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CD8$^+$ T lymphocytes enhance the anabolic effect of intermittent parathyroid hormone on cementoblasts

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ABSTRACT

Root resorption is usually inflammatory in nature and has a tight link with immune system. Intermittent parathyroid hormone (iPTH) could promote cementum regeneration. The cross-talk of immune cells and cementoblasts may play an important role in the regeneration which stayed to be elucidated. In this study, a CD8$^+$ T cells-OCCM-30 cells coculture system was established in vitro to investigate whether CD8$^+$ T cells could enhance the anabolic effect of iPTH on cementoblasts and to find out the potential link of the effect with Wnt signal pathway. Determined by real-time PCR and Western Blot, we found an amplified cementogenesis in the OCCM-30 cells from coculture system, including increased mRNA and protein expression of *Alp*, *Opn* and *Runx2*, ALP activity and mineralization. We also found iPTH could increase the expression of *Wnt10b* in CD8$^+$ T cells by ELISA. In addition, *Wnt10b* would promote the proliferation of OCCM-30 cells, while the effect on differentiation was various in different culture medium. These results demonstrated that the stimulating effect of iPTH on cementoblasts could be mediated through an interaction with CD8$^+$ T cells, and T-cell-induced *Wnt10b* might be a key mechanism in the mediation.

1. Introduction

Root resorption is a common side effect during orthodontic treatment. It is usually inflammatory in nature and involves loss of the dental root structure (cementum and/or dentin) [1,2]. Cementoblast is a unique phenotype and plays an important role in the repairment process of cementum [3].

Parathyroid hormone (PTH) has different influence on bone remodeling depending on the interval of administration. Continuous delivery of PTH leads to bone loss, while intermittent PTH (iPTH) treatment increases bone turnover and promotes bone formation [4]. As a result, intermittent administration of PTH (1–34) as a therapeutic for postmenopausal osteoporosis is approved by Food and Drug Administration (FDA) [5]. Similarly, iPTH treatment could also enhance cementum regeneration by elevating cementum formation [6].

Research in osteoimmunological field indicated a tight link of regulatory process between immune cells and bone remodeling related cells [7–9]. T cells, especially CD8$^+$ T cells, were involved in the anabolic effect of iPTH on osteoblasts [10,11]. Similarly, CD8$^+$ T cells could amplify the regenerative effect of PTH on human periodontal ligament (hPDL) cells [12]. Studies showed that PTH could promote expression of *Wnt10b* in T cells, and this might be the key mechanism underlying the amplified anabolic effect of PTH by T cells. However, the PTH administration in these in-vitro studies did not imitate the "intermittent" stimulation. And the influence of Wnt signaling on cementum formation is controversial [13–15].

Since root resorption and repairment is a process involved inflammation in nature, the cross-talk of immune cells and cementoblasts may play an important role which stayed to be elucidated. The purpose of this study was to investigate whether CD8$^+$ T cells could enhance the anabolic effect of iPTH on cementoblasts by establishing a coculture model in vitro and to find out the potential link of the effect with Wnt signal pathway.

2. Materials and methods

All experimental protocols were reviewed and approved by the ethics committee of West China School of Stomatology, Sichuan University.

2.1. Cell culture

2.1.1. T cell

Spleen T lymphocytes were isolated from wild-type BALB/c mice

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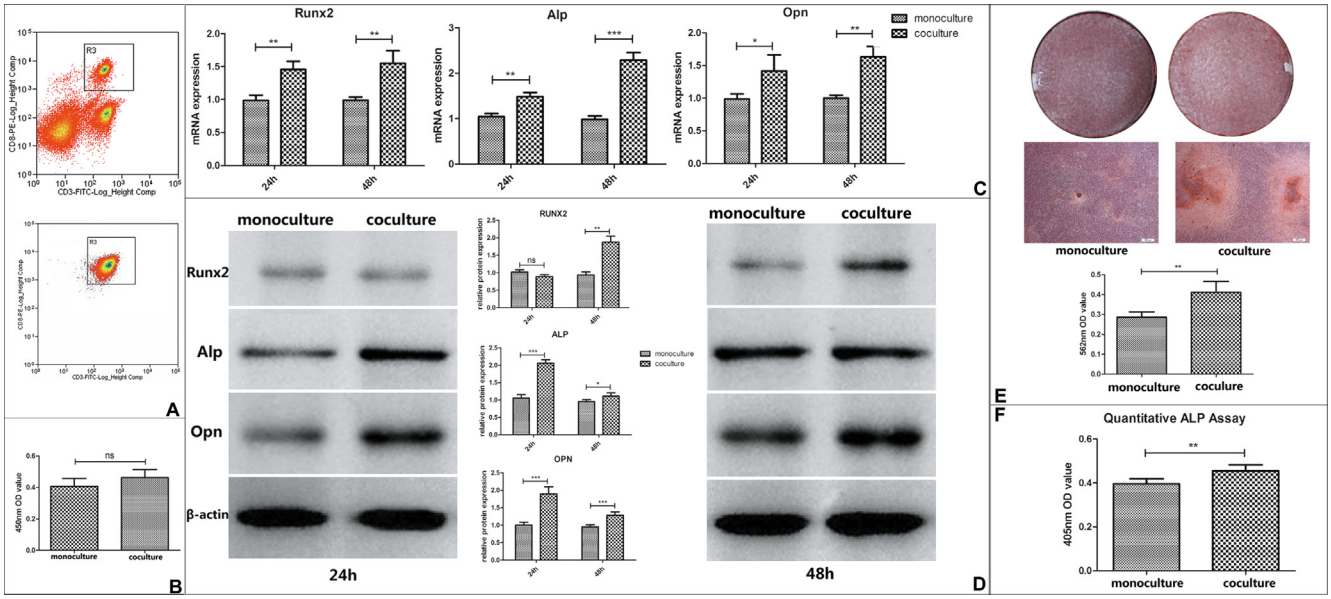

Fig. 1. The effect of interaction of CD8$^+$ T cells on proliferation and differentiation of OCCM-30 cells under iPTH stimulation after two cycles, these results indicated that under iPTH treatment, coculture with CD8$^+$ T cells could promote the differentiation of OCCM-30 cells, but not the proliferation. A. Preparation of CD8$^+$ T cells. The upper figure showed cell plot in the flow cytometry analysis for the sort of CD8$^+$ T cells and the lower one revealed CD8$^+$ T cells were purified to be 99.38%. B. Result of CCK-8 Assay showed no significant difference of viability of OCCM-30 cells between coculture and monoculture groups. C. Results of RT-PCR showed increased gene expression of *Runx2*, *Alp* and *Opn* in coculture group after 24 h and 48 h. D. Results of Western blot showed a similar trend of protein expression to gene expression, although there was no significant difference on *Runx2* between coculture and monoculture groups after 24 h. E. After 2 cycles of iPTH treatment, Alizarin Red S stain was performed at 14d. Mineralized nodules in coculture group were larger in comparison with that of the monoculture group. F. After 2 cycles of iPTH treatment, quantitative ALP assay was conducted. It revealed an enhanced ALP activity in the coculture group. All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (Microscopic sale bar:200um. ns = no significance,*P < 0.05,**P < 0.01,***P < 0.005).

(Chengdu Dashuo Biological Technology, Chengdu, China) and then resuspended in phosphate-buffered saline. In brief, fluorescein isothiocyanate(FITC)-conjugated monoclonal antibodies against mouse CD3 and phycoerythrin(PE)-conjugated monoclonal antibodies against mouse CD8a (eBioscience, Thermofisher Scientific, San Diego, CA, USA) were incubated with the isolated cells at 4 °C for 20 min. Cells were washed and then purified to be 99% by flow cytometry (Fig. 1A). The isolated CD8$^+$ T cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified chamber with 5% CO_2 and 95% air. 40 IU/mL IL-2 (PEPROTECH, Rocky Hill, USA) was added thereafter, two days before the coculture.

2.1.2. Cementoblast

The immortalized mouse cementoblast cell line OCCM-30 was a kind gift from Prof. Somerman (NIH, Bethesda, USA) maintained as described previously [16], it was isolated from developing molars of OC-Tag transgenic mice and used to provide a model to study cementogenesis in vitro. Briefly, OCCM-30 cells were cultured in Dulbeco's modified Eagle medium (DMEM) containing the same supplements as stated for CD8$^+$ T cells above.

2.2. Establish the CD8$^+$ T cells-OCCM-30 cells coculture system in vitro

To determine whether the cementogenetic effect of iPTH on cementoblasts would be enhanced under interaction of CD8$^+$ T lymphocytes, CD8$^+$ T cells-OCCM-30 cells coculture system was established in vitro by using 6-well plate with 0.4 μm pore size transwell inserts. OCCM-30 cells were seeded in 6-well plate at the amount of 2×10^5 cells/well while 10^6 CD8$^+$ T cells were seeded in the transwell inserts. In control groups (monoculture groups), only RPMI 1640 medium with no T cells were contained in the inserts. PTH (1–34) (Bachem, Torrance, CA, USA) was dissolved in 0.1% acetic acid according to the manufacturer's protocol. To mimic iPTH incubation, the coculture/monoculture system was exposed to 40 ng/ml PTH (1–34) for the first 6 h in each 24 h incubation cycle, and cultured for the remainder of the cycle in vehicle culture medium without PTH. After 1 and 2 cycles of treatment, both OCCM-30 cells and DMEM medium were collected for further examination.

2.2.1. Proliferation experiments

To investigate the effect on the proliferation of OCCM-30 cells, 24-well plate with 0.4 μm pore size transwell inserts was used to establish a coculture system. Five replicate wells were set in each group. After 2 cycles of iPTH treatment, Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to examine proliferation of OCCM-30 cells according to the manufacturer's protocol.

2.2.2. Quantitative alkaline phosphatase (ALP) assay

After 2 cycles of iPTH treatment, OCCM-30 cells from both groups were evaluated for ALP activity with a quantitative ALP assay kit (Beyotime, Shanghai, China). The prepared samples were distributed at 100 μL per well on a 96-well plate, and were incubated for 30 min at 37 °C. The optical density (OD) was measured at 405 nm.

2.2.3. Alizarin red S staining

For differentiation induction, culture medium of OCCM-30 cells was replaced with DMEM containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone (Sigma, St. Louis, MO, USA). The transwell inserts were removed after 2-cycle treatment and then culture medium was changed every 3d. Alizarin red S stain (Sigma-Aldrich, St. Louis, MO, USA) were performed at 14d. Mineralized nodules were observed under an Olympus IX70 microscope (Olympus, Tokyo, Japan) and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). For quantitative calcium

measurement, 10% cetylpyridinium chloride (J&K CHEMICA, Beijing, China) solution was used for elution of the dye. After incubation at room temperature for 1 h, OD value was measured at 562 nm.

2.3. Effect of PTH treatment on $CD8^+$ T cell

To find out the potential effect of iPTH on $CD8^+$ T cells, 2×10^7 $CD8^+$ T cells were seeded on a dish. The same protocol of iPTH stimulation with the coculture process was performed. After 6 h, 1 and 2 cycles of treatment, $CD8^+$ T cells and culture medium were harvested for further examination.

2.4. Effect of WNT10B on OCCM-30 cells

2.4.1. Proliferation of cells

To investigate the influence of WNT10B on the proliferation of OCCM-30 cells, 0 ng/ml, 25 ng/ml, 50 ng/ml, 75 ng/ml and 100 ng/ml WNT10B (R&D system, MN, USA) protein were used to stimulate OCCM-30 cells, respectively. The cells were seeded in 96-well plate (10^3 cells/well) with six replicate wells for each group. CCK-8 assay was used to examine proliferation of OCCM-30 cells at 48 h.

2.4.2. Cementogenesis experiment

To investigate the effect of WNT10B on the cementogenesis of OCCM-30, cells were stimulated with 50 ng/ml WNT10B protein, while vehicle-treated cultures (PBS) served as control groups. Cells were harvested at 24 h and 48 h for further examination.

2.4.3. ALP staining & Alizarin red S staining

Osteogenic culture medium was for differentiation induction, which was changed every 3d. ALP activity was measured at 7d by an ALP staining kit (Jiancheng, Nanjing, China) and Alizarin red S staining was performed at 14d.

2.5. Real-time quantitative polymerase chain reaction (RT-PCR)

Isolation of total RNA, cDNA synthesis and RT-PCR were performed for gene expression analysis. Total RNA was extracted from cultured OCCM-30 cells by Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Baltics, Lithuania) as indicated by the manufacturer. RT-PCR was performed on an ABI QuantStudio® 3 instrument (Applied Biosystems, Carlsbad, CA, USA) with SYBR® Premix Ex TaqTMII kit (Takara, Bio Co Ltd, Kyoto, Japan). Target gene expression was normalized to GAPDH. The primer pairs were listed as follows:

(1) *Gapdh*-forward:5′-AGGTCGGTGTGAACGGATTTG-3′;*Gapdh*-reverse:5′-TGTAGACCATGTAGTTGAGGTCA-3′ (2) *Alp*-forward:5′-ACTGATGTGGAATACGAACTGG-3′;*Alp*-reverse:5′-AGTTCAGTGCGGTTCCAG-3′ (3) *Opn*-forward:5′-GAGCGAGGATTCTGTGGA-3′; *Opn*-reverse: 5′-TCGACTGTAGGGACGATTG-3′ (4) *Runx2*-forward:5′-GACTGTGGTTACCGTCATGGC-3′; *Runx2*-reverse: 5′-ACTTGGTTTTTCATAACAGCGGA-3′ (5) *Wnt10b*-forward:5′-GCAGGCATTGTGTGGAGTCA-3′; *Wnt10b*-reverse: 5′-ATTGTCACCCGAGGTCCCAT-3′ (6)*β-Catenin* -forward: 5′-AGGAATGAAGGCGTGGCAACA-3′; *β-Catenin* -reverse: 5′-GCACCAATGTCCAGTCCGAGAT-3′ (7) *Lef1*-forward: 5′-GAAATGAGGGCGAATGTCGT-3′; *Lef1*-reverse: 5′-CAGCTGTCATTCTGGGACCT-3′

2.6. Western blot analysis

Equivalent amounts of protein (20–40 μg) were extracted from OCCM-30 cells from different groups. The detailed process was performed in accordance with the previous description [6]. Subsequently, immunoreactive proteins were detected with ECL Prime Western blotting detection reagents and the intensity of each band was quantified using Image-Pro Plus 6.0 after normalization to β-actin (dilution 1:10000). For immunoblotting, anti-RUNX2 antibody (Abcam, Cambridge, UK, dilution 1:1000), anti-OPN antibody (Abcam, Cambridge, UK, dilution 1:1000) and anti-ALP antibody (Biorbyt, Cambridge, UK, dilution 1:2000) were used.

2.7. ELISA

According to the manufacturer's instructions, the protein level of the secretion of WNT10B protein in culture medium of OCCM-30 cells from different culture systems, including control group (monoculture system without PTH stimulation), monoculture and coculture system with iPTH stimulation was analyzed by a specific ELISA kit (USCN Life Science Inc., Wuhan, China) at 6 h, 24 h and 48 h. Absorbance was measured at a 450 nm wavelength.

2.8. Statistic analysis

All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). Data analysis was conducted with SPSS (version 16.0, SPSS Inc.). Student's *t* test was performed for the data analysis between 2 group comparisons and One-way ANOVA was conducted for more groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effect of $CD8^+$ T cells interaction on OCCM-30 cells under PTH stimulation

To explore the possible effect of coculture with $CD8^+$ T cells on the proliferation of OCCM-30 cells, we employed CCK-8 assay after 2 cycles of iPTH treatment. The results of OD values at 450 nm in monoculture and coculture groups were presented in Fig. 1B, which indicated proliferation of OCCM-30 cells would not be significantly influenced by $CD8^+$ T cells interaction under iPTH stimulation within 48 h, though there seemed to be an increasing trend. ($P = 0.172$).

To find out whether $CD8^+$ T cells could enhance the cementogenesis effect of iPTH on OCCM-30 cells, expression of skeletogenic markers (*Alp* and osteopontin (*Opn*)) and osteogenic transcription factor runt-related transcription factor 2 (*Runx2*) were examined by RT-PCR and Western Blot on gene and protein level, respectively. After 24 h and 48 h treatment, gene expression of *Runx2*, *Alp*, and *Opn* was increased in coculture group (Fig. 1C, $P < 0.05$). Compared with monoculture group, the mRNA levels of *Runx2*, *Alp* and *Opn* in coculture group were significantly increased by 1.48-, 1.43-, 1.44- fold after one cycle and 1.56-, 2.33-, 1.63- fold after two cycle of iPTH treatment ($P < 0.05$). However, the enhanced effect did not show a clear time dependence.

As for the protein expression, the result of Western Blot also showed $CD8^+$ T cells an enhanced protein expression of *Alp* and *Opn* by 1.95- and 1.89-fold, with the exception that no significant differences were observed in *Runx2* expression after one cycle of iPTH treatment, and an increased expression of *Runx2*, *Alp* and *Opn* by 2.01-, 1.21 and 1.35-fold (Fig. 1D), which indicated $CD8^+$ T cells could amplify the cementogenesis effect of iPTH on OCCM-30 cells in 48 h.

To investigate the effects of $CD8^+$ T cells interaction on mineralization of OCCM-30 cells under iPTH condition, quantitative ALP assay, Alizarin red S staining and quantitative calcium assay were performed. The results were presented in Fig. 1E&F. Compared with monoculture group, the mineralized nodules were bigger, and the OD value of dissolved nodules was significantly higher (nearly 1.44-fold) in coculture group, which showed 48 h interaction of $CD8^+$ T cells could promote the mineralization of OCCM-30 cells. The results of quantitative ALP assay also expressed a slightly but statistically significant increased ALP activity (1.15-fold) in the coculture group.

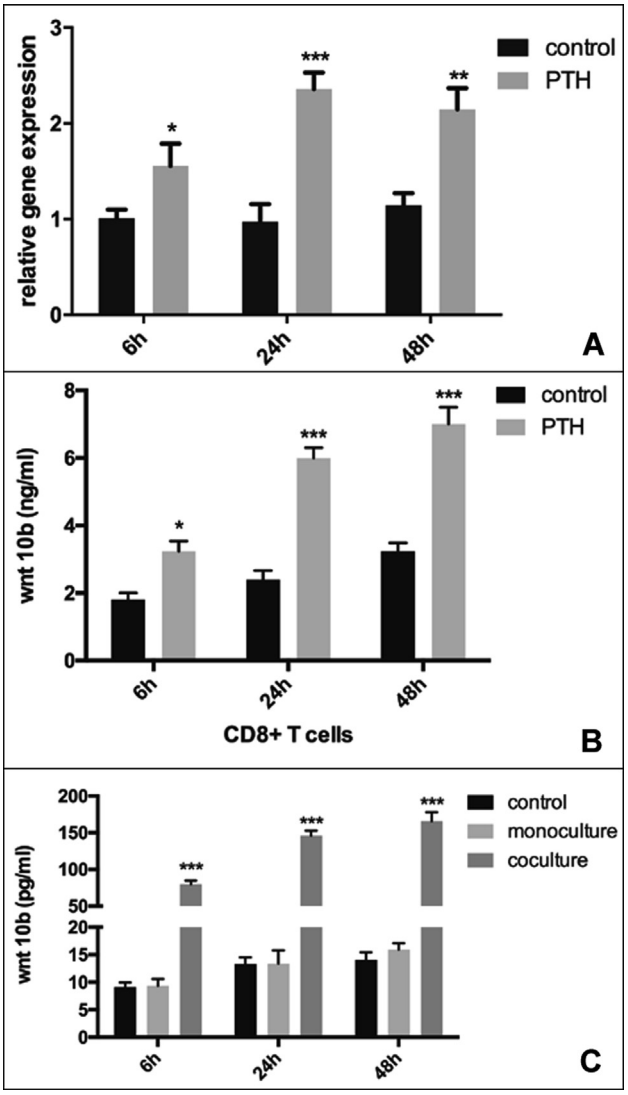

Fig. 2. The effect of iPTH stimulation on expression of *Wnt10b* in CD8$^+$ T cells and expression of *Wnt10b* in the culture medium of OCCM-30 cells from different culture systems, these results showed iPTH treatment could increase the expression of *Wnt10b* in CD8$^+$ T cells. A. Results of RT-PCR showed a significant increase of *Wnt10b* expression in CD8$^+$ T cells in iPTH group compared to control group. B. Results of ELISA revealed that iPTH could promote secretion of WNT10B in CD8$^+$ T cells at 6 h, one cycle and two cycles. C. WNT10B in the culture medium of OCCM-30 cells was quantified by ELISA and the results indicated increased expression of *Wnt10b* in coculture system. All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (*P < 0.05, ***P < 0.005).

3.2. The effect of iPTH on expression of Wnt10b in CD8$^+$ T cells

To explore the effect of iPTH on CD8$^+$ T cells, RT-PCR and ELISA were performed to test the gene expression and protein secretion level of *Wnt10b*. The results showed stimulation of PTH for 6 h could significantly increase the gene expression of *Wnt10b* in CD8$^+$ T cells (Fig. 2A), and treatment of iPTH for one and two cycles also promoted the gene expression of *Wnt10b*, by 1.54-, 2.24-, 1.87- fold after 6 h, one cycle and two cycles, separately. Results of quantified test by ELISA also revealed PTH could significantly promoted the secretion of WNT10B in CD8$^+$ T cells at 6 h, one and two cycles (Fig. 2B).

3.3. The expression of Wnt10b in coculture and monoculture system

To determine whether the enhanced cementogenesis in coculture system was the result of increased expression of *Wnt*10b of CD8$^+$ T cells under iPTH stimulation, WNT10B in medium of OCCM30 cells from different culture system were quantified by ELISA. As presented in Fig. 2C, the concentration of WNT10B from control group (monoculture system without PTH stimulation) was about 9.16 pg/ml, 9.35 pg/ml in monoculture system with stimulation and 80 pg/ml from coculture medium at 6 h. After one cycle, it was about 13.29 pg/ml, 13.39 pg/ml and 145.8 pg/ml in control group, monoculture system and coculture system. And after two cycles, the concentration was 14.08 pg/ml, 15.92 pg/ml and 165.67 pg/ml in the three culture systems, separately. These results revealed iPTH could significantly promote the secretion of WNT10B of CD8$^+$ T cells in the transwell inserts, thus increased the concentration of WNT10B in the culture medium of OCCM-30 cells in the wells. However, there was no significant difference of WNT10B concentration between control group and monoculture group, which revealed iPTH could not significantly change the secretion of WNT10B of OCCM-30 cells. These results indicated WNT10B secreted from CD8$^+$ T cells was involved in the amplified anabolic effect of iPTH on OCCM-30 cells.

3.4. The effect of WNT10B on OCCM-30 cells

As the concentration of secreted WNT10B was depend on many factors, such as the number and status of CD8$^+$ T cells. To explored the effect of WNT10B on OCCM-30 cells, we used the recombination protein of WNT10B. After 48 h treatment, the result of CCK-8 assay test showed that 50 ng/ml, 75 ng/ml and 100 ng/ml WNT10B could promote proliferation of OCCM-30 cells, while 25 ng/ml WNT10B could not (Fig. 3), and no significant difference was found in the condition of proliferation among the three groups.

To determine the influence of WNT10B on the cementogenesis on OCCM-30 cells, RT-PCR showed in the first 24 h of 48 h, the gene expression of *Alp* and *Opn* was increased by 1.53-fold and 1.38-fold in 10b group, while the gene expression of *Runx2* was decreased. The gene expression of *β-Catenin* and *Lef-1*, which were important members of Wnt signal pathway, were also increased in wnt10b group by 7.12-fold and 1.63-fold. However, the results at 48 h were different. Gene expression of *Runx2*, *Alp* and *Opn* were decreased in wnt10b group, so did that of *β-Catenin* and *Lef-1* (Fig. 4).

Interestingly, when the medium was changed every day or OCCM-30 cells were cultured in osteogenic medium for 48 h, the results of RT-PCR showed a significant increase of *Runx2*, *Alp*, *Opn*, *β-Catenin* and *Lef-1* in wnt10b group (Figs. 5 and 6A).

As for the protein expression, western blot presented a similar trend with the gene expression in all different culture medium (Figs. 4 and 5).

The results of ALP staining at 7d and Alizarin red S staining at 14d

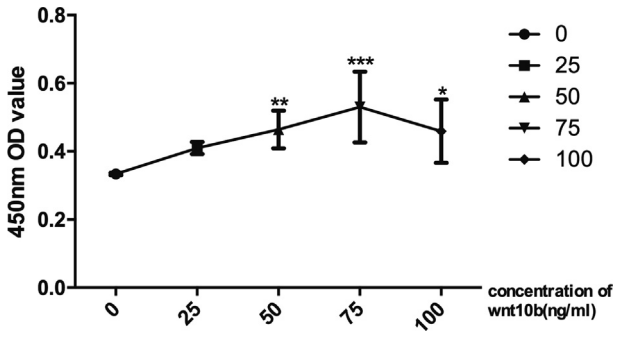

Fig. 3. The influence of WNT10B on proliferation of OCCM-30 cells at 48 h. CCK-8 test showed that 50 ng/ml, 75 ng/ml and 100 ng/ml WNT10B could promote the viability of OCCM-30 cells, while 25 ng/ml WNT10B could not, and there was no significant difference in the condition of proliferation among the three groups. Experiment was performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (*P < 0.05, **P < 0.01, ***P < 0.005).

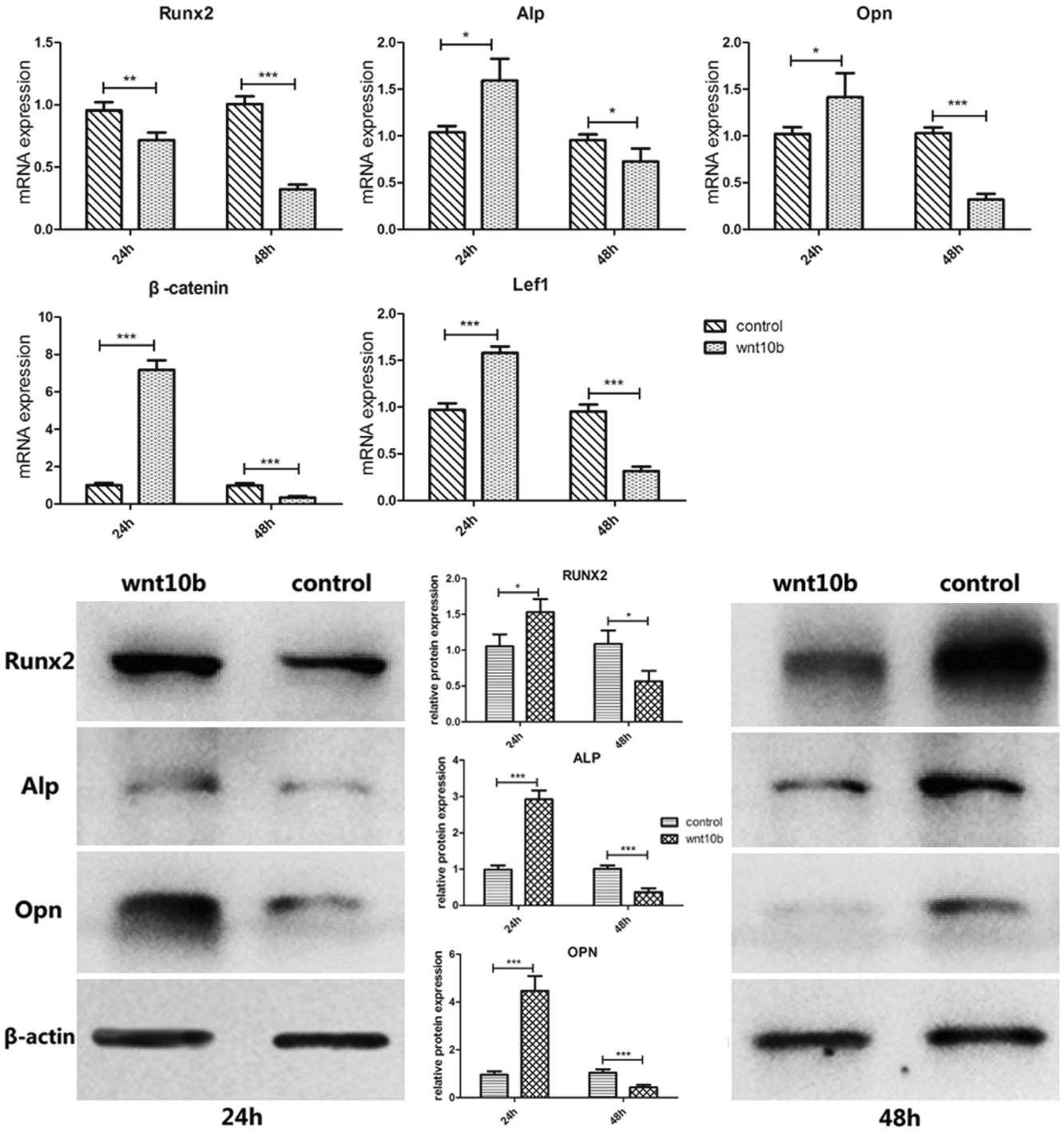

Fig. 4. The influence of WNT10B on differentiation of OCCM-30 cells. In the first 24 h, the gene expression of *Alp*, *Opn*, *β-Catenin* and *Lef-1* was increased in wnt10b group, while the gene expression of *Runx2* was decreased. However, the results at following 24 h were different. The proteins expression had a similar trend by Western Blot. All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (*P < 0.05, **P < 0.01, ***P < 0.005).

were presented in Fig. 6. ALP staining showed an increased ALP activity in wnt10b group. There were significantly more mineralized nodules in wnt10b group and the nodules were bigger when compared with control group, and the OD value of dissolved nodules was significantly higher (nearly 1.46- fold) in wnt10b group Both of them indicated that WNT10B could promote the mineralization of OCCM-30 cells.

4. Discussion

As a crucial regulator of the immune system, T cells, especially of the produced cytokines, were usually reported to be a stimulator of bone resorption [17,18,19], and some findings suggested that immune response also regulated osteoblastic bone formation [20]. In the present study, we provided evidence that $CD8^+$ T cells could promote the cementogenesis effect of iPTH on cementoblasts in a short period time (48 h) through establishing a cell coculture system in vitro, and showed the potential link with increased expression of *Wnt10b*. First, compared with monoculture of OCCM-30 cells, the cementogenesis effect of iPTH was enhanced in the $CD8^+$ T cells-OCCM-30 cells coculture system, on both the expression of related gene and protein. Second, iPTH could

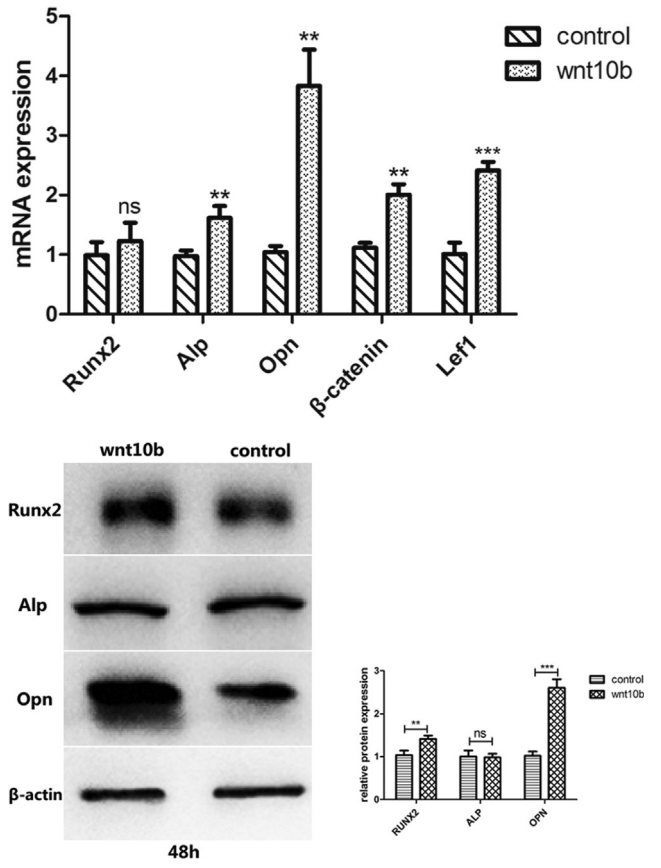

Fig. 5. The influence of WNT10B on differentiation of OCCM-30 cells. When the medium was changed every day, the gene and protein expression at 48 h showed a significant increase trend in wnt10b group. All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (ns = no significance, **P < 0.01,***P < 0.005).

increase the expression of *Wnt10b* in $CD8^+$ T cells, thus the secreted WNT10B from $CD8^+$ T cells in inserts increased the concentration of WNT10B of the culture medium of OCCM-30 cells in wells. Third, WNT10B could promote the cementogenesis process of cementoblasts, especially in a culture medium of differentiation induction. These results demonstrated that the stimulating effect of iPTH on cementoblasts could be mediated through an interaction of $CD8^+$ T cells and cementoblasts, and T-cell-induced *Wnt10b* might be a key mechanism in the mediation.

Many studies showed PTH played an important role in bone homeostasis. However, studies showed that nude mice deficient of T cells were protected from the catabolic effects of hyperparathyroidism [21,22], whereas T cell deficient mice exhibited a blunted anabolic response to iPTH [11]. These reports indicated the potential effect of celluar interaction between bone cells and T cells on the response to stimulation of PTH. Although there was research showed T cells contribute to the actions of PTH on some cells, such as osteoblasts, hPDLs and hemopoietic stem cells [22], the effects of PTH in other cells might be T cell-independent.

Research previously identified that iPTH could promote the cementogenesis process [6,23,24]. Review of literature implied that T cells might mediate the cementogenesis induced by iPTH. Therefore, the lack of information on whether T cells contribute to the influence of iPTH on cementoblasts prompted the present study. In this study, a $CD8^+$ T cells-OCCM-30 cells coculture system was established to investigate the role of T cells and the potential mechanism. Although cementoblasts are unique phenotypes [3], they share many similar characteristics including gene expression profiles and cell morphologies [6]. Consistent to the previous studies on the interaction of $CD8^+$ T cells and osteoblasts/hPDLs, $CD8^+$ T cells could enhance the cementogenesis effect of iPTH on OCCM-30 cells. The expression of skeletogenic markers (*Alp* and *Opn*) and osteogenic transcription factor *Runx2* was increased in the coculture system during 48 h treatment of iPTH.

Previous studies [11,12] identified iPTH could increase the expression of *Wnt10b* in $CD8^+$ T cells in vitro, however, since the different dose and time of exposure to PTH, we still examined the expression of *Wnt10b* in the present study. Our results were consistent with the previous studies, iPTH promoted the expression of *Wnt10b* in $CD8^+$ T cells.

Wnt10b could activate canonical Wnt/β-catenin signaling and increase osteoblastogenesis [25,26]. However, previous studies showed Wnt signaling inhibited cementoblast differentiation [15,26]. Given the results showed $CD8^+$ T cells could enhance the cementogenesis effect of iPTH on OCCM-30 cells, we investigated the effect of *Wnt10b* on OCCM-30 cells. In the present study, *Wnt10b* at a concentration of 50 ng/ml could promote the proliferation of OCCM-30 cells in 24 h, and increase the expression of *Alp*, *Opn*,*β-Catenin* and *Lef1* in OCCM-30 cells in the first 24 h, and decreased the expression of related gene during the following 24 h. As for *Runx2*, RT-PCR showed a decreased gene expression while Western Blot indicated an increased protein expression at 24 h, this might be a result of post-transcriptional modification. This result at 48 h was basically consistent with the previous studies [12,15], which indicated Wnt signaling inhibited cementoblasts differentiation and promoted proliferation. However, the result at 24 h indicated WNT10B could promote cementogenesis in a short reaction time. The expression of *β-Catenin* and *Lef1* at 48 h decreased which indicated there might be other signaling mediated by *Wnt10b*, thus the reduction might be a comprehensively result.

In addition, we found WNT10B could promote cementoblasts differentiation and mineralization when the culture medium was changed everyday or in an osteogenic medium, which was different from the results reported by Cao et al. [27]. We thought both of the results were reasonable. There were some factors might lead to this controversy. Firstly, it was reported that LiCl and *Wnt3a* might target different genes, and in the present study, we used recombined *Wnt10b* protein to stimulate OCCM-30 cells, while Cao and colleagues used LiCl [15,28]. Secondly, although both *Wnt10b* and *Wnt3a* were canonical Wnt ligands, there was some difference between them. For example, they bound to different domains of LRP5/6 [29,30]. In U2OS osteosarcoma cells, different from *Wnt3a* which was Wnt-specificity, *Wnt10b* could stimulate the Notch pathways [31], which was identified to affect cementogenesis [32]. Thirdly, different concentration and frequency of stimulation could bring different effect. The results in our study indicated when OCCM-30 were exposed in *Wnt10b* 24 h/24 h (results at 24 h and 48 h when changed medium everyday), it could promote cementogenesis, whereas it would decrease the differentiation of cementoblasts in an environment of *Wnt10b* 24 h/48 h (results at 48 h when changed medium every two days). In addition, it was indicated that Dexamethasone (Dex) induced mesenchymal cell differentiation could be mediated by activating Wnt/β-catenin signaling [33,34], and a level of Dex similar to 10 nM is the optimal concentration for mineralized nodule formation in vitro [35]. In the present study, we used 10 nM Dex rather than 100 nM which used in Cao's study, and recombined *Wnt10b* protein might directly enhance the cementogenesis effect of Dex in an osteogenic medium. Therefore, *Wnt10b* could increase the proliferation of OCCM-30 cells and in an osteogenic medium it could promote the differentiation and mineralization. Nevertheless, *Wnt10b* produced in the coculture system could not be quite the same with recombined protein, both in the concentration and the stimulation time, thus it could only be suggested $CD8^+$ T cells might enhance the anabolic effect of iPTH via *Wnt10b*, more precise evidence should be collected from further study, such as experiments in knock-out mice model.

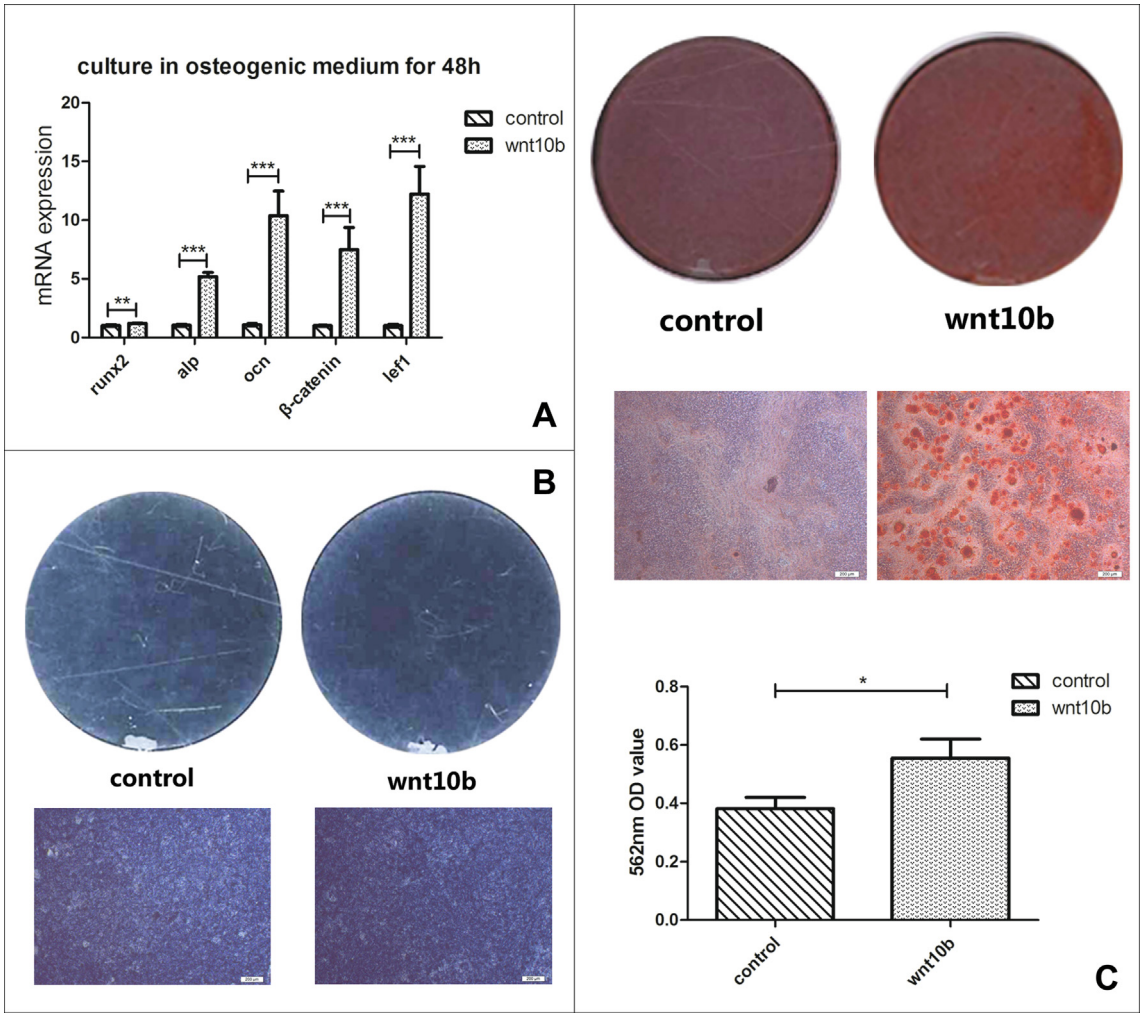

Fig. 6. The influence of WNT10B on mineralization of OCCM-30 cells. A. When cultured in osteogenic medium for 48 h, the gene expression of *Runx2*, *Alp*, *Opn*, *β-Catenin* and *Lef-1* was significantly increased in wnt10b group. B. After stimulated by WNT10B for 7d, ALP staining was conducted. It showed an enhanced ALP activity in the wnt10b group. C. After treatment with WNT10B for 14d, Alizarin Red S stain was performed. Enhanced mineralization was observable in wnt10b group. All experiments were performed three times and the results were presented as the mean ± standard deviation (Mean ± SD). (Microscopic sale bar:200um. ns = no significance, *P < 0.05,**P < 0.01,***P < 0.005).

5. Conclusions

This in-vitro study investigated the role of T cells in the stimulating effect of iPTH on cementoblasts. The results showed the anabolic effect of iPTH could be regulated through an interaction of $CD8^+$ T cells and cementoblasts, and T-cell-induced *Wnt10b* might be a key mechanism underlying the mediating process.

Declaration of Competing Interest

The authors declare no conflict of interest.

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