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Name(s) of Principal Investigator(s) **Sumit Yadav**

Institution: **University of Connecticut Health Center**

Title of Project: **PTH Mediated regulation of the Chondrogenic Lineage in the Mandibular Condyalr Cartilage**

Period of AAOF Support (e.g. 07-01-18 to 06-30-19): 07-01-17 to 12-31-18

Amount of Funding: **$30,000**

Summary/Abstract :

Objective: To characterize the long-term effects of intermittent Parathyroid Hormone (I-PTH) on the mandibular condylar cartilage (MCC) and subchondral bone of the temporomandibular joint (TMJ), in vivo and in vitro. Materials and Methods: For the in vivo experiments, 16 10-week-old mice were divided into two groups: (1) I-PTH (n=8): subcutaneous daily injection of PTH; (2) Control Group (n=8): subcutaneous daily injection of saline solution. Experiments were carried out for four weeks. Mice were injected with calcein, alizarin complexone and cell proliferation marker before euthanasia. For the in vitro experiments, primary chondrocyte cultures from the MCC of eight 10-week-old mice were treated with I-PTH for 14 days. Results: There was a significant increase in bone volume, tissue density, mineral deposition, osteoclastic activity, cell proliferation in the cartilage and cartilage thickness in the I-PTH treated mice when

compared to control group. In addition, immunohistochemistry in cartilage revealed that I-PTH administration led to a increase in expression of Vascular Endothelial Growth Factor (VEGF) and to a decreased expression of Sclerostin (SOST), Matrix Metallopeptidase 13 (MMP13) and Aggreganase-1 (ADAM-TS4). Quantitative Polymerase Chain Reaction (QPCR) analysis of the I-PTH treated chondrocytes revealed significantly decreased relative expression of Collagen Type X (Col10a1), Alkaline Phosphatase (Alp) and Indian Hedgehog (Ihh) and remarkable increased expression of Sox9, Fibroblast Growth Factor 2 (Fgf2) and Proteoglycan 4 (Prg4). Conclusion: I-PTH administration causes anabolic effects at the subchondral region of the mandibular condyle while triggers anabolic and protective effects at the MCC.

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Cartilage

Intermittent PTH (1-34) augments chondrogenesis of the mandibular condylar cartilage of the Temporomandibular Joint

Journal:	*Cartilage*
Manuscript ID	CART-18-0129.R1
Manuscript Type:	Original Article
Keywords:	Mandibular Condylar Cartilage, Temporomandibular Joint, Parathyroid Hormone
Abstract:	Objective: To characterize the long-term effects of intermittent Parathyroid Hormone (I-PTH) on the mandibular condylar cartilage (MCC) and subchondral bone of the temporomandibular joint (TMJ), in vivo and in vitro. Materials and Methods: For the in vivo experiments, 16 10-week-old mice were divided into two groups: (1) I-PTH (n=8): subcutaneous daily injection of PTH; (2) Control Group (n=8): subcutaneous daily injection of saline solution. Experiments were carried out for four weeks. Mice were injected with calcein, alizarin complexone and cell proliferation marker before euthanasia. For the in vitro experiments, primary chondrocyte cultures from the MCC of eight 10-week-old mice were treated with I-PTH for 14 days. Results: There was a significant increase in bone volume, tissue density, mineral deposition, osteoclastic activity, cell proliferation in the cartilage and cartilage thickness in the I-PTH treated mice when compared to control group. In addition, immunohistochemistry in cartilage revealed that I-PTH administration led to a increase in expression of Vascular Endothelial Growth Factor (VEGF) and to a decreased expression of Sclerostin (SOST), Matrix Metallopeptidase 13 (MMP13) and Aggreganase-1 (ADAM-TS4). Quantitative Polymerase Chain Reaction (QPCR) analysis of the I-PTH treated chondrocytes revealed significantly decreased relative expression of Collagen Type X (Col10a1), Alkaline Phosphatase (Alp) and Indian Hedgehog (Ihh) and remarkable increased expression of Sox9, Fibroblast Growth Factor 2 (Fgf2) and Proteoglycan 4 (Prg4). Conclusion: I-PTH administration causes anabolic effects at the subchondral region of the mandibular condyle while triggers anabolic and protective effects at the MCC.

Intermittent PTH (1-34) augments chondrogenesis of the mandibular condylar cartilage of the Temporomandibular Joint

Abstract

Objective: To characterize the long-term effects of intermittent Parathyroid Hormone (I-PTH) on the mandibular condylar cartilage (MCC) and subchondral bone of the temporomandibular joint (TMJ), *in vivo* and *in vitro*. **Materials and Methods:** For the *in vivo* experiments, 16 10-week-old mice were divided into two groups: (1) I-PTH (n=8): subcutaneous daily injection of PTH; (2) Control Group (n=8): subcutaneous daily injection of saline solution. Experiments were carried out for four weeks. Mice were injected with calcein, alizarin complexone and cell proliferation marker before euthanasia. For the *in vitro* experiments, primary chondrocyte cultures from the MCC of eight 10-week-old mice were treated with I-PTH for 14 days. **Results:** There was a significant increase in bone volume, tissue density, mineral deposition, osteoclastic activity, cell proliferation in the cartilage and cartilage thickness in the I-PTH treated mice when compared to control group. In addition, immunohistochemistry in cartilage revealed that I-PTH administration led to a increase in expression of Vascular Endothelial Growth Factor (VEGF) and to a decreased expression of Sclerostin (SOST), Matrix Metallopeptidase 13 (MMP13) and Aggreganase-1 (ADAM-TS4). Quantitative Polymerase Chain Reaction (QPCR) analysis of the I-PTH treated chondrocytes revealed significantly decreased relative expression of Collagen Type X (*Col10a1)*, Alkaline Phosphatase (*Alp)* and Indian Hedgehog (*Ihh*) and remarkable increased expression of *Sox9*, Fibroblast Growth Factor 2 (*Fgf2*) and Proteoglycan 4 (*Prg4)*. **Conclusion:** I-PTH administration causes anabolic effects at the subchondral region of the mandibular condyle while triggers anabolic and protective effects at the MCC.

Keywords: Mandibular Condylar Cartilage, Temporomandibular Joint, Parathyroid Hormone

Introduction

Parathyroid hormone (PTH) is one of the key hormones that regulate bone and cartilage growth [1]. It is known that PTH produces both anabolic and catabolic effects in bone depending on the mode of administration. Continuous treatment of PTH stimulates catabolic modeling of bone, whereas intermittent PTH (I-PTH) increases anabolic bone modeling [2,3]. The action of PTH is mediated by the parathyroid hormone receptor 1 (PTH1R) which is expressed in the chondrocytes of the mandibular condylar cartilage (MCC) [4].

Previous studies have demonstrated that I-PTH not only prevents the degeneration of the articular cartilage but also retains the ultrastructure of the subchondral bone [5,6]. Despite its established mechanisms whereby PTH stimulates anabolic bone formation; the molecular mechanism, which leads to anabolic role of PTH in the MCC, is not fully understood. Our short-term studies on the effects of I-PTH administration in growing and adult mice suggested an anabolic effect at the mandibular condyle, characterized by an increase in cartilage thickness and enhanced mineralization in the subchondral region [7,8]. However, the effects of longer I-PTH administration are not known.

The aim of this study was to characterize the long-term effects of I-PTH on the MCC and the subchondral bone of the temporomandibular joint of mice and also to investigate the molecular mechanisms by which I-PTH exerts it effects, *in vitro*.

Materials and methods

Ethical Statement

The Institutional Animal Care Committee of the University of Connecticut Health Center approved the experimental protocol involving the mice in this study. The mice were obtained from Jackson Labs (Bar Harbor, ME, USA). Mice were group housed in individually ventilated

cages (Thoren Caging, Hazleton, PA, USA) with a photoperiod of 12:12. The room temperature and humidity were maintained at 22 °C and 30–70% respectively.

***In vivo* studies**

Study design

We used 10-week-old male C57BL/6J mice for this study. The treatment group received daily subcutaneous injections of PTH [1-34] for 4 weeks, while control animals were injected with saline. The mandibular condyles were assessed by micro-computed tomography (micro-CT), histomorphometric and immunostaining analyses.

All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Connecticut Health Center. The mice were randomly divided into two groups: (1) I-PTH Group (n=8): 80μg/Kg of the body weight PTH [1-34] (Prospec-Tany TechnoGene Ltd., Ness Ziona, Israel) was injected subcutaneously daily for four weeks; (2) Control Group (n=8): saline was injected subcutaneously daily for four weeks. The animals were fed a standard diet over the entire experimental period. All mice were injected with alizarin complexone (2μg/kg body weight) on the 24th day and calcein (2μg/kg body weight) on the 27th day. Furthermore, mice were injected with 5-ethnyl-2'-deoxyuridine (EdU, Life Technologies, Grand Island, NY, USA), in a concentration of 30mg/kg per body weight, 48 and 24 hours before euthanization. Mice were euthanized 24 hours after the last injection of PTH or saline.

Micro-CT

We further evaluated the microstructure of the subchondral bone and calcified cartilage were scanned (SCANCO Medical AG, Brüttisellen, Switzerland) in 70% ethanol and serial tomographic projections were acquired at 55kV and 145μA, with a voxel size of 6μm and 1000 projections per rotation collected at 300000μs. In order to distinguish calcified tissue from non-calcified tissue, an automated algorithm using local threshold segmented the reconstructed grey scale images. Our region of interest was the mushroom shaped head of the condyle and within the region of interest

we recorded the bone volume and tissue volume to calculate the bone volume fraction (BVF), tissue density, trabecular spacing and trabecular thickness.

Histomorphometry

Mandibular condyles were fixed for 24 hours in 10% formalin and placed in 30% sucrose overnight before embedding in cryomedium (Thermo Shandon, Pittsburgh, PA, USA) using disposable molds (Thermo Shandon, Pittsburgh, PA, USA). The medial surfaces of the samples were embedded against the base of the mold, parallel to the floor of the mold. Specimens were stored at -20°C before sectioning. Histological sections (5 -7 µm thickness) were performed using a Leica Cryostat (Nussloch, Germany). Sections were transferred to slides using a tape transfer method. Sequential sections were mounted using 50% glycerol buffered in Phosphate Buffered Saline (PBS) and were stored in the dark at 4°C. Sections were examined with an observer ZI fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) using appropriate filters (Chroma Technology, Bellow Falls, VT, USA).

Histological Staining

Histological sections were stained following a previously described protocol [9]. The 5µm-7µm MCC sections remain adherent to glass slides through all of the process of staining and imaging. The first step was to image the bone labels alizarin complexone (red) and calcein (green). Baseline imaging of the sections was performed with the observer ZI fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) using appropriate fluorescent protein filters. Subsequently, coverslips were removed by soaking slides in PBS, and sections were stained for Tartrate Resistant Acid Phosphatase (TRAP) using the ELF97 substrate (Life Technologies, Grand Island, NY). After imaging for TRAP, coverslips were removed again and sections were stained for EdU (ClickiT® EdU Alexa Fluor 555HCS kit, Life Technologies, Grand Island, NY, USA) and DAPI (Thermo Fisher Scientific, Waltham, MA, USA) and re-imaged.

Additional slides were used for Safranin O staining (IHC WORLD, LLC; Ellicott, MD, USA)

and immunostaining for Sclerostin (SOST, R&D Systems, Minneapolis, MN, USA), Vascular Endothelial Growth Factor (VEGF), Matrix Metallopeptidase 13 (MMP13) and Aggreganase-1 (ADAM-TS4) (ABCAM, Cambridge, MA, USA).

Histological analysis and quantification

We examined mineralization and TRAP activity in the subchondral bone by counting the number of red (alizarin complexone), green (calcein) and yellow (TRAP) pixels and dividing it by the total number of pixels in the subchondral region. Cellular proliferation was quantified by counting EdU and DAPI positive pixels in the proliferative zone of the MCC and calculating the percentage of EdU positive pixels over DAPI positive pixels. Distance mapping (cartilage thickness) in Safranin O stained sections was analyzed using Digimizer® Image software (MedCalc Software, Ostend, Belgium) and measurements were performed from the outer cellular layer of MCC to the tidemark (in three different locations in the entire MCC).

***In vitro* studies**

Primary chondrocyte micro mass culture

Eight C57BL/6J male mice were euthanized at 10 weeks of age by CO_2 asphyxiation. Mandibles were dissected and the MCC (outer layer of mandibular condyle) was removed from the subchondral region by carefully cutting it using surgical blades. Isolated MCCs were placed in PBS with 50 U/ml penicillin-streptomycin (P/S; Thermo Fisher Scientific, Waltham, MA, USA). In order to retrieve chondrocytes, MCCs were incubated at 37° C in the P/S solution with collagenase D at 3 mg/ml (Sigma-Aldrich, St. Louis, MO, USA) and dispase at 2 mg/ml (Thermo Fisher Scientific, Waltham, MA, USA). Cells released from the tissue were transferred to media (DMEM with high glucose and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), 50 U/ml P/S, and 10% fetal bovine serum (BSA; Thermo Fisher Scientific, Waltham, MA, USA) and kept on ice. Cells were centrifuged for 5 minutes at 1200 rpm, at 4° C. Cells were then

resuspended in media and counted using a hemocytometer. Cells were plated (50 µl) for micro mass culture, adding 50,000 cells per well, using 6-well polystyrene flat bottom microplates (Fisher Scientific, Hampton, NH, USA). Three ml of media was added to each well 2.5 hours after plating cells. Media was changed daily and cells were treated for 14 days. Cells were kept in a 5% oxygen incubator. There were 3 biological replicates for each group. Human PTH (1-34) (Prospec-Tany TechnoGene Ltd., Ness Ziona, Israel) dissolved in 1 mg/ml in 4mM HCl with 0.1% BSA was stored at minus 20°C. The stock PTH was diluted in PBS to make a working concentration of 25µg/ml. The final concentration of PTH used in media was 50 µg/ml, and PBS was used for control cells.

RNA isolation and gene expression

RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. For qPCR analysis, 1ug of RNA was used from each sample for reverse transcription using Superscript II (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer instructions, with oligo dT 12-18 primer and RNase OUT RNase inhibitor. Sequences of primers (IDT) used in qPCR are found in Supplementary Table S1. QPCR was carried out using a BioRad CFX instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We used the Sybr Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with a 20 µl reaction volume. The qPCR protocol used was: 50°C for 2 minutes, followed by a 95°C step for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation curve was then run and the temperature cycled from 95°C to 65°C and back to 95°C. Melting curves were examined to determine product specificity. We used 96-well plates and samples were run in technical duplicate and averaged. The delta-delta Ct method was used for analysis. GAPDH was used to normalize the expression values. Data is expressed as fold-change versus the control ± the standard error of the mean (SEM). The primers for the genes analyzed are in Table 1.

Statistical Analysis

Descriptive statistics were used to examine the distribution of bone volume fraction, tissue density, trabecular thickness, trabecular spacing, histological analysis and gene expression. A one Sample Kolmogorov-Smirnov test was used to examine the normality of data distribution. Outcomes were compared between the I-PTH and control groups. Statistically significant differences among means were determined by unpaired t-test (Student t-test). All statistical tests were two sided and a p-value of <0.05 was deemed to be statistically significant. Statistical analyses were computed using Graph Pad Prism (San Diego, CA, USA)

Results

In vivo results

MicroCT analysis

Our goal was to compare the calcified tissue mass after long-term treatment with I-PTH (Fig.1A). We observed there was a significant increase in the BVF in the experimental group (24.08%) when compared to the control group (Fig. 1B). Similarly, there was significant increase in the tissue density (6.13%; Fig 1C) and trabecular thickness (9.75%; Fig. 1D) in the experimental group when compared to the control group. However, there was a significant decrease (14.71%; Fig. 1E) in trabecular spacing in the experimental group when compared to the control group. These results suggest that I-PTH treatment can trigger an anabolic effect, increasing mineralization at the MCC and subchondral region.

Histological findings

In general, histological analysis showed that the MCC had a smooth surface and normal cellularity with no cellular or matrix abnormalities in both I-PTH and control groups. The mineralization label that separates the calcified and non-calcified cartilage (Tidemark) was present in both the I-PTH and the control group (Fig. 2A). However, the quantity of mineral deposition was significantly higher in the I-PTH group, as represented by an increased uptake of

fluorochrome markers into the mineralizing matrix, when compared to the control group. The amount of alizarin complexone in the subchondral bone was approximately 242% higher in the I-PTH group, whereas the amount of calcein was approximately 160% higher in the I-PTH group when compared to control (Fig. 2B and 2C). Additionally, we noticed there was a faint line of alizarin complexone in the tidemark of the in I-PTH group, whereas the control group had higher intensity of alizarin complexone in that region (Fig. 2A). This finding suggests an increased dynamic remodeling in the I-PTH group, given that alizarin complexone was the first bone label injected. In addition, I-PTH stimulated the catabolic remodeling of the subchondral bone as observed by a significant increase in the TRAP stained region (127% increase) in the subchondral bone (Fig. 2D and 2E). We also observed TRAP positive cells in the hypertrophic zone of the MCC in the I-PTH group, but none in the control group (Fig. 2D).

Regarding the non-mineralized portion of the cartilage, we observed that I-PTH induced a significant increase in thickness compared to control group (Fig. 3A). The cartilage distance mapping was measured on Safranin-O stained sections and our measurements revealed that the I-PTH group presented with a 21.4% increase in cartilage thickness when compare to the control group (Fig. 3B). Furthermore, we observed that there was a significant increase in cellular proliferation in the I-PTH group (Fig. 3C) as evidenced by an increase in EdU positive cells (126% more EdU positive pixels when compared to control group; Fig. 3D).

In order to further understand the *in vivo* changes in the mandibular condyle correlated with I-PTH, we performed immunohistochemistry assays for SOST, VEGF, MMP13 and ADAMST4 after I-PTH treatment (Fig. 4).

I-PTH decreased the expression of SOST, a negative regulator of mineralization, at the hypertrophic region of the MCC (Fig. 4A). In addition, we observed an increase in the expression of the angiogenesis promotor VEGF, in both MCC and subchondral region of the I-PTH group (Fig. 4B). Furthermore, the expression of MMP13, a collagenase involved in extracellular matrix breakdown, was decreased in the MCC of I-PTH treated mice (Fig. 4C). Similarly, I-PTH

decreased the expression of ADAMTS4, a major proteinase that degrades proteoglycans (Fig. 4E).

***In vitro* results**

We examined the RNA expression of genes relevant to chondrogenesis and endochondral ossification in primary chondrocyte micro mass cultures treated with I-PTH by qPCR**.** There was no significant difference between I-PTH treated chondrocytes and control groups when the expression of *Sost*, *Bmp2*, *Noggin*, *Col2a1*, *Opn* and *Runx2* were analyzed by qPCR. However, we found a remarkable decrease in *Col10a1* (Fig. 5A) and *Alp* (Fig. 5B), important markers for cartilage mineralization. In addition, we observed a significant increase in *Sox9* (Fig. 5C) and *Fgf2* (Fig. 5D) and a significant decrease in *Ihh* (Fig.5E), suggesting I-PTH affects chondrocyte proliferation and differentiation. Interestingly, we observed a substantial increase (more than 5-fold) in *Prg4* (Fig. 5F), a novel finding suggesting that I-PTH may improve lubrication and chondrocyte survival in the MCC.

Discussion

To our best knowledge this is the first study to demonstrate the long-term effects of I-PTH on the MCC and the subchondral bone of the TMJ. This study chiefly demonstrates that although I-PTH showed increased mineralization of the calcified cartilage and the subchondral bone but the cartilage thickness and metabolism of cartilage were better preserved by I-PTH.

The anabolic action of I-PTH in bone has been studied for many years, yet the molecular mechanisms underlying its anabolic action in the MCC of the TMJ are still incompletely elucidated. Our *in vivo* results have shown increased mineralization and turnover in the calcified cartilage and the subchondral bone region, accompanied by increased cellular proliferation and cartilage thickness, as a result of 4 weeks of daily I-PTH injection. These results are consistent with our previous reports in younger and older mice when I-PTH was administrated for shorter periods of

time [7,8]. In addition, we found decreased expression of SOST and increased expression of VEGF at the MCC of I-PTH injected mice. SOST is a mineralization inhibitor and a classic target of PTH in bone [3,10], while VEGF is known to promote the endochondral vascularization [11] and has been linked to the I-PTH anabolic effects in long bones of rats [12]. The altered expression of these proteins correlates with the increased bone volume, tissue (mineral) density and mineralization after I-PTH administration. Our data is consistent with the literature on the anabolic effects of SOST and VEGF on bone after I-PTH. However, can this enhanced mineralization of the subchondral bone and the calcified cartilage lead to degeneration of the unmineralized portion of the cartilage and subsequently result in osteoarthritis? This question prompted us to analyze, if in addition to increase the mineralization at the hypertrophic and subchondral bone regions, I-PTH treatment could cause the destruction of the extracellular matrix. Our immunostaining suggested a reduced expression of MMP13, a collagenase involved in extracellular matrix breakdown and osteoarthritis [13,14]. Furthermore, I-PTH administration induced an inhibition of ADAMTS4, a proteoglycan-degrading enzyme (aggrecanase-1) correlated with degradation [14]. This is also consistent with the increased cartilage thickness, proteoglycan distribution and chondrocyte proliferation observed in our *in vivo* results. Taken together, these results suggest that I-PTH seems to impair the extracellular matrix catabolism and treatment with I-PTH seems to induce a protective effect against extracellular matrix degradation at the MCC, despite the enhanced anabolic mineralization in the calcified cartilage and the subchondral bone region of the TMJ. Similar findings in vertebral disc of rats have been reported by Zhou et al, who tested the effects of intermittent treatment of PTH in ovariectomized rats [15]. The authors found not only an improvement of bone volume and density at vertebral body, but also an enhancement of disk extracellular matrix and a decrease in the expression of MMP13 and ADAMTS4 [15]. The dual effect of I-PTH in cartilage and bone have also been described in articular cartilage studies; I-PTH has been shown to prevent and repair osteochondral defects by stimulating both articular cartilage and subchondral bone regeneration [5,16]. Furthermore, a reduction of SOST and MMP13 in the

articular cartilage of an osteoarthritis model after treatment with PTH has been reported [16], which is consistent with our present results.

Our *in vitro* results only partially replicated the *in vivo* findings. The primary chondrocyte micro mass cultures consisted of cells extracted from the MCC only, which could explain the different behavior in response to I-PTH. Although we found increased expression of markers for chondrocyte proliferation *in vitro* (upregulation of *Fgf2*), we observed decreased markers for mineralization (reduced *Col10a1* and *Alp*), which contradicts our *in vivo* findings.

We found increased relative gene expression of *Sox9* in I-PTH treated chondrocytes. SOX9 is a transcription factor with important roles for chondrocyte survival and inhibiting of differentiation [17,18] and its phosphorylation has been associated with activation of the PTH receptor [19]. Furthermore, we observed significant suppressed expression of *Ihh* in our I-PTH treated chondrocytes in *in vitro* experiments. IHH is another essential player in chondrogenic differentiation, which has also been implicated with activation of the PTH receptor [19]. These results suggest that I-PTH increases mandibular cartilage thickness by increasing chondrocyte proliferation (by increasing *Fgf2*), delaying chondrocyte differentiation (by increasing *Sox9) and* inhibiting chondrocyte differentiation *(*by decreasing *Ihh).* Finally, the decrease in chondrocyte terminal differentiation leads to a decrease in *Col10a1* and *Alp* in the MCC.

An interesting novel finding was that the relative gene expression of Proteoglycan 4 (*Prg4*) was substantially increased in the *in vitro* I-PTH treated chondrocytes. *Prg4,* which is highly expressed in bone and articular joints and induce a protective and anti-inflammatory effect at the articular joints [20-22], and has been identified as a new target of PTH for skeletal anabolism [23]. We suggest that I-PTH may induce a protective effect at the mandibular condyle by stimulating *Prg4*. One of the limitations of our study is that the experiments were only done in male mice. However, our future studies are focusing on both male and female mice. Furthermore, we are studying the effects of I-PTH in repair and regeneration of the cartilage in an injury model (Partial disectomy).

Conclusions

In summary, the present research showed that long-term administration of I-PTH leads to anabolic bone formation at the mandibular condyle, while maintains the integrity of the unmineralized portion of the MCC and increases the thickness of the cartilage. In addition, I-PTH seems to induce a chondroprotective effect at the MCC. Moreover, long-term I-PTH exhibited better performance in balancing the anabolic and catabolic metabolism of the extracellular matrix. Our future directions include using a mouse model with degeneration of the TMJ in order to investigate whether I-PTH could be used as a therapeutic treatment to improve this condition.

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Conflict of Interest:

None to Declare

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Figure Legends

Figure 1: Increased bone volume and density at the mandibular condyle after long-term I-PTH administration. Coronal micro-CT images of condyles of Control (CTRL) and I-PTH injected mice (A). Quantification of bone parameters: B) BVF - bone volume fraction, C) Tissue Density, Trabecular Thickness (D) and Trabecular Spacing (E). Histograms (B-E) represent means ± SD for n = 8 per group. Statistically significant difference between groups: *p < 0.05. Region of interest is illustrated by dotted lines in (A). Scale bar = 500μm.

Figure 2: Increased mineralization and bone remodeling at the subchondral region of mandibular condyle after long-term I-PTH administration. Sagittal sections of mandibular condyles of Control (CTRL) and I-PTH injected mice illustrating alizarin complexone and calcein labeling (A). Dotted lines in (A) in the alizarin complexone images in CTRL and PTH images represent the tidemark: faint line of alizarin complexone is observed in the PTH image. Quantification of alizarin complexone (red, B) and calcein (green, C) percentage of positive pixels over the subchondral bone area. Sagittal sections of mandibular condyles of Control (CTRL) and I-PTH injected mice stained for TRAP (D). Quantification of percentage of TRAP positive pixels

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Figure 3: Increased cartilage thickness and chondrocyte proliferation at the MCC after long-term I-PTH administration. Sagittal sections of mandibular condyles of Control (CTRL) and I-PTH injected mice stained for Safranin O (A). Mandibular condylar cartilage (MCC) and subchondral bone area (Sub. Bone) are labeled. Quantification of cartilage thickness (B). Sagittal sections stained for EdU (C). Quantification of EdU positive pixels (yellow), representing the amount of cellular proliferation, over DAPI positive pixels (blue) at the proliferative zone (D). Histograms (B and D) represent means ± SD for n = 5 per group. Statistically significant difference between groups: *p < 0.05. Scale bar = 50μm.

Figure 4: Decreased expression of SOST, MMP13 and ADAMTS4 and increased expression of VEGF at the mandibular condyle of I-PTH injected mice. Immunohistochemistry for SOST (A), VEGF (B), MMP13 (C) and ADAMTS4 (D) in sagittal sections of condyles. Scale bar = 50μm.

Figure 5: Gene expression changes in chondrocyte micro mass cultures treated with I-PTH. Histograms represent relative gene expression tested by quantitative polymerase chain reaction (qPCR) 14 days after I-PTH treatment. The relative expression of *Col10a1* (A), *Alp* (B) and *Ihh* (E) was markedly decreased, while the expression of *Sox9* (C), *Fgf2* (D) and *Prg4* (F) was significantly increased. Statistically significant difference between groups: *p < 0.05

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
Overall Review: This manuscript investigates the effect of long-term intermittent PTH on the cartilage layer and subchondral region of the mandibular condyle. The authors did a good job in designing the in vivo and in vitro study. The main issue is the title and the comparisons to previous publication. 4 weeks is not really considered long term, and no differences were apparent to 2 or 3 weeks of PTH administration (previous results). The authors should consider changing 'long term' to '4 weeks' and highlight the in-vitro results in the title. The in-vitro results are truly unique to this manuscript. There is also a lack of explanation on the amounts of PTH used between the in vivo and in vitro study.

Detailed Concerns:
Abstract
1) In the "Results", please specify in which area(s) were the changes seen.
Authors response: The Changes were seen both in the cartilage and the subchondral bone and as suggested by the reviewers we have specified that.

2) Change "while triggering" to "while triggers".
Authors response: modification made.

Introduction
1) Please give in-depth introduction on PTH, e.g. how does it affect chondrocytes activity, bone metabolism, etc?
Authors response: In-depth introduction on how PTH affects bone metabolism is out-of-scope of this manuscript. In addition, how PTH affects chondrocyte activity is still uncertain and one it is of the objectives of the present manuscript, so we could not describe it in-depth in the introduction.

Materials and Methods
1) Is the PTH used in the in vivo and in vitro study the same? How were the concentrations chosen between the two systems?
Authors response: Yes, the same PTH [1-34] from Prospec-Tany TechnoGene was used for both *in vivo* and *in vitro* studies. The concentrations were choosen based on the prior published literature. (Comparison of the action of transient and continuous PTH on primary osteoblast cultures expressing differentiation stage-specific GFP. J Bone Miner Res. 2005 Jan;20(1):5-14. Epub 2004 Oct 25)

2) What is the rational for the PTH concentration for the in vitro study? Is it relative to the serum PTH concentration after the injection in the in vivo study? How representative is the in vitro study for the in vivo study?
Authors response: The PTH concentration was based on previous published literature. In vitro study was done to understand the mechanism. Reviewer made a good point and in future study we will look at the serum concentration of the PTH to do in vitro studies.The concentration of PTH in in vitro experiments were choosen based on the published literature. (Comparison of the action of transient and continuous PTH on primary osteoblast cultures expressing differentiation stage-specific GFP. J Bone Miner Res. 2005 Jan;20(1):5-14. Epub 2004 Oct 25)

3) Is daily administration in vivo considered intermittent?
Authors response: Yes, daily injection is considered intermittent administration, while continuous infusion through a pump is considered continuous administration (Hock, J. and Gera, I. (1992), Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. J Bone Miner Res, 7: 65-72. doi:10.1002/jbmr.5650070110)

4) The number of samples used in vivo is no clear. 12 animals per group were tested, but the break down per test is unclear. N=8 for CT and n=5 for histology is 13 animals
Authors response: A total of 12 animals per group were used for the study, however, only 8 were sent to microCT (due to elevated cost of microCT) and 5 were used for histology (5 animals would be enough to allow proper statistical analysis). So, we changed the number of animals in the second paragraph of the study design to 8 animals per group because this is the actual number of animals analyzed.

5) The break down for in-vitro is not clear. 10 mice were pooled but the biological replicate was n=3?
Authors response: Chondrocytes from 10 mice were pooled, but cells were divided equally in 3 cell culture wells to receive independent treatments and represent different biological replicates.

6) Post hoc tests are not needed if you are not doing the ANOVA test.
Authors response: We apologize for the confusion. We did not do Post hoc as we used T-test as statistical analysis. This correction was made (post hoc analysis was removed).

Results

1) In fig. 1, please outline the region of interest with dotted line in the CTRL and PTH images instead of the third image, and give explanation in the legend. Please add scale bars to the CT images.
Authors response: Suggested changes in Figure 1 were made. ROI was outlined in CTRL and PTH microCT images and scale bars were placed.

2) In fig. 2, the y axis of the graphs can't be only "%" (% of what?). In the legend, change the "quantification of … positive pixels" to "percentage of …. positive pixels" since you are presenting the data in percentage.
Authors response: Suggested changes in Figure 2 were made. Instead of only %, we added "% of positive pixels". Changes in the legend were made as well.

3) In fig. 2A, please illustrate the tidemark and the "faint line of alizarin complexone" mentioned in the result.
Authors response: Lines to illustrate the tidemark and alizarin complexone line in Figure 2A were placed.

4) In fig. 3, please add annotations for different layers, especially the cartilage layer since you are measuring the cartilage thickness.
Authors response: Labels for mandibular condylar cartilage (MCC) and subchondral bone were placed.

5) Fig. 4 A) PTH seems to be over exposed compared to CTRL, which makes it difficult for the readers to compare.

Authors response: Histological sections were exposed for the same time. CTRL section seems to have a stronger hematoxylin background (used for counterstaining), but the immunostaining positive signal (brown color) is of same intensity between sections.

6) There was no quantification for Figure 4, so result can only be descriptive. Meaning, "observed" or "seen" should be used when describing increases or decreases
Authors response: We have removed the term "significantly" from the description of the immunostaining results, mentioning only "decreased" or "increased".

7) In the in vitro results, what is "an important decrease"?
Authors response: We change the word "important" for "significant".

Discussion
1) How does the study provide mechanical characterization (Young's modulus, viscosity, stiffness, etc.) of the MCC as mentioned at the second sentence in the discussion?
Authors response: We did not perform any mechanical characterization, except the microCT analysis which provided bone density evaluation, giving a suggestion on bone quality. Since bone density does not provide a definite mechanical characterization, we removed the second sentence of the discussion.

2) The expression of "4 weeks after I-PTH injection" in page 10 line 7 is confusing, because PTH was injected daily during the 4-week period as mentioned in the method.
Authors response: We changed the phrase to "as a result of 4 weeks of daily I-PTH injection".

3) In page 10, please delete "can" in line 16. Change "increasing" in line 18 to "increase the", and "be causing" to "cause the" in line 19.
Authors response: Corrections were made.

4) If the chondrocytes are not maturing and mineralizing, then what is responsible for new bone deposition? What is maintaining the height of the condyle?
Authors response: We observed increased bone turnover and mineralization at the subchondral region after intermittent administration of PTH. As stated in your discussion and conclusion, PTH seems to induce distinct effects at the cartilage and subchondral bone. We believe that most of the mineralization of the subchondral region is possibly independent from endochondral ossification. That's why we see an increase in subchondral mineralization, in face of a decrease in chondrocyte maturation.

5) The in vivo and in vitro study cannot be compared directly, because 1) the PTH concentration used in the in vitro study was not validated to be the same as the local PTH concentration at the condyle in the in vivo study; 2) the in vitro study only involved the cells from MCC, but not the subchondral bone.
Authors response: We agree with the reviewer. The *in vivo* study was done to understand the phenotypic changes and *in vitro* study was done to understand the mechanism

Conclusion
1) In page 12 line 10, what does it mean by "…balancing the anabolic and catabolic metabolism of the extracellular matrix"? How did the study prove the balancing effect of PTH on the extracellular matrix metabolism?
Authors response: We made this conclusion based on the fact that the cartilage thickness and extracellular matrix are increased with intermittent administration of PTH, although we have an increase in subchondral mineralization.

Reviewer: 2

Comments to the Author
- Abstract and later: Please explain abbreviations at first use or give a table/pargraph with all abbreviation as there are several.
Authors response: Abbreviations were explained in the abstract and later in the first time mentioned.

- Introduction: No comments
- Material and Methods:
o Please always give manufacturer, town and state for non-American readers, e.g.: (Bar Harbor, ME, USA)
Authors response: Complete manufacturer information was added.

o The Material and Methods section is sound.
- Results:

o P.8, l. 46: compared
Authors response: correction made.

o P.9, l. 37: Interestingly
Authors response: correction made.

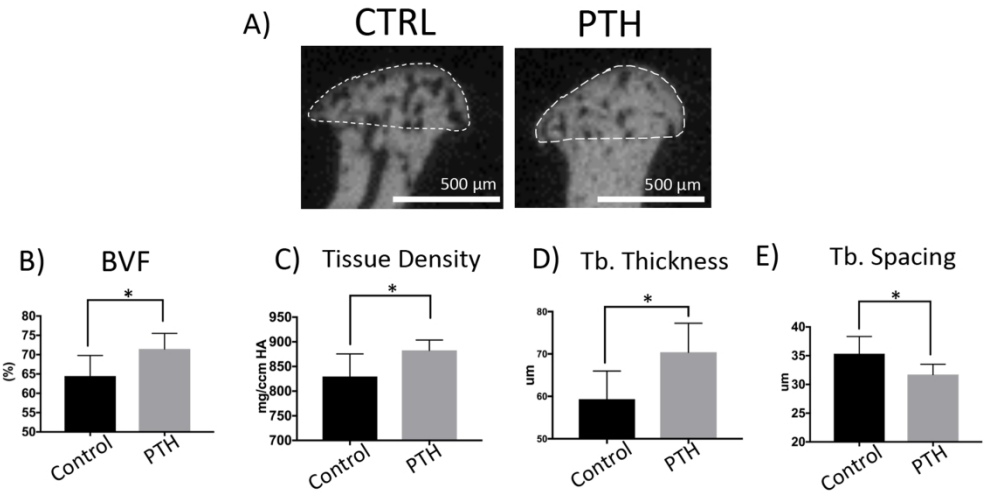
o P.9, l.39: 5-fold
Authors response: correction made

o The Results section is sound.
- Discussion:
o Please add “limitations” of your study and on what you will lie your future focus, e.g. translational research in a larger animal model etc.
Authors response: One of the limitations of our study is that the experiments were only done in male mice. However, our future studies are focusing on both male and female mice. Furthermore, we are studying the effects of I-PTH in repair and regeneration of the cartilage in an injury model (Partial disectomy).

P.10, l. 25: … while VEGF is known…
o P.10, l. 35: … cartilage lead…
o P.11, l. 12: disc
o P.11, l. 41 and 47: activation of the PTH receptor.
- Authors response: correction made.

Conclusion:
o Please move limitations to “Discussion” and see the comment above
o P.12, l. 20-22: … while maintaining the integrity of the unmineralized portion of the MCC and increasing the thickness of the cartilage…
- Acknowledgements
o P.12, l. 39: We would like to thanks Li Chen
- References: No comments
- Images: No comments

- Authors response: correction made.



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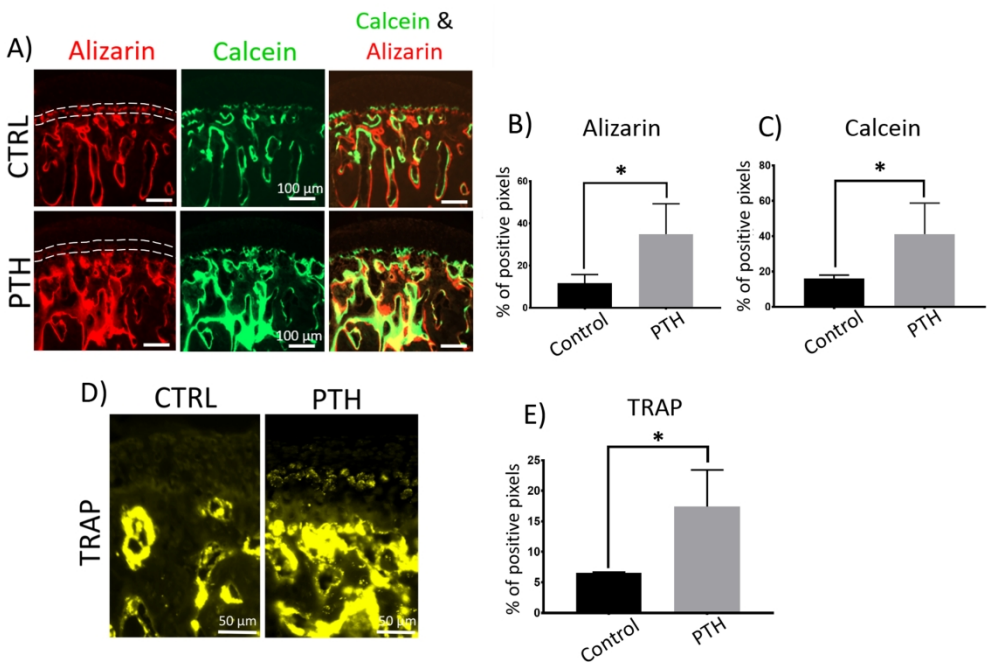

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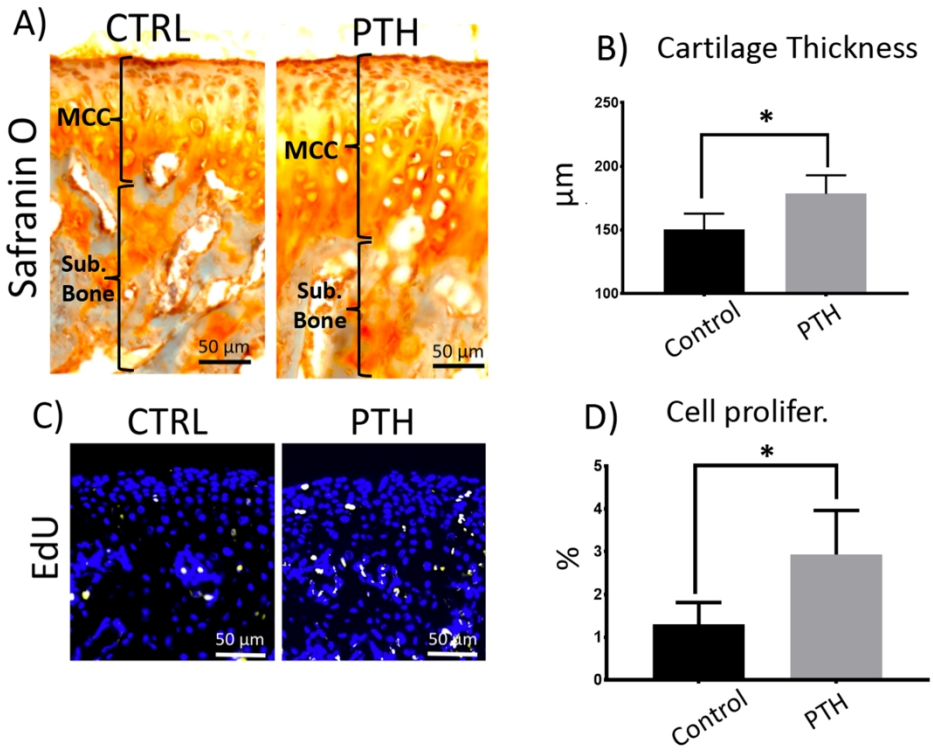

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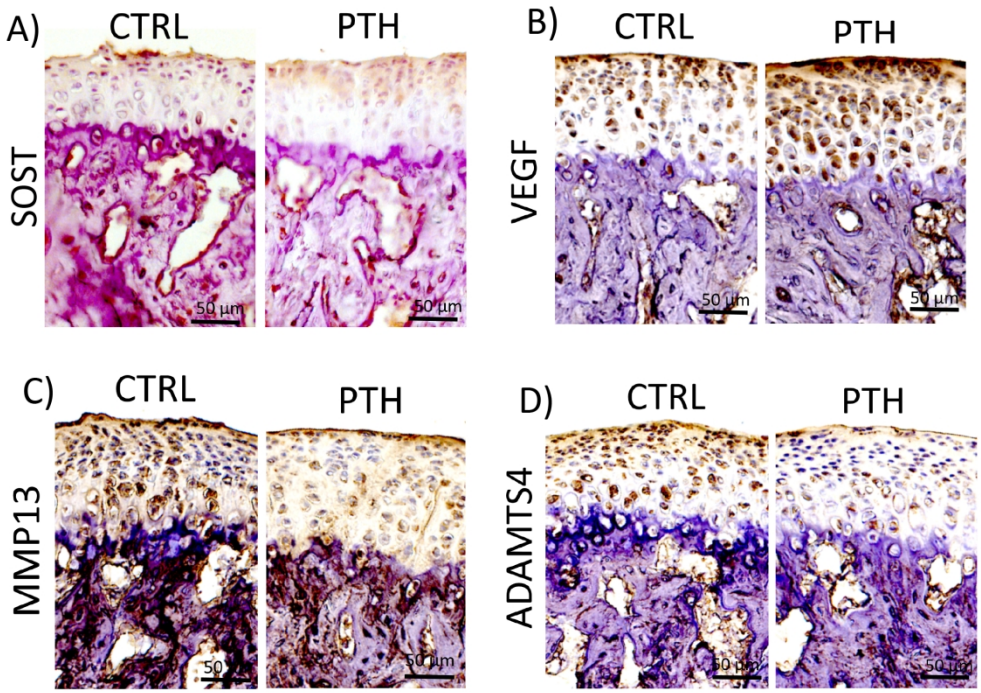

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788x561mm (72 x 72 DPI)

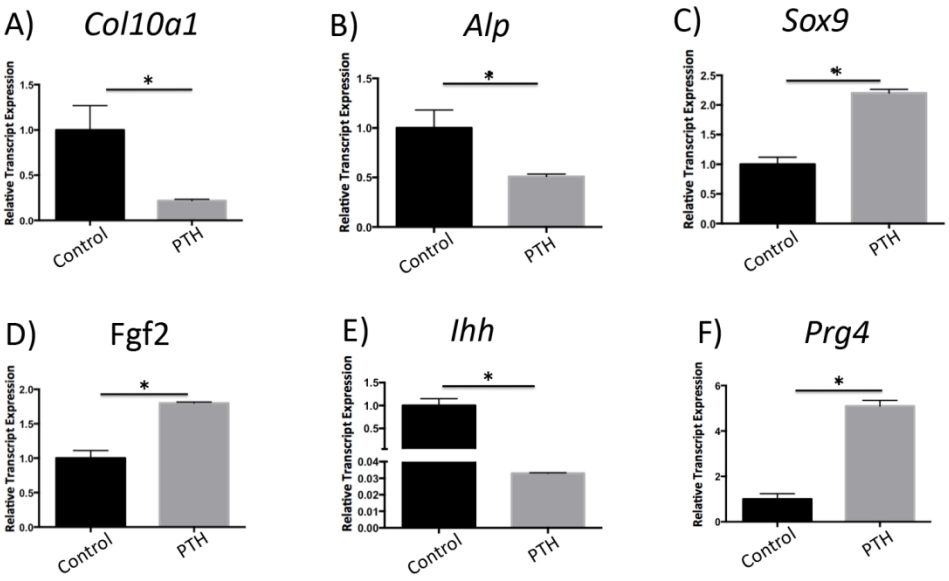

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Table 1: Primers for the genes analyzed

Col10a1
Alp
Sox9
Fgf2
Ihh
Prg4

Supplementary Table S1.

Primer Name	Sequence
F GAPDH	5' AGGTCGGTGTGAACGGATTTG '3
R GAPDH	5' GGGGTCGTTGATGGCAACA '3
F Col10a1	5' CAATACTTCATCCCATACGC '3
R Col10a1	5' AGGAATGCCTTGTTCTCC '3
F ALP	5' AATGAGGTCACATCCATCC '3
R ALP	5' CGAGTGGTAGTCACAATGC '3
F Sox9	5' AGTACCCGCATCTGCACAAC '3
R Sox9	5' TACTTGTAATCGGGGTGGTCT '3
F Fgf2	5' GGCTGCTGGCTTCTAAGTGTG '3
R Fgf2	5' TTCCGTGACCGGTAAGTATTG '3
F IHH	5' GACTCATTGCCTCCCAGAACTG '3
R IHH	5' CCAGGTAGTAGGGTCACATTGC '3
F Prg4	5' TTTTGGCCGGGAGACTCAATC'3
R Prg4	5' CAGCGTAGTCAGTCCATCCAC'3