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Vitamin A metabolite inhibits proliferation, migration and osteogenic differentiation in a human periodontal ligament stem cells subpopulation

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Abstract: The periodontal ligament stem cells (PDLSCs) are regarded as the promising seed cells for in situ facilitated endogenous periodontal tissue regeneration. Nevertheless the patients with hypervitaminosis A and alcoholism become very difficult to repair periodontal defects and guide bone regeneration. The purpose of this study was to evaluate the effect of all-trans retinoic acid (ATRA) on the recruitment and osteogenic differentiation in human PDLSCs. The periodontal ligament tissues were obtained from healthy premolars extracted for orthodontic reasons and used to isolate and characterize the hPDLSCs subpopulation. Following various concentrations of ATRA treatment, cell proliferation and migration of hPDLSCs were significantly inhibited, and the cell cycle progression was significantly arrested with the high-dose of ATRA group. Furthermore, with the increase of ATRA concentration, both the early and late stages of osteogenic differentiation were suppressed in hPDLSCs. Our results provide further evidences that excessive ATRA hinders facilitated endogenous periodontal tissue regeneration mediated by hPDLSCs.

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1. Introduction

Although mesenchymal stem cells (MSCs) mediated traditional regenerative medicine has been applied in wide fields, limited to the tissue biopsies, expansion in vitro, rejection reaction [1,2], hence an innovative concept named facilitated endogenous tissue engineering (FETE) has been presented [3,4]. Endogenous approaches focus on the recruitment and homing of stem cells or progenitor cells with high proliferation and differentiation potential to the tissue damage sites, and their subsequent regenerative function. Many influencing factors have already been reported in MSCs, including positive and negative factors [5,6]. However, in the periodontal tissue regeneration the effect on the recruitment of tooth-derived stem cells (TDSCs) is poorly understood.

Up to date, five types of dental stem cells have been successfully isolated and identified [7,8]. Human periodontal ligament stem cells (hPDLSCs) from the periodontal ligament tissues reside in the perivascular space of the periodontium and possess characteristics of mesenchymal stem cells [9,10]. hPDLSCs are thought to be progenitor/ stem cells which have the capacity to differentiate into cementoblast and osteoblasts. hPDLSCs show the multilineage differentiation capacity to undergo osteogenic, adipogenic and chondrogenic differentiation cultured with the appropriate inductive medium. When transplanted into periodontal defects with some scaffold materials, hPDLSCs are able to maintain self-renewal and form a typical cementum-PDL-like complex structure expressing osteogenesis and dentin-specific markers such as alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP). hPDLSCs are the promising seed cells for in situ facilitated endogenous periodontal tissue regeneration [11].

All-trans retinoic acid (ATRA) is a derivative of vitamin A that exerts effects by binding to retinoic acid receptors (RARs), which heterodimerize with retinoid X receptors (RXRs) [12]. As well known, Vitamin A plays a crucial role in embryonic development and multiple biological processes [13-15], including vision, the immune system, bone metabolism, the proliferation and differentiation of cells, so vitamin A supplementation is becoming more common dietary. On the other hand, researchers have found that excessive vitamin A intake could cause accelerated bone fragility, periosteal bone resorption, spontaneous hip fracture and decreased bone mass in humans [16-18]. Moreover, the increased ATRA in serum caused by alcoholism maybe a potential mechanism of ethanol toxicity [19]. Therefore, the study of the involvement of ATRA how regulates the biological behavior of hPDLSCs to affect the periodontal tissue regeneration and implant osteointegration is very meaningful for patients with alcoholism and hypervitaminosis A.

In this study, we investigated the proliferation, migration and osteogenesis potential of hPDLSCs induced by ATRA. For the first time we demonstrated that high-dose ATRA could hinder cell growth, cell cycle, migration and suppress osteogenic differentiation of primary hPDLSCs. Our results suggest that excessive ATRA is a negative regulator of the periodontal regenerative potentials mediated by hPDLSCs.

2. Materials and Methods

2.1. Isolation and culture of primary hPDLSCs

The protocol of human periodontal ligament tissues collection performed in this study was approved by the Medical Ethics Committee of College of Stomatology, Chongqing Medical University and written informed consent was obtained from the donors. Teeth were collected from clinically healthy premolars extracted for orthodontic reasons aged between 12 and 16 years old. Periodontal ligament was scraped from the middle third of the root surface and digested with 0.1% collagenase type Ⅰ (Sigma, USA) and 0.25% trypsin for 30 min at 37 ℃. After centrifugation, the precipitate was seeded about 6 hours in the inverted 25-ml flasks (Corning, USA) coating the fetal bovine serum (FBS, Gibco, USA). The digested tissue mass was cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco) Supplemented with 10% FBS and 1% penicillin/ streptomycin (P/S, Gibco) as the basic medium in a 5% CO_2 humidified atmosphere at 37 ℃. Generally cultured for 5 days, some cells would climb out around the tissue mass. The colony-forming cells were trypsinized and serially subcultured, the third generation of cells were used for identification and subsequent experiment, as described below.

2.2. Colony formation test

The cells were trypsinized to obtain single cell suspension and seeded at a density of 200 cells into a 10-cm dish. Then the cells were incubated in a 5% CO_2 humidified atmosphere at 37 ℃, and the medium were renewed every 5 days. After cultured 10 days, cells were washed twice with PBS, stained with 0.1% crystal violet and captured under a microscope. Aggregates of 50 or more cells were scored as one colony.

2.3. Immunofluorescent (IF) Staining

PDL single cell colonies were cultured on 24-well chamber slides until 80% confluence. After rinsed and fixed with 4% paraformaldehyde for 30 min, the cells were blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature, then with diluted primary antibodies overnight at 4 ℃, and finally with fluorescein-conjugated secondary antibody at 37 ℃ in the dark for 30 min. Afterwards, the cell nuclei were counterstained with DAPI in the dark for 5 min. The images were acquired with Laser Scanning Confocal Microscope (LSCM, Leica TCS SP8).

2.4. Osteogenic and Adipogenic Differentiation Potentials of hPDLSCs

Osteogenic differentiation was induced as previously described [20]. The cells were seeded in 24-well plates (Corning) and cultured with the basic DMEM/F12 medium supplemented with ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) for 21 days. For calcium deposition, cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.4% Alizarin Red S (Sigma) for 5 min at room temperature.

Adipogenic differentiation medium was composed of 10 μg/ml insulin, 1μM dexamethasone (Sigma), 500 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma), and 200 μM indomethacin (Sigma). The cells were seeded in 24-well plates and cultured with adipogenic differentiation medium for 14 days. The cells were fixed with 4% paraformaldehyde for 10 min and stained with Oil Red O (Sigma) for formation of lipid droplet

at room temperature. The staining of calcium mineral deposits and lipid-laden fat cells were recorded under bright field microscopy.

2.5. Cell proliferation assay

The proliferation of hPDLSCs was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). The cells were seeded in a 96-well plate (Corning, USA) and treated with 10 nM or 10 μM ATRA in the basic DMEM/F12 medium (DMSO treatment as a control). The analysis (3 wells per sample) was performed on day 1st, 3rd and 5th. The each sample was incubated with 10 μl CCK-8 solution at 37 ℃ for 2 hours. Finally, the cell viability was assessed as OD values measured at the wavelength of 450 nm by a microplate reader (BioTek ELX800, USA). ATRA (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored in light protected vials at -20 ℃. The stock solution was diluted to the desired concentrations immediately prior to use and all experiments were performed under low-light conditions to minimize ATRA photoisomerization.

2.6. Cell cycle analysis using flow cytometry (FCM)

hPDLSCs were seeded at a density of 1 x106 cells in 10-cm dish (Corning, USA) with the treatment of 0, 10 nM or 10 μM ATRA for 48 hours. Trypsinized and collected the single cell suspension, then fixed with ice-cold 70% ethanol overnight at 4 ℃. The cells were washed twice with PBS, and centrifuged at 800 rpm for 5 min. Next, 100 μl of RNAase A was added for 1hour at 37 ℃, following which 300 μl of propidium iodide (PI) staining solution (0.1% Triton X-100, 100 μg/ml PI in PBS) was added for 30 min at 37 ℃ in dark. The stained cells were passed through a 30 μm nylon mesh filter. The cell cycle in each sample was analyzed using flow cytometry (FCM, BD Influx, USA). The proliferation index (PI) was used to indicate the proliferation level, according to the following formula: PI = (S + G2/M) / (G0/G1 + S + G2/M) x 100%.

2.7. Scratch wound healing assay

To study the effects of ATRA treatment on cell migration, a monolayer wound healing assay was performed. The hPDLSCs were seeded at a density of $1x10^5$ cells/well in 6-well culture plates (Corning, USA). When reaching complete confluence, scratch wounds were created mechanically in a straight line using a sterile 1000-μl pipette tip. Detached cells and debris were washed away three times with phosphate-buffered saline (PBS). The cells were incubated in the basic DMEM/F12 medium with 10nM or 10 μM ATRA and the control group was treated with DMSO in culture medium. Photomicrographs were taken at time 0 (immediately following the scratch wound) and 24 hours under a phase-contrast microscope.

2.8. Alkaline phosphatase (ALP) activity assay

ALP activity was assessed by histochemical staining assay (using a mixture of napthol AS-MX phosphate and Fast Blue BB salt, sigma) and/or a modified Great Escape SEAP Chemiluminescence assay (BD Clontech, CA) as described previously [20]. ALP activity was normalized by total cellular protein concentrations among the samples.

2.9. Statistical analysis

All values were presented as the mean ± SD (standard deviation). Student's T-test for two groups was performed to evaluate the statistical significance. For all quantitative assays, each result was performed in triplicate, and repeated in at least three independent experiments. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of hPDLSCs

To isolate the PDL cells, the periodontal ligament tissue suspension was obtained by enzymatic digestion and placed into the basic DMEM/F12 culture medium. Generally cultured for 5 days, some spindle-shape cells continuously climbed out around the PDL tissue mass (Fig.1A). We performed a limiting dilution assay using the first-generation PDL cells. After 10 days of culture, single cell colonies generated which formed adherent clonogenic cell clusters of typical fibroblastic-like cells, confirming the self-renewal and colony forming capacity (Fig.1B). The colony-forming cell population was termed as hPDLSCs. The cells were serially subcultured, the third generation of cells were used for subsequent experiment (Fig.1C). hPDLSCs positively expressed MSC markers STRO-1, confirming the stromal stem cell status (Fig.1D). Immunofluorescence staining of hPDLSCs showed positive for vimentin but negative for cytokeratin, confirming their mesodermal origin (Fig.1E and 1F). To assess their multipotent capability, with the osteogenic or adipogenic inductive

medium respectively, hPDLSCs could form mineralized calcium nodules, as was shown by staining with Alizarin Red S (Fig. 1G), or accumulate lipid droplets within the cytoplasm, as was confirmed by Oil Red O staining (Fig. 1H).

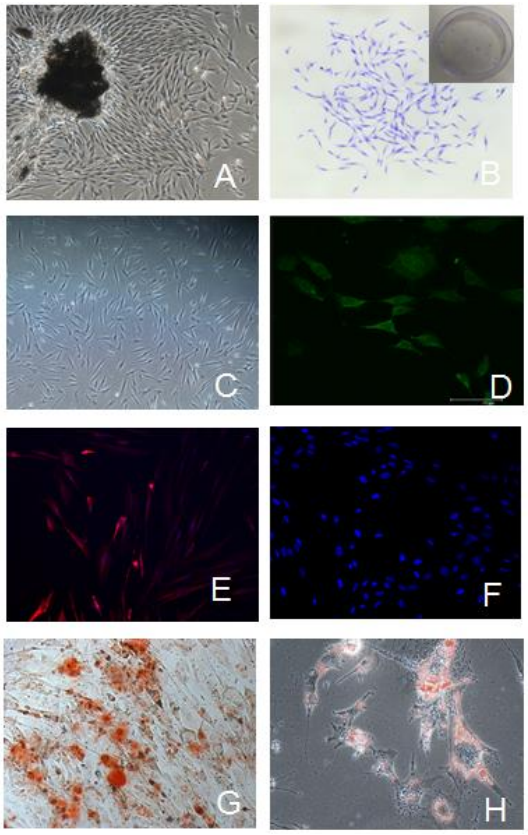

Fig.1. Characterization of hPDLSCs. (A) A number of spindle-shape primary cells grew from PDL culturing for about 5 days (40x). (B) Single cell colonies formed after 10 days of culture by a limiting dilution. Cell clusters derived from a single colony were stained with 0.1% crystal violet. (C) Serially subcultured hPDLSCs exhibited typical fibroblast-like morphology, and the third or fourth passage of cells were used for subsequent experiment (40x). (D) Cultured hPDLSCs exhibited positive staining for STRO-1 (400x). IF staining of hPDLSCs showed positive for vimentin (E) and negative for cytokeratin (F), confirming their mesodermal origin (100x). (G) Cultured in osteogenic induction media for 4 weeks, calcified nodules formed positive for Alizarin Red S staining (100x). (H) Cultured in adipogenic induction media for 3 weeks, lipid droplets formed positive for Oil Red O staining (400x).

3.2 Inhibition of cell growth and cell cycle progression of hPDLSCs by excessive ATRA

The effect of ATRA on cell proliferation of hPDLSCs was evaluated by CCK-8 assay. As shown in Fig.2A, cell viability of hPDLSCs was decreased by incubation with 10 nM or 10 μM ATRA for 3 and 5 days. The proliferation of hPDLSCs treated with 10 μM ATRA was significantly slower compared to that of 10 nM ATRA treated cells at day 3 and 5, indicating the decreased proliferation rate of hPDLSCs caused by ATRA in a dose-dependent manner. In next step we investigated the effects of ATRA on cell cycle progression for 24 h treatment. The results showed that ATRA arrest cells in G0/G1 accompanied with decrease of cell population in the S and G2/M phases (Fig.2B and 2C). Interestingly, the cell proportion of S and G2/M phase with 10μM ATRA treatment was as low as 13.08%, in contrast the DMSO control group was 46.52% and 10 nM ATRA treatment group was 44.81%. The PI of the 10 μM ATRA treatment group was significantly decreased ($p < 0.05$). Collectively, these results demonstrated that the high dose of ATRA could significantly inhibit cell growth and cell cycle progression in hPDLSCs.

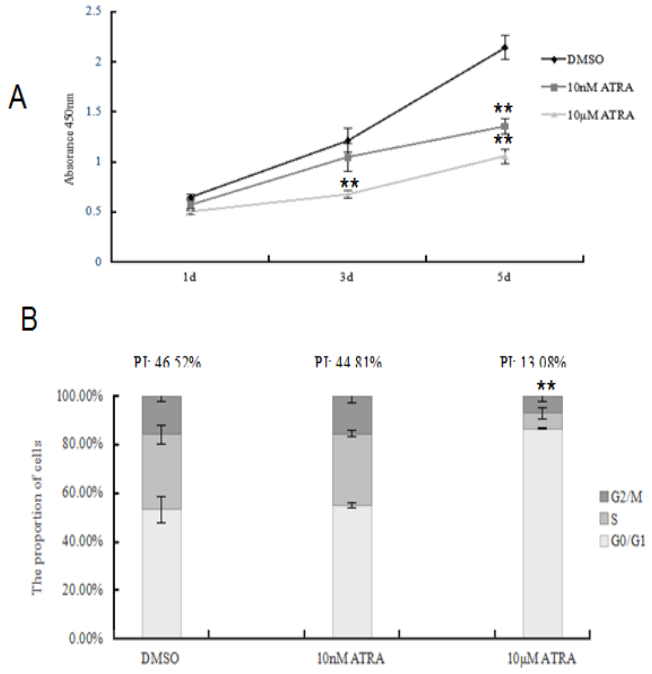

Fig.2. ATRA inhibited cell proliferation and cell cycle of hPDLSCs. (A)The cell proliferation treated with 10nM and 10μM ATRA respectively for 1, 3, and 5 days was measured by CCK-8 assay, DMSO treatment as control. (B) The cell cycle distribution treated with 10nM and 10μM ATRA respectively for 48h was performed by flow cytometry. Distribution of hPDLSCs in various phases of cell cycle was exhibited. The PI decreased with the increase of ATRA concentration. The data represented the mean ± SD of 3 independent experiment performed in triplicate. ** indicates $p < 0.05$ vs. DMSO. Proliferation index (PI) = (S + G2/M)/(G0/G1 + S + G2/M) x100%.

3.3 ATRA postponed migration capability in hPDLSCs

The movement of stem cells and the capacity to migrate to injury sites also determine their regenerative effects. In our cell migration analysis, by the scratch wound healing assay, ATRA treated hPDLSCs moved slower than the control hPDLSCs (Fig.3). When 10 μM ATRA treated hPDLSCs, no or very less number of cells were observed to grow across the wound even after 24 h. However, the DMSO control group were grown throughout the culture plate and were found to migrate across the wound after 24 h. This result suggested that excessive ATRA could reduce the migration ability of hPDLSCs.

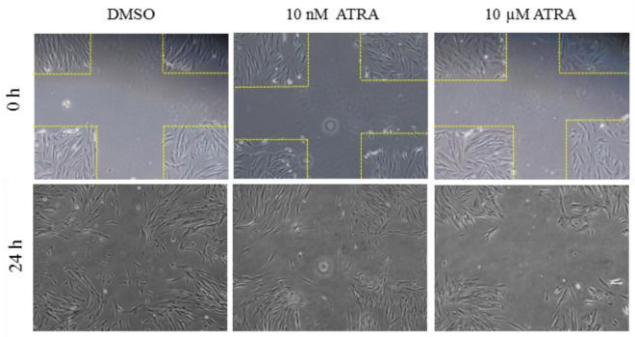

Fig. 3. ATRA postponed the migration capability in hPDLSCs. The effect of ATRA on the migration of hPDLSCs was evaluated by scratch wound healing assay. For the upper panel, images were taken at time 0 hour (100x); for the lower panel, images were taken at time 24 hours (100x).

3.4 ATRA suppressed the osteogenic differentiation of hPDLSCs

There have been several conflicting reports about the role of retinoic acids in osteogenic differentiation. Here, we tested the effects of different concentrations of ATRA on osteogenesis in hPDLSCs. By treating the hPDLSCs with ATRA (1 nM, 10 nM, 100 nM, 1 μM and 10 μM), we found that the early osteogenic marker ALP staining was significantly decreased with increasing ATRA concentrations at day 7 (Fig. 4A and 4B). Coherently, the quantity of ALP activity showed the identical tendency with the ALP staining (Fig. 4C). Compared with the DMSO group, ALP activity of 1nM ATRA treated hPDLSCs had no significant difference ($p > 0.05$), while which from 10nM to 10 μM ATRA treated group exhibited the significant reduce ($p < 0.01$). Likewise, the late osteogenic marker calcium deposition detected by Alizarin Red S staining was remarkably reduced in the high concentration of ATRA treated hPDLSCs (Fig. 4D). It was noteworthy that calcium deposition of 1nM ATRA treated hPDLSCs was significantly reduced, nevertheless ALP activity of 1nM ATRA treated hPDLSCs was normal compared with the DMSO control. Taken together, the above results strongly suggest that excessive ATRA treatment may inhibit both early and late stages of osteogenic differentiation of hPDLSCs.

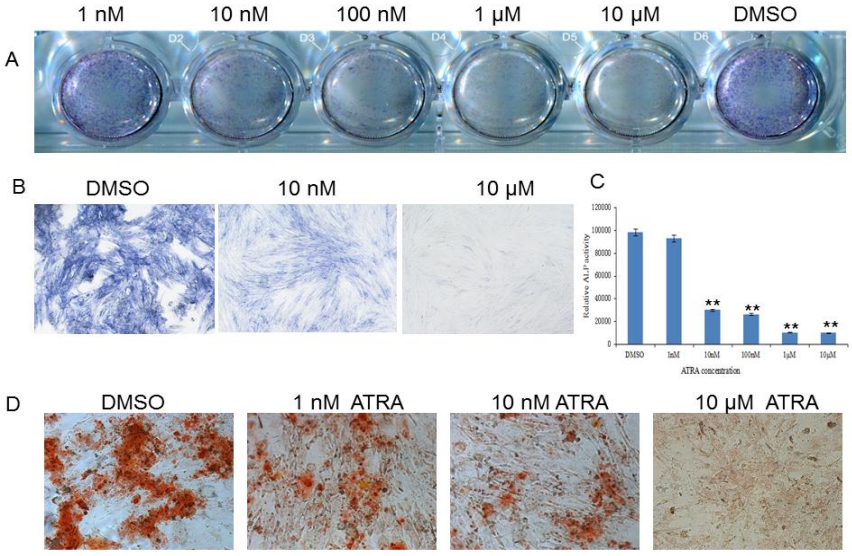

Fig. 4. ATRA suppressed the osteogenic differentiation of hPDLSCs. (A) and (B) The early osteogenic marker Alkaline phosphatase (ALP) staining of hPDLSCs treated with different concentrations of ATRA (0, 1nM, 10nM, 100nM, 1 μM and 10 μM) for 7 days (100x). (C) The quantity of ALP activity treated with different concentrations of ATRA respectively for 7 days was performed by single pipe chemiluminescence detection system. The data represented the mean ± SD of 3 independent experiment performed in triplicate. ** indicates $p<0.01$ vs. DMSO. (D) The late osteogenic marker calcium deposition was detected by Alizarin Red S staining for 21 days (100x).

4. Discussion

Clinically, the patients with hypervitaminosis A and alcoholism become very difficult to repair the periodontal defects and guide bone regeneration. PDLSCs are regarded as residential candidates for in situ periodontal tissue regeneration, consequently to study the effect of ATRA, an active metabolite of vitamin A, on the biological activity of PDLSCs may be helpful to illuminate this phenomenon.

It was reported that periodontal ligament cells (PDLCs) should be a heterogeneous cell pool [21], including cells at different stages of differentiation and lineage commitment, just a small population of self-renewing and multipotent stem cells were identified as periodontal ligament stem cells (PDLSCs). In this study, a human PDLSCs subpopulation was successfully isolated and characterized. The single cell-derived colonies were positive for vimentin and negative for cytokeratin, and they had the differentiation potential to form calcified nodules and lipid vacuoles with the inductive medium. These cells displayed many characteristics that were similar to the mesenchymal stem cells, including a typical fibroblastic appearance, the mesodermal origin, the expression of the MSCs markers STRO-1, and multilineage differentiation potential. This hPDLSCs subpopulation are currently exploited as the main acting cells for facilitated endogenous periodontal bone tissue reconstruction and regeneration. However, various adverse microenvironment can significantly affect the new bone regeneration, such as cigarette smoking, alcoholism and the hypervitaminosis A [18,22,23]. Although Vitamin A and related compounds play important regulatory roles in development and diseases therapy, the association between concentration and regeneration effects of ATRA on hPDLSCs remain unclear.

The facilitated endogenous tissue regeneration is determined by three critical processes: stem cell proliferation, migration and differentiation. Subsequently, we examined the potential effect of ATRA on the recruitment and regulation of hPDLSCs in vitro. Cell proliferation determines the amount of stem cells capable of exerting a regenerative effect. Our results demonstrate that ATRA treatment significantly inhibited the proliferation of hPDLSCs in a dose-dependent manner. In consistency with the previous studies, the similar result was reported that high concentrations of ATRA caused proliferation reduction and cell growth arrest in pre-osteoblasts MC3T3-E1 cells [24]. Furthermore, it has been reported that Runx2 could promote a transition from a proliferative to a post-proliferative stage prior to osteoblast differentiation [25]. It remains to be elucidated whether the significant up-regulation of Runx2 by ATRA could partially account for the significant down-regulation of cell proliferation. Cell migration allows stem cells to actively move towards the injury sites and contribute to the healing process. In this study, we showed that high concentrations of ATRA treatment significantly inhibited the migration of hPDLSCs, which was consistent with the cell growth assay. The reduced migration of hPDLSCs might be related to the downregulation of focal adhesion kinase expression. Further investigation is needed to confirm the proposition.

Stem cell differentiation into tissue-specific cells is one of the key mechanisms for stem cell repair at the injury site during the healing process. Alkaline phosphatase and matrix mineralization are recognized as the early and late stage markers respectively that reflects osteoblastic activity of PDLSCs [26]. Our results further proved that the alkaline phosphatase activity and the calcium deposition were reduced in hPDLSCs with high concentrations of ATRA treatment, interestingly the influence of the concentration on the late matrix mineralization was more significant (Fig.4). Excessive ATRA could affect the osteogenic differentiation potential of hPDLSC, which might be related to the delayed healing processes seen clinically. Coordinate results showed that ATRA treatment slowed the proliferation and inhibited the osteogenic differentiation, which may be due to the induction and maintenance of a partially differentiated non-proliferating state.

In summary, our study showed that the proliferation, migration and differentiation abilities of hPDLSCs with high concentrations of ATRA treatment were significantly inhibited. Vitamin A and related compounds may have a dual role in development and tissue repair, however excessive dose is a negative influence factor in the periodontal regeneration mediated by hPDLSCs. Therefore, the optinal physiological doses of Vitamin A should be taken into account for the periodontal defect and dental implant patients.

Acknowledgments

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