

Original Research Article

Triiodothyronine participates in odontoblast differentiation of apical papilla stem cells through regulation of ERK and p38MAPK signaling pathways

Jiali Xu, Lei He, Haibing Yang*

Department of Stomatology, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, Changzhou 213000, Jiangsu Province, China

*For correspondence: **Email:** yanghaibing2@163.com

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Abstract

Purpose: To investigate the effect of triiodothyronine (T3) in odontoblast differentiation of apical papilla stem cells, and the mechanism of action involved.

Methods: Apical unclosed permanent molars extracted from patients due to orthodontics and impaction were selected. The extracted teeth were cultured in the isolation stage of SCAP cells. The cells were exposed to different concentrations of T3. The effects of ERK and p38 MAPK signaling pathways on activity of alkaline phosphatase (ALP) were determined. Calcium deposition was measured using a calcium determination kit, while the expression of BMP - 2 protein by T3 was determined by Western blot assay. Fluorescence quantitative polymerase chain reaction (FqPCR) method was used to determine the mRNA expression of BMP.

Results: The ALP activities were significantly higher in T3 groups than in control group. Relative to control, there were marked differences in ALP activity and calcium deposition in T3 group, T3 + PD group and T3 + SB group ($p < 0.05$). Relative to control, the mRNA and protein expressions of BMP-2 in T3 group were increased significantly ($p < 0.05$).

Conclusion: Triiodothyronine regulates the differentiation of apical papilla stem cells into dentin through ERK and p38MAPK signaling pathways. This provides the mechanism underlying odontoblast differentiation of apical papilla stem cells.

Keywords: Triiodothyronine, Differentiation, Apical papilla stem cells, Dentin, p38MAPK signal pathway, Phosphorylation

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INTRODUCTION

Apical dental papillary stem cells (SCAP) have strong proliferation and differentiation capacity, and they are the source of odontoblast cells during tooth root development. Indeed, abnormal differentiation of SCAP leads to abnormal tooth

root development [1]. The differentiation of apical papilla stem cells and dentin is important in the formation of root pulp and dentin, and the regeneration of pulp and dentin is closely related to the progressive differentiation of SCAP dentin [2]. At present, SCAP has good prospects in

tissue regeneration due to its good proliferation and differentiation potential [3].

Abnormal endocrine states affect the differentiation of stem cells to varying degrees. The thyroid hormone acts on most tissues and cells in human body, and is essential for tooth growth [4]. Studies have found that serum free thyroid hormone is associated with the occurrence of osteoporosis and other diseases, and that the rate of dental malformation in children is closely related to the secretion of thyroid hormone [5]. The main bioactive form of thyroid hormone in the body is triiodothyronine (3,5,3'-triiodothyronine or T3), which is formed by de-iodization and transformation in peripheral tissues [6]. At present, there are few clinical studies on the effect of T3 on the odontogenic differentiation of SCAP. Previous studies have found that MAPK signaling pathway is a major route for the direct proliferation and differentiation of bone marrow mesenchymal stem cell osteoblasts [7].

The P38 MAPK pathway which comprises extracellular signal-regulated kinase (ERK) and P38 MAPK, plays a key role in promoting osteoblast differentiation. The ERK pathway inhibits osteoblast differentiation. The synergistic balance between these two pathways determines whether bone marrow mesenchymal stem cells can differentiate into osteoblasts [8]. However, there are limited reports on implication of ERK and P38 MAPK signal route on the differentiation of SCAP odontoblast.

Therefore, this study was aimed at investigating the effects of T3 on the proliferation and differentiation of human SCAP cells, and the implication of ERK and P38 MAPK signaling pathways in the process. This was with a view to laying a foundation for further development and application of T3 in clinical practice.

METHODS

Materials

Permanent molars without apical closure due to orthodontic and impacted causes were extracted from patients aged 7 - 20 years in the Surgical Outpatient Department of The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, between December 2019 and January 2021.

Ethical approval

This research received approval from the ethical authority of The Affiliated Changzhou No. 2

People's Hospital of Nanjing Medical University (approval no. 20200321), and it was carried out in line with the Helsinki declaration of 1964 and its later amendments [9].

Main reagents and instruments

Triiodothyronine (T3) was purchased from Tianjin Xidan Chemical Technology Co. Ltd. Phosphate buffered saline (PBS) was purchased from Wuhan Shane Biotechnology Co. Ltd. Fetal bovine serum was product of Thermo Fisher Technology Co. Ltd, China. Trypsin, double antibody, FBS, and α -MEM culture medium were bought from Gibco, USA, while DMEM for cell culture was purchased from Sigma Company in the United States. Ultra-clean workbench, centrifuge, and cell culture incubator were purchased from Thermo Company (USA). Cell culture dish and cell culture plates were purchased from Corning Company (USA).

Isolation of SCAP

The extracted teeth were kept in PBS buffer solution. The teeth were washed with 1 % dual-antibody PBS buffer solution. The apical papillae were cut off with ophthalmic scissors and washed with 1 % dual-antibody PBS buffer solution, followed by transfer to petri dishes and addition of 200 μ L of α -MEM culture medium. The apical tooth papilla tissue was cut into pieces and placed in a centrifuge tube containing trypsin and collagenase. The tube was maintained at 37 °C in a 5 % CO₂ incubator for 20 min, after which 2 mL of α -MEM was used to terminate the digestion. The digest was spun at 900 rpm for 15min. The supernatant was discarded, and 3ml of FBS and complete culture medium with 2 % double antibody were added. The cells were cultured in the culture dish in the cell culture incubator at 37 °C and 5 % CO₂ for 3 days, followed by rinsing with double antibody PBS buffer. When the cells attained 80 % confluence, they were passaged in trypsin digestion solution. The resultant P3-p5 cells were used for subsequent experiments.

SCAP cell culture

The SCAP cells were seeded in a 6-well plate and incubated separately with the prepared 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M solutions of T3 for 5 days, with medium refreshed every 48 h. After 5 days, the culture medium was washed with PBS culture medium, and the cells were lysed with PIPA lysis solution. The cell lysate was centrifuged at 12000 rpm. Lysate protein content was measured with BCA method.

Evaluation of parameters/indices

Alkaline phosphatase (ALP) activity and differentiation of SCAP cells

Four groups of cells were used: control, 10^{-10} M T3 group, 10^{-9} M T3 group, 10^{-8} M T3 group and 10^{-7} M T3 group. After washing with PBS buffer solution, the cells were digested with protease E. Para-nitrophenyl phosphate was added and placed in the reaction solution at 37 °C and pH 10.3 for 30 min, after which the reaction was halted with NaOH solution. The T3 concentration with the highest ALP activity was used in follow-up investigations.

Cell proliferation

Logarithmic growth-phase cells were inoculated in five 96-well plates and cultured at 37°C in a 5 % carbon dioxide and saturated-humidity incubator for 1 day, and then changed to serum-free medium. After culturing for 24 h, the cells entered the stationary growth phase. Then, each well was incubated with 5 µL of MTT solution at 37 °C for 4 h, followed by discarding of the medium and solubilization of the resultant formazan crystals in each well with 100 µL DMSO, and shaking for 15 min. The absorbance value (OD value) of each formazan solution was measured at 490 nm. The MTT assay was done on the 1st and 7th days of culture.

Effects of ERK and p38MAPK signaling pathways on changes in ALP activity

In the control group, T3 group, T3+PD group (T3+ERK blocker PD8089 group) and T3+SB group (T3+ P38 inhibitor SB203580 group), changes in ALP activity of the cells were determined after culture.

Calcium deposition

Following washing using PBS buffer solution and decalcification using 200 µL hydrochloric acid, calcium concentration was determined using calcium determination kit in strict accordance with the kit protocols.

mRNA and protein expressions of T3 to BMP-2

Total protein was extracted from logarithmic growth phase cells in each group by lysing with RIPA, followed by centrifugation at 12000 g for 5 min, and lysate protein quantitation with BCA procedure. Then, after boiling in water, 30-µg protein portions were subjected to 10 % SDS-polyacrylamide gel electrophoresis and

transferred to PVDF membranes which were blocked by incubation with non-fat milk solution for 1h. Then, the membranes were incubated overnight at 4°C with the appropriate 1° immunoglobulins. Thereafter, the membranes were rinsed thrice with TBS, and incubated with horse radish peroxidase-linked secondary antibody at room temperature for 1 h. The bio-RAD exposure system was used to select the appropriate exposure time for the corresponding strips, while Image Lab software was employed for gray value analysis.

mRNA expression of BMP-2

TRIzol was used to extract total RNA from cells, and reverse transcription of total RNA was carried out according to the instructions of RNA reverse transcription kit. Transcriptional amplification was done strictly in line with directions in the fluorescence real-time quantitative PCR kit. The relative expression of BMP-2 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data were analyzed using SPSS 21.0 software. Measured results are expressed as mean \pm SD. Inter-group data comparison was done using independent sample *t*-test, while numerical data are presented as numbers and percentages [n (%)]. Comparison between two groups was done with χ^2 test. Values of $p < 0.05$ implied statistical significance.

RESULTS

Impacts of different concentrations of T3 on SCAP cell proliferation

Table 1 shows no significant difference in absorbance value among groups on day 1 after T3 addition ($p > 0.05$). However, on day 7, absorbance value was highest in 10^{-9} M T3 group.

Table 1: Influence of T3 on SCAP proliferation (mean \pm SD)

Group	Absorbance (Day 1)	Absorbance (Day 7)
Control	0.122 \pm 0.020	0.639 \pm 0.014
T3 (M)		
10^{-10}	0.125 \pm 0.015	0.684 \pm 0.029
10^{-9}	0.127 \pm 0.014	0.711 \pm 0.032
10^{-8}	0.122 \pm 0.014	0.648 \pm 0.024
10^{-7}	0.128 \pm 0.018	0.641 \pm 0.026
<i>t</i>	0.43	22.74
<i>P</i> -value	0.786	<0.001

Optimal T3 concentration for ALP activity

Figure 1 shows that relative to control group, ALP activities in T3 groups were significantly increased, with highest ALP activity in 10⁻⁹ M T3 group).

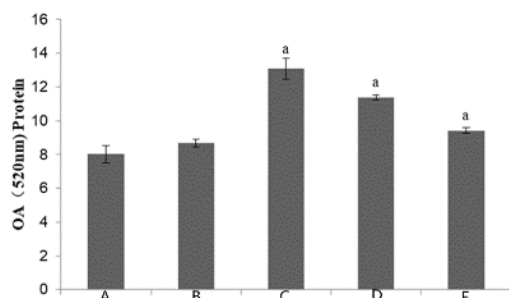


Figure 1: Optimal T3 concentration for ALP activity. **Key:** A: Control; B: 10⁻¹⁰ M T3; C: 10⁻⁹ M T3; D: 10⁻⁸ M T3; E: 10⁻⁷ M T3

Changes in ALP activity

Compared with the control group, ALP activities in T3 group, T3+PD group and T3+SB group are higher significantly ($p < 0.05$).

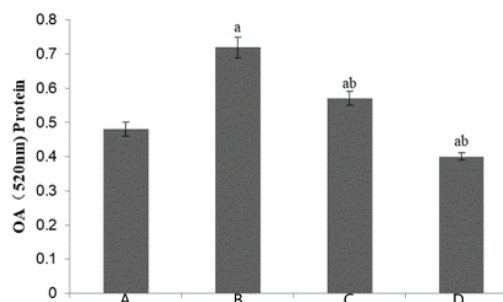


Figure 2: Changes in ALP activity in the various groups. **Key:** A: Control group; B: ALP activities in T3 group; C: T3+PD group; D: T3+SB

Influence of ERK and p38MAPK signaling pathways on calcium deposition

Calcium deposition in T3 group, T3+PD group and T3+SB group differed significantly from that in the control group ($p < 0.05$). There were significantly decreased calcium depositions in T3+PD and T3+SB groups, when compared to T3 group ($p < 0.05$; Figure 3).

Effects of ERK and p38MAPK signaling pathways on mRNA and protein expressions of BMP-2

Protein and mRNA expressions of BMP-2 mRNA in T3 group were markedly increased, relative to control, but were decreased significantly in T3+PD group ($p < 0.05$). However, the mRNA

and protein expressions of BMP-2 were significantly decreased, when compared to T3 group ($p < 0.05$; Figure 4).

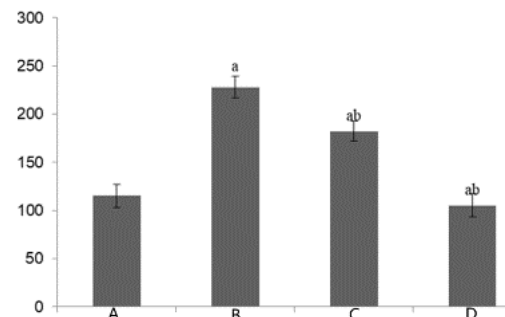


Figure 3: Influence of ERK and p38MAPK signaling pathways on calcium deposition. **Key:** A: Control group; B: ALP activities in T3 group; C: T3+PD group; D: T3+SB

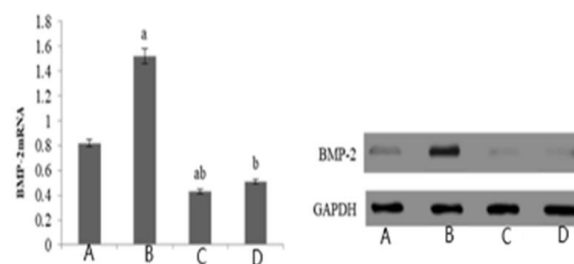


Figure 4: Effects of ERK and p38MAPK signaling pathways on BMP-2 mRNA and protein expressions. **Key:** A: control group; B: ALP activities in T3 group; C: T3+PD group; D: T3+SB

DISCUSSION

Dental caries and dental trauma are common oral diseases of children. If not treated in time, pulp necrosis and periapical lesions may result, leading to delayed growth and arrested development of permanent teeth [10]. The main characteristics of these diseases are short root, weak tooth tissue, open apical mouth and weakened tooth resistance, which have serious impacts on chewing quality and physical development in children [11]. The main clinical treatment involves induction of apical development with appropriate drugs after infection control. However, drug-induced apical development often and sometimes fails to increase the thickness of root canal wall and tooth resistance, resulting in poor therapeutic effect [12]. Therefore, there is need to develop a biological therapy that can promote the growth of tooth root and regeneration of dentin.

The SCAP, odontogenic stem cells with mesenchymal stem cell characteristics in undeveloped apical papillary tissues, were

discovered by Sonoyama *et al* [13]. Compared with dental pulp stem cells, SCAP has stronger proliferation and differentiation potential [14]. Thyroxine plays an important role in bone growth, development and metabolism, but its effects on cells are different under different conditions [15]. It promotes the activity of ALP in SCAP osteoblasts, and this effect is positively correlated with T3 concentration and duration of stimulation [16]. It has been reported that T3 promoted osteogenic differentiation of some stem cells [17].

Bone suture osteogenesis plays an important role in the process of bone formation, and the development of maxillofacial bones, especially the face and skull, is very important [18]. Animal studies have shown that skull fusion in mice is caused by overexposure to thyroxine *in utero* [19]. Premature closure of mouse skull sutures may be due to enhancement of differentiation of osteoblasts at bone sutures due to excessive thyroxine [20]. On day 1 following T3 addition, OD was comparable among the groups. The OD value was highest in 10^{-9} M T3 group. Relative to control, ALP activities in T3 groups were significantly increased, and the activity of ALP in 10^{-9} M T3 group was the highest. These results suggest that T3 enhances the proliferation and differentiation of SCAP cells.

Signaling pathways play key roles in various types of biological behaviors through regulation of cell proliferation, migration and differentiation [21]. The MAPK signal route involves ERK, JNK and P38 proteins. Following phosphorylation, MAPK enters the nucleus and regulates the rate of gene transfer, thereby influencing the biological behavior of cells [22]. The bioactive substance MTA promotes tooth/bone transformation of stem cells of periodontal ligament by activating the MAPK pathway. Studies have confirmed that inhibition of ERK pathway suppresses chondrogenic differentiation of dental pulp stem cells [23].

In this study, compared with the control group, there were statistically significant differences in ALP activity and calcium deposition in T3, T3+PD and T3+SB groups. Compared with T3 group, ALP activity and calcium deposition among T3+PD and T3+SB groups were significantly decreased. The mRNA and protein levels of BMP-2 in T3+PD and T3+SB groups were significantly decreased, when compared to T3 group. These data reveal that T3 might be implicated in odontogenic differentiation of SCAP cells by regulating the ERK and p38MAPK signaling pathways.

CONCLUSION

The ERK and p38MAPK signaling pathways play regulatory roles in the odontogenic differentiation of apical papillary stem cells. This perhaps unravels a mechanism for odontoblast differentiation of apical papilla stem cells.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Haibing Yang designed the study, supervised the data collection, and analyzed the data. Jiali Xu and Lei He interpreted the data and prepared the manuscript for publication. Jiali Xu supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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