 putative Cbfa1/Runx2-binding elements (osteoblast-specific element 2—OSE₂) were presented in human OPG promoter sequence and Runx2 could bind to the proximal OSE₂ to increase OPG expression level [36]. Further, when doxycycline was used to induce increased expression of Runx2 in ST2 cells (a bone marrow-derived mesenchymal pluripotent cell line), the differentiation of co-cultured splenocytes into osteoclasts was enhanced along with the inhibition of OPG [37], which result was consistent with our data. Thus, it can be supposed that Runx2 may directly regulate the expression of OPG in PDLSCs. However, the regulative mechanism and regulative effect of RANKL and OPG may differ among different cells, which need further research.

Besides, we demonstrated that the proliferation of PDLSCs from resorbed primary teeth (M and S group) was lower than that from unresorbed primary teeth (UN group and CDD group). The proliferative ability was decreased and osteogenic capacity was increased during root resorption. These results suggest that during root resorption the

proliferation of PDLSCs might be inhibited and prefer to differentiate to osteoblast for bone remodeling.

Summary

In this study, we isolated PDLSCs from primary teeth from different resorption period and permanent teeth from normal patient and primary teeth from CCD patient. Through qRT-PCR, western blot, RNAi, overexpression, and co-culture assay, we demonstrated that PDLSCs promote osteoclast differentiation via Runx2 upregulating RANKL and downregulating OPG, leading to enhanced root resorption that results in physiological exfoliation of primary teeth. Furthermore, our result also clarified the mechanism of retained primary teeth due to no root resorption in pathological condition, such as CCD.

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Author Disclosure Statement

No competing financial interests exist.

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