Am J Transl Res 2020;12(5):1976-1984
www.ajtr.org /ISSN:1943-8141/AJTR0102424

Original Article

Effects of the multifunctional hormone leptin on orthodontic tooth movement in rats

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Received September 17, 2019; Accepted April 10, 2020; Epub May 15, 2020; Published May 30, 2020

Abstract: This study aims to investigate the effects of leptin, which is a multifunctional hormone, on orthodontic tooth movement (OTM) and the underlying mechanism. Sprague-Dawley rat OTM models were established and divided into two groups with the administration of vehicle or leptin respectively. Stereomicroscope and microcomputed tomography were used to evaluate the amount of OTM. TRAP staining, immunohistochemical and immunofluorescence staining were used to detect osteoclasts and relative protein expressions. After treated with compression force, human periodontal ligament cells (hPDLCs) were co-cultured with human peripheral blood mononuclear cells (hPBMCs) with the presence or absence of leptin. Small interfering RNA (siRNA) was transfected to knock down the leptin receptor (LepR). The mRNA expressions of the targeted genes were evaluated by quantitative real-time polymerase chain reaction. We found that leptin receptors were expressed on both rat periodontal ligament cells and hPDLCs. OTM was significantly attenuated in the leptin-treated group comparing to the control group. The number of osteoclasts was reduced in the periodontal ligament tissues *in vivo* and *in vitro* co-cultured system when treated with leptin. The expression of RANKL was inhibited by leptin administration either *in vivo* and *in vitro*. Leptin administration also inhibited the force-induced up-regulation of RANKL expression in hPDLCs, which was rescued by LepR siRNA transfection. The osteoclastogenesis was attenuated by leptin administration which was reversed by the LepR siRNA transfection. Taken together, leptin was able to attenuate OTM by inhibiting osteoclastogenesis which can be attributed to the reduced expression of RANKL in the periodontal ligament. Leptin may possess the potential for reinforcing anchorage clinically.

Keywords: Leptin, orthodontic tooth movement, osteoclastogenesis, rat

Introduction

There has been a dramatic increase in the incidence of obesity and overweight worldwide [1, 2] and among those patients with high body mass index (BMI), the circulating leptin was found elevated [3]. Leptin identified as a 167 amino-acid peptide, was mainly produced by adipose tissues [4]. Leptin is revealed as a multifunctional hormone and played an important role in regulating food intake, energy expenditure, neuroendocrine and immune function [5, 6]. Moreover, in recent years, it was found that leptin could influence bone metabolism either through the hypothalamus [7, 8] or directly to the bone peripherally [9]. However, the effects of leptin on bone metabolism remained controversial [10].

Bone metabolism which involves bone formation and bone resorption, is the basis of orthodontic tooth movement (OTM). During this process, constant force applied to the periodontal ligament (PDL) would induce osteoclasts accumulation at the compression side and give rise to bone remodeling [11]. Recent studies suggested that OTM was not only a local biological reaction within the periodontal tissue but also regulated by systemic reactions, such as the immune system and sympathetic nervous system [12-15]. Furthermore, recent clinical studies have identified that the tooth movement rate varied between high BMI and normal-weight patients with a different salivary level of leptin [16, 17]. Previously we showed that the serum level of leptin was elevated in high-fat induced obese mice which may affect osteo-

clastogenesis [18]. However, the effects of leptin on OTM and the underlying mechanism remains to be investigated. Therefore, in this study we aimed to determine whether leptin can affect OTM in a rat tooth movement model and tried to reveal the underlying mechanisms.

Materials and methods

Animals and experimental tooth movement

Sixteen 6 to 8 week-old rats (Weitong Lihua Experimental Animal Center, Beijing, China) were purchased and housed in cages under certain conditions of temperature (23°C ± 1°C), humidity (50% ± 10%), and a 12:12-hour light-dark cycle. The experimental animals were provided with water and standard laboratory chow. Orthodontic nickel-titanium coiled springs (0.2 mm in thickness, 1 mm in diameter, 10 mm in length; Smart Technology, Beijing, China) were ligated between the left maxillary first molar and central incisors of the rats. 60 N of orthodontic force was delivered by the activated springs (**Figure 2A**). Leptin (20 μg/100 g, PeproTech, USA) or vehicle (PBS) were injected intraperitoneally every other day since one day before force application. Rats were sacrificed by pentobarbital sodium overdose. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University (LA2013-92).

Measurement of OTM and MicroCT analysis

We used a stereomicroscope (SWZ1000, Nikon, Japan) to take photo of the occlusal surface of the maxillae. The OTM distance was measured between the midpoint of the distal-marginal ridge of the first molar and the midpoint of the mesial-marginal ridge of the second molar. Then the measurement of OTM distance was carried out by using Image J (version 1.37) software (National Institutes of Health, USA).

A micro-computed tomography (MicroCT, Skyscan1174, Bruker micro CT, Belgium) was used to scan the maxillae of the rats. To analyze the bone mass indicators, we chose the furcation of the first molar as the measurement area due to its reproducible morphological landmarks.

Tartrate-resistant acid phosphatase staining

After collection and fixation with 4% paraformaldehyde, rat maxillae tissues were demineralized in 15% EDTA and embedded in paraffin. The transverse cutting method is employed to get serial sections from the corresponding group at a thickness of 4 μm. Then the sections were deparaffinized and performed tartrate-resistant acid phosphatase (TRAP) with a leukocyte acid phosphatase kit (387A, Sigma, USA) according to the manufacturer's protocol. The TRAP- positive multinucleated (> 3 nuclei) cells that were attached to the alveolar bone surface mesial to the middle palatal roots were counted.

Human periodontal ligament cells (hPDLCs) culture and treatment

The protocols were performed with appropriate informed consent (PKUSSIRB-201311103). Human periodontal ligament cells (hPDLCs) were isolated from PDL of normal orthodontic extracted bicuspids. The hPDLCs from 3 different individuals were pooled together and used in this study with the 4th to 6th passages. Cell suspensions (1×10^4 cells) were seeded in 10 cm diameter culture plates (Costar, Cambridge, MA, USA). The seeded cells were cultured with α-MEM medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 15% fetal calf serum (Equitech-Bio Inc, Kerrville, TX, USA) and incubated at 37°C supplied with 5% CO_2. Mechanical compression stress was applied to the hPDLCs as previously reported [19]. Briefly, a glass coverslip was placed on the 80%-confluent cell layer in 6-well plates (**Figure 4A**). Metal weights were subsequently placed over the coverslip to exert continuous compressive stress of 1 g/cm^2 on the hPDLCs for 24 h with the presence or absence of leptin (100 ng/ml). To knock down leptin receptor (LepR) expression, hPDLCs were transfected with specific small interfering RNA (siRNA) targeting at LepR and scramble siRNA (Santa Cruz Biotechnology, USA) for 48 hours before treated with leptin (Figure S1).

Peripheral blood mononuclear cells and hPDLCs co-culture

The human peripheral blood mononuclear cells were isolated (National Infrastructure of Cell Line Resource, Beijing, China) and co-cultured

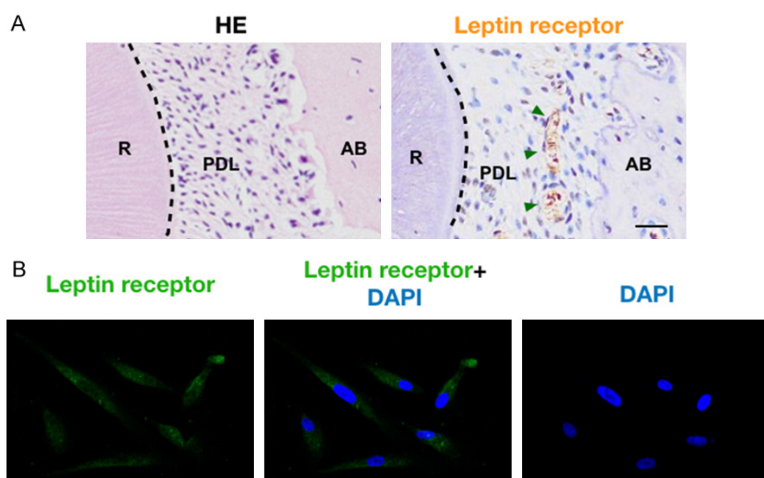

Figure 1. The expression of leptin receptor on periodontal ligament (PDL). A. Immunohistochemical staining showed that leptin receptor was expressed on PDL in rats. B. Confocal microscopy images showed that the expression of leptin receptor on human PDL. Scale bar, 50 μm.

with hPDLCs in α-MEM containing 10% FBS supplemented with antibiotics (100 u/ml of penicillin and 100 ug/ml of streptomycin). Cells were cultured at 37°C in a 5% CO_2 incubator. Osteoclasts differentiation was induced by M-CSF and sRANKL (R&D Systems, USA) at concentration of 10 ng/ml in the presence or absence of leptin (100 ng/ml, PeproTech, USA) for 21 days. Immunofluorescence Staining.

Cells were incubated with primary antibodies against LepR or RANKL (LepR, 1:100, RANKL, 1:100, Santa Cruz, USA) to detect the leptin receptor or RANKL expression on PDLCs and then washed, incubated with FITC-conjugated secondary antibodies (Zhongshan Golden Bridge Biotechnology, Beijing, China). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopic images were processed with LSM 5 Release 4.2 soft- ware after acquisition by a laser-scanning microscope (LSM 510; Zeiss, Germany).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from cell lysate the with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription and real-time PCR were performed following previously described protocol [20]. Briefly, the sequences of primers were from previously published reports [14] and commercially synthesized are listed below: human RANKL sence/antisence: 5'-AGAGCGCAGATGGATCCTAA-3'/5'-TTCCTTTTGCACAGCTCCTT-3'; human OPG sence/antisence: 5'-GGAACCCCA GAGCGAAATACA-3'/5'-CCTGAAGAATGCCTCCTCACA-3'; human GAPDH sence/antisence: 5'-ATGGGGAAGGTGA AGGTCG-3'/5'-ATGGGGAAGGTGAAGGTCG-3'; human PPAR-γ sence/antisence: 5'-CTCCTATTGACCCAGAAAGC-3'/5'-GTAGAGCTGAGTCTTCTC G-3'; human GSK-3β sence/antisence: 5'-TTGAAAATCCAGCGTGGACA-3'/5'-TCGAGTCATTGCATACTGTC-3'.

Statistical analysis

All data are presented as the means ± SD, and statistical analysis was carried out by T-test or one-way ANOVA (Tukey multiple comparison test). All statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA); $P<0.05$ was considered to be significant.

Results

Periodontal ligament cells express leptin receptors

In order to determine whether leptin is involved during the OTM process, we tested the expression of leptin receptors on both the rat and human PDL. Immunohistochemical staining showed that leptin receptor was expressed on PDL tissue in rats (**Figure 1A**), and immunofluorescence staining showed that leptin receptor was also expressed on hPDLCs (**Figure 1B**). These data suggests that leptin could participated the process of OTM.

Administration of leptin attenuated orthodontic tooth movement along with reduction of osteoclastogenesis in rats

In order to evaluate the effects of leptin on OTM, we administrated leptin to rats every other day before force application (**Figure 2B**). After 7 days of force application, intra-oral photographs showed that the distance between first (M1) and second (M2) molars in the leptin treated group were reduced when compared to the control group. To further confirm these find-

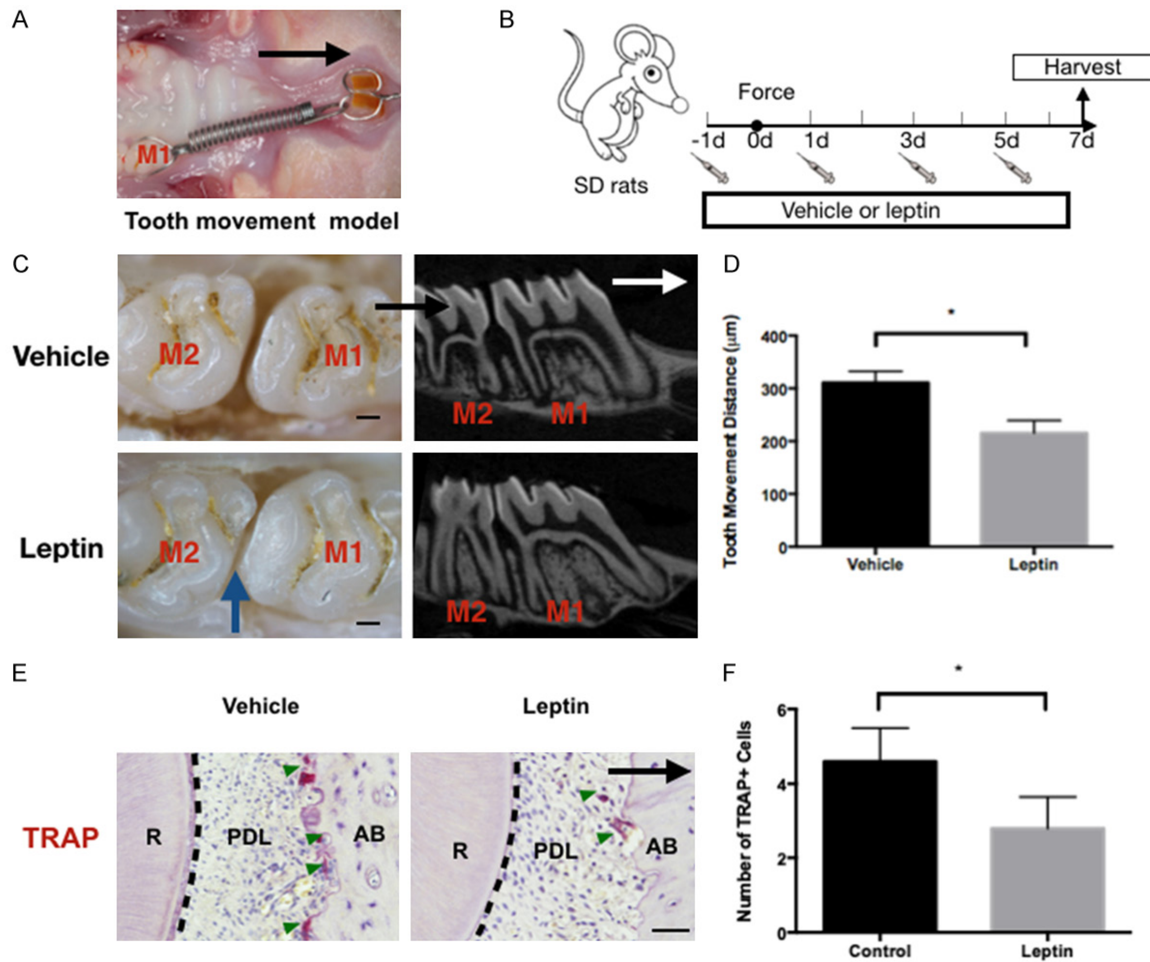

Figure 2. Effects of leptin on OTM in rats. A. The OTM model in rat. B. The schedule of the experiment. C and D. Stereomicroscope and MicroCT representative images of tooth movement after 7 days of force application. The arrows indicated the direction of tooth movement. The amount of tooth was significantly attenuated after 7 days of force application in rats treated with leptin when comparing to the control group. E and F. TRAP staining for osteoclasts on histological slides indicated that the number of osteoclasts in rats treated with leptin was significantly reduced after 7 days of force application when compared to the vehicle-treated group. M1: first molar. M2: second molar; n=5, error bars, means ± SD, n=5; scale bar: 500 μm; *denotes significant difference, $P<0.05$.

ings, microCT scans were introduced to measure the distance between M1 and M2. We found that the distances between M1 and M2 were decreased in leptin treated group when comparing to the control group (**Figure 2C** and **2D**). These data revealed that OTM rate was significantly decreased by the leptin administration. Since osteoclasts played a crucial role in OTM, we then investigated whether osteoclastogenesis was affected in the leptin administration group. TRAP staining showed that the number of TRAP$^+$ cells was reduced in the leptin-treated group when compared to the control group (**Figure 2E** and **2F**). These results indicated that leptin administration was able to attenuate OTM along with the reduction of osteoclasts.

Leptin reduced the RANKL expression

RANKL produced by PDL tissues plays an essential role of osteoclastogenesis in the process of OTM [11], therefore, we tested whether the RANKL expression was affected by leptin administration. The immunohistochemical staining showed that the expression of RANKL was reduced in the leptin administration group after 7 days of force application when comparing to the control group (**Figure 3A** and **3B**).

Leptin reduced the RANKL expression and RANKL/OPG ratio through leptin receptor

To further confirm that leptin could directly downregulate the RANKL expression in the PDL

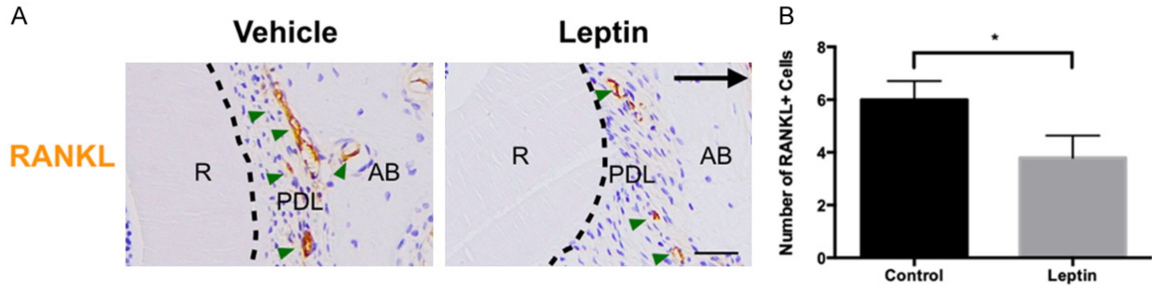

Figure 3. The RANKL expression was inhibited by leptin in rats. A. Immunochemical staining for RANKL on histological slides. B. The number of RANKL positive cells was significantly reduced by leptin administration. *denotes significant difference, $P<0.05$.

A
compressive force
Cover glass
Culture medium
hPDL

B
Vehicle
Leptin
Leptin+LepR siRNA
DAPI+RANKL

C
Fold induction of RANKL mRNA
Force
Leptin
Leptin+LepR siRNA

D
Fold induction of RANKL/OPG mRNA
Force
Leptin
Leptin+LepR siRNA

E
Fold induction of PPAR-γ mRNA
NS
Force
Leptin
Leptin+LepR siRNA

F
Fold induction of GSK-3β mRNA
NS
Force
Leptin
Leptin+LepR siRNA

Figure 4. The RANKL expression was downregulation by leptin in human periodontal ligament cells with force application. A. Schema of the *in-vitro* compression force application to hPDLCs. B. Immunofluorescence staining showed that showed that number of the RANKL positive cells was reduced by leptin administration and reversed by LepR siRNA transfection. C. Quantitative PCR results showed that the expression of RANKL mRNA was significantly elevated after compression force application and reduced by leptin administration which was reversed by LepR siRNA transfection. D. Quantitative PCR results showed that administration of leptin to hPDLCs was able to significantly reduce RANKL/OPG ratio under compression which was reversed by LepR transfection. E. Quantitative PCR results showed that the expression of PPAR-γ mRNA was significantly inhibited by leptin which was reversed by LepR siRNA transfection. F. Quantitative PCR results showed that the expression of GSK-3β mRNA was significantly inhibited by leptin which was reversed by LepR siRNA transfection. **denotes significant difference, $P<0.01$; *denotes significant difference, $P<0.05$; NS denotes no statistical significance; scale bar: 20 μm.

cells, we cultured hPDLCs *in vitro* and loaded the compression force onto these cells for further experiments. The schema showed that static compression force was applied to hPDLCs (**Figure 4A**). The immunofluorescence staining showed that the RANKL expression in hPDLCs with compression force application was inhibited by the leptin administration, which was partially rescued by LepR siRNA transfection (**Figure 4B**). Moreover, real-time PCR results showed that the RANKL expression and was significantly elevated with compression force application, which was reduced by leptin administration. However, after the transfection of LepR siRNA, the RANKL expression level in hPDLCs was partially rescued when treated by leptin under the loading condition (**Figure 4C**). The real-time PCR results also showed the elevation of RANKL/OPG ratio after force application was significantly reduced by the leptin administration which was rescued by LepR siRNA transfection (**Figure 4D**). These data suggested that, leptin could downregulate RANKL expression through the leptin receptor in the hPDLCs. Moreover, RANKL/OPG ratio, which determined the osteoclasts activation decrease significantly.

PPAR-γ and Wnt signaling were reported to regulate the RANKL expression [21, 22], and we evaluated whether the administration of leptin could affect PPAR-γ expression and Wnt signaling. We found that there was a trend of up-regulation of PPAR-γ expression with force application, which was inhibited by leptin administration and rescued by LepR siRNA (**Figure 4E**). Moreover, there was a down-regulated trend of GSK-3β by leptin administration, which was also reversed by LepR siRNA treatment (**Figure 4F**). These data indicated that PPAR-γ and Wnt signaling might be affected by leptin to contribute to the RANKL regulation.

Leptin attenuated osteoclastogenesis through leptin receptor

Next, we tried to confirm that leptin administration was not only able to decrease the expression of RANKL but can also affect the osteocalstogenesis *in vitro*. Thus, we co-cultured hPBMCs with hPDLCs in the osteoclastogenesis induction medium and explore whether leptin administration could inhibit osteoclastogenesis. After 21 days of induction, TRAP staining showed that leptin administration could decrease the osteoclasts numbers. However, LepR siRNA transfection could partially rescue the induction osteoclasts numbers. These data suggested that leptin could inhibit hPDLCs inducted osteoclasts activation (**Figure 5A** and **5B**).

Discussion

With the increasing prevalence of obesity and overweight, the chances of high-BMI patients encountered in orthodontic clinics are rising. A previous clinical study has suggested that increased BMI may exert influences on OTM [23]. More interestingly, previous clinical studies also indicated that there was a significant difference in the salivary leptin level between normal weight and high BMI patients [16, 17]. Leptin was first discovered to have role in regulating food intake and energy expenditure through the neuroendocrine pathways [6]. In recent decades, the other effects of leptin, including the bone effects, were uncovered. Leptin was reported could reduce bone mass through the hypothalamus [7]. However, this phenotype was challenged in these years, since leptin was mainly produced by the adipose tissue, and could pass through the blood brain barrier. Leptin could directly upregulate the bone mass, which indicated that the peripheral pathway might play the leading role [9]. Whe-

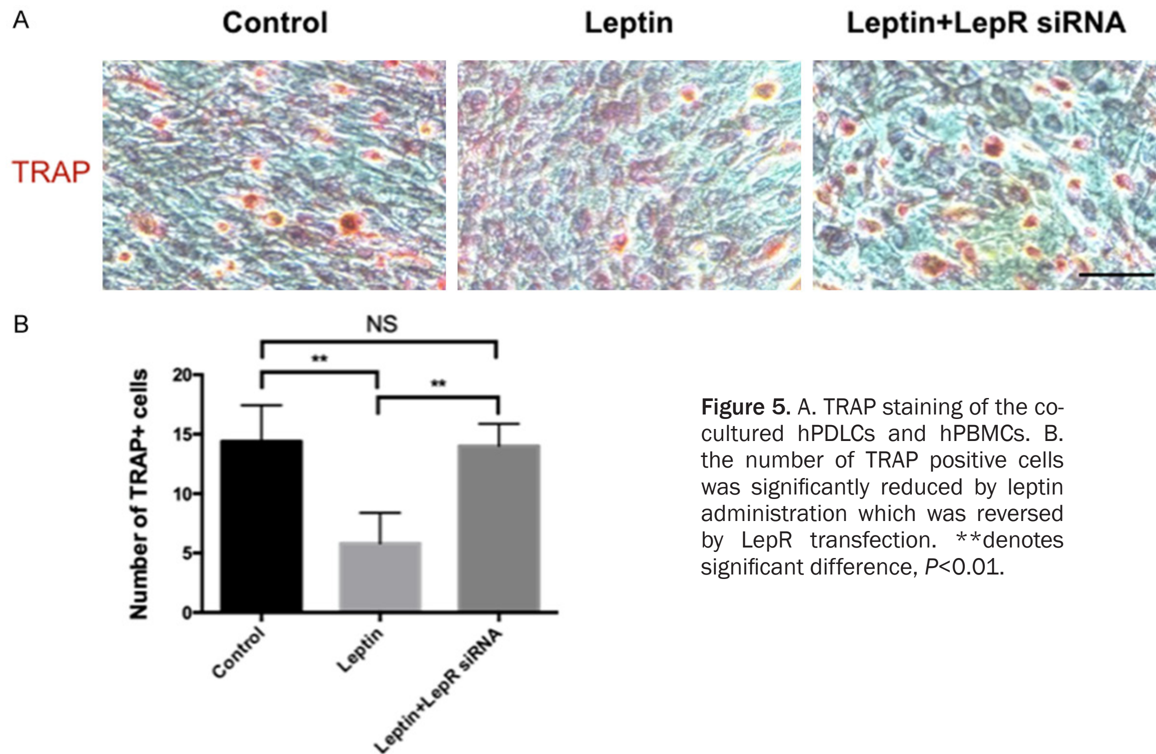

Figure 5. A. TRAP staining of the co-cultured hPDLCs and hPBMCs. B. the number of TRAP positive cells was significantly reduced by leptin administration which was reversed by LepR transfection. **denotes significant difference, *P*<0.01.

ther leptin can affect OTM remains to be verified.

In this study, we first investigated the effects of leptin on OTM in a rat tooth movement model. We found that OTM would be attenuated in rats when treated by leptin. Then we confirmed that the leptin receptors were expressed on both rat and human PDL cells, which suggested that leptin was able to act directly to PDL to exert its biological effects. Next, we tested whether the osteoclastogenesis, which plays a critical role in OTM, was affected by leptin administration. We found that the number of osteoclasts was reduced in the leptin-treated rats comparing to the control group. Leptin administration was also able to inhibit osteoclastogenesis in hPDLCs and hPBMCs co-culture system. These findings were consistent with previously published results [24, 25]. RANKL and OPG were critical regulators of osteoclastogenesis in the process of OTM [11], we found that leptin was able to inhibit RANKL expression either *in vivo* or *in vitro*, which could be the mechanism of the attenuated OTM caused by leptin. Previous studies showed that PPAR-γ and Wnt signaling played an important role in the regulation of osteoclastogenesis [26, 27], and may participate in the regulation of RANKL signaling [21], we next tried to evaluate the effects of leptin on PPAR-γ and Wnt signaling in hPDLCs. We found that PPAR-γ and GSK-3β expression was down-regulated by leptin administration, which was reversed by LepR siRNA interference. These data suggested that PPAR-γ and Wnt signaling may be involved in the process of leptin regulating OTM. However, the underlying mechanisms remains to be elucidated.

Unwanted tooth movement, such as unexpected posterior tooth mesial movement, the so-called anchorage loss, was encountered frequently in clinical situations, enforcing anchorage remains an essential issue. For instance, administration of bisphosphonate was considered to be an effective way to reinforce anchorage, yet some adverse events such as osteonecrosis may occur [28]. Unlike other chemical drugs, leptin is a natural hormone produced by adipose tissues, which are rather safe with rare adverse effects, which may possess the potential to serve as a method for reinforcing anchorage.

Conclusion

In summary, we identified that leptin, a multifunctional hormone was able to attenuate OTM

by inhibiting osteoclastogenesis by down-regulation of the RANKL expression through the leptin receptor and possess the potential serving as an enforcing anchorage method.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81970909, D.L.), (No. 51903003, T.Y.), (No. 81470717, Y.Z.), (No. 81600893, D.H.), (No. 81600865, R.Y.), (No. 81571815, Y.L.) and (No. 81871492, Y.L); The Capital Health Research and Development of Special (No. 2016-1-4102, Y.Z.); The Young Scientists Fund of PKUSS (No. 20110203, No. 20170109, D.L.); Peking University Medicine Seed Fund for Interdisciplinary Research (No. BMU2018MX007, D.L.); The Beijing Natural Science Foundation (No. 7182182, R.Y.); the Beijing New-star Plan of Science and Technology (No. Z171100001117018, Y.L.); the Beijing Nova Programm Interdisciplinary Cooperation Project (No. Z181100006218135, Y.L.); The Young Elite Scientist Sponsorship Program by CAST (YESS, 2017QNRC001, R.Y).

Disclosure of conflict of interest

None.

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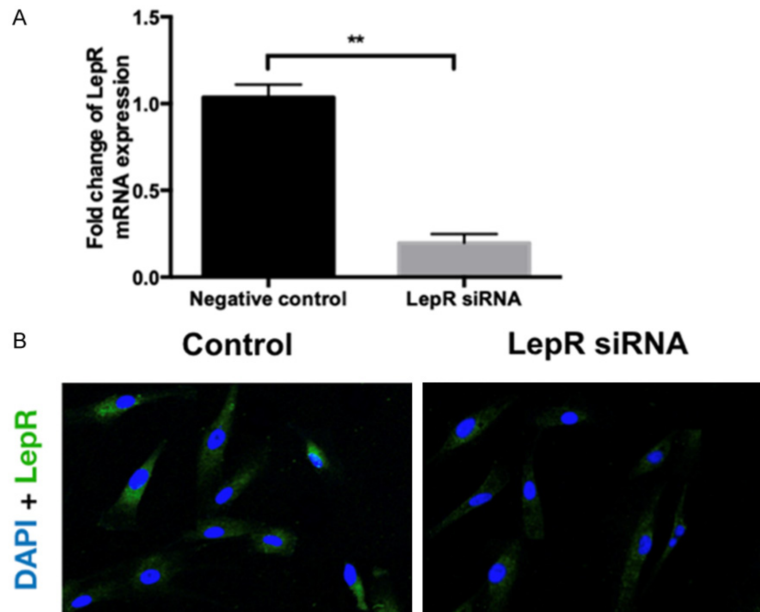

Figure S1. The leptin receptor was knocked-down by the LepR siRNA. A. Quantitative PCR results showed LepR expression was significantly reduced by LepR siRNA transfection. B. Immunofluorescence staining showed that the number of LepR positive cells was reduced by LepR siRNA transfection. **denotes significant difference, *P*<0.01.