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**14. ABSTRACT**

The goal of this research project is to characterize how disruptor peptide blocks beta-catenin interaction with PTH receptor, inhibits chondrocyte hypertrophy and prevents osteoarthritis (OA) progression. In the first year, we completed the most work in the Aim 1 and initiated some work in Aim 2. We designed a disruptor peptide corresponding to the carboxyl-terminal region of PTH receptor, and found this disruptor peptide inhibited beta-catenin binding to PTH receptor by GST-pull down assay. We also confirmed that disruptor peptide conjugated to penetratin can enter cells. Importantly, disruptor peptide can reverse the beta-catenin-mediated PTH receptor signaling switch by increasing Gs/cAMP signaling and inhibiting Gq/PLC activation in chondrocytes. In addition, we successfully induced ATDC5 cell differentiation from proliferating chondrocytes to the hypertrophic stage, and generated mouse OA model surgically induced by destabilization of the medial meniscus. These results provide the foundation for further studies whether disruptor peptide can inhibit chondrocyte hypertrophy in vitro and protect cartilage damage in a mouse OA model.

**15. SUBJECT TERMS**

Osteoarthritis**;** Parathyroid hormone-related protein; PTH receptor; Beta-catenin; Chondrocyte hypertrophy

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## Table of Contents

**Page**

## 1. INTRODUCTION:

Cartilage pathology in osteoarthritis (OA) is associated with extracellular matrix collagen destruction, which is accompanied by phenotypic changes in articular chondrocytes. Chondrocyte hypertrophy-like changes have been well known in both human OA and experimental OA, which are characterized by expression of type X collagen (Col X) and matrix metalloproteinase 13 (MMP13), suggesting the hypertrophic program is activated in OA conditions. The growing evidence indicates that inhibition of the hypertrophic differentiation is able to attenuate the severity of cartilage lesions in mouse OA models. These findings offer new approaches for the treatment of OA by targeting the chondrocyte phenotype to reduce its hypertrophic differentiation. The application of a disruptor peptide to interfere with protein-protein interaction represents a novel therapeutic strategy for inhibition of chondrocyte hypertrophy and treatment/prevention of OA. The purpose of our proposal is to design and develop a novel disruptor peptide and test its efficacy in relevant cellular and in vivo OA models, thus accomplishing most pre-clinical goals necessary for ultimate human subject testing.

## 2. KEYWORDS:

Osteoarthritis

Parathyroid hormone-related protein

PTH receptor

β-catenin

Cell signaling

Chondrocyte hypertrophy

Destabilization of the medial meniscus

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

The goal of this research plan is to characterize how disruptor peptide blocks beta-catenin interaction with PTH receptor, inhibits chondrocyte hypertrophy and prevents OA progression.

### What was accomplished under these goals?

The grant project entitled “Inhibition of chondrocyte hypertrophy of osteoarthritis by disruptor peptide” started from July 1, 2016 and will end on December 31, 2018. During the second year, we completed the work in Aim 1 and most of work in Aim 2 studies according to the time line in this Discovery Award.

### 3.1. Major activities.

The application of disruptor peptide to target the β-catenin interaction with PTH receptor represents a novel therapeutic strategy for the inhibition of chondrocyte hypertrophy and treatment/prevention of OA.

### 3.2. Specific objectives

Aim 1 will develop and characterize a disruptor peptide specifically blocks the interaction of beta-catenin with PTHR and inhibits the pathogenic beta-catenin-mediated PTHR signaling switch. In Aim 2, we will define the role of disruptor peptide in inhibiting chondrocyte differentiation in a mouse DMM (destabilization of the medial meniscus) model.

### 3.3. Significant results in second year

**a. Disruptor peptide reverses the β-catenin-mediated PTHR signaling switch in chondrocytes.**

Mouse primary rib chondrocytes express both β-catenin and PTHR. Cellular delivery of peptides is facilitated by peptide conjugation with the carrier, cell-penetrating peptides. To assess disruptor peptide (6-cdp) permeability into cells, the effects of the disruptor peptide conjugated to: 1) penetratin (Pen); 2) nine arginine residues (9R), or 3) transactivator of transcription (TAT), were compared. Primary chondrocytes were pretreated with 10 μM Pen-6-cdp, 9R-6-cdp, TAT-6-cdp, 6-cdp, Pen, 9R, or TAT for 2 h. cAMP formation induced by 10 nM human PTHrP(1-34) (hereafter referred to as PTHrP) (Bachem, Torrance, CA) was measured using the cyclic AMP direct EIA kit (Arbor assays, Ann Arbor, MI). **Figure 1A** data demonstrate that the 6-cdp itself, Pen and 9R had

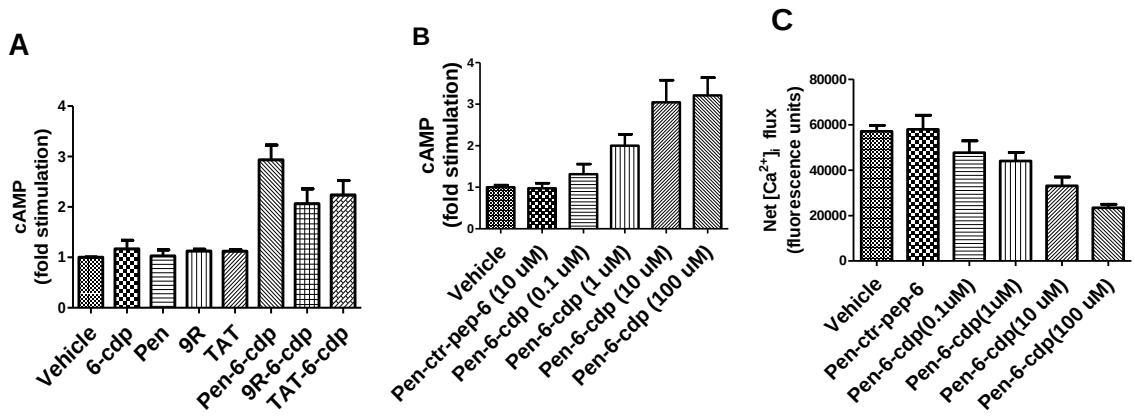


**Figure 1. Disruptor peptide (6-cdp) switches PTHR signaling from Gαq/PLC to Gαs/cAMP activation. A**, Effects of Pen-6-cdp, 9R-6-cdp, TAT-6-cdp, 6-cdp, Pen, 9R or TAT on PTHrP-induced cAMP formation. **B**, Pen-6-cdp increases PTHrP-induced cAMP formation. **C**, Pen-6-cdp inhibits PTHrP-stimulated intracellular calcium.

no effect on PTHrP-induced cAMP formation. However, the stimulatory effect of PTHrP together with Pen-6-cdp was higher than that of 9R-6cdp and TAT-6cdp. Therefore, the 6-cdp conjugated with Pen was used in the following experiments. As expected, Pen-6-cdp concentration-dependently increased 10 nM PTHrP-induced cAMP formation (**Figure 1B**), while control peptide (Pen-ctr-pep-6) had no effect on PTHrP-stimulated cAMP formation. Pen-6-cdp itself had no effects on cAMP formation without PTHrP treatment (data not shown). To evaluate PTHR-mediated Gαq/PLC signaling, intracellular calcium mobilization ($[Ca^{2+}]_i$), an index of PLC activity, was measured. Pretreatment with Pen-6-cdp for 2 h concentration-dependently inhibited PTHrP (100 nM)-induced $[Ca^{2+}]_i$ (**Figure 1C**). Collectively, these data demonstrate that the disruptor peptide limits the PTHR signaling switch by increasing Gαs/cAMP activation while inhibiting Gαq/PLC signaling.

**b. Disruptor peptide has no effect on Wnt3a-induced β-catenin/ T-cell factor (TCF) activation.**

ATDC5 cells (chondrogenic cell line) were transfected with TOPflash luciferase reporter plasmid, whose promoter contains a TCF binding region responsive to β-catenin signaling. Cells transfected with the FOPflash reporter plasmid (containing mutated TCF binding region) serve as a control. After 36 h, cells were pretreated with Pen-6-cdp (10 μM) for 2h, and then Wnt3a (50 ng/ml) was added for 8 h. After treatment, the TOPflash and FOPflash were measured. Our data demonstrated disruptor peptide did not affect the canonical Wnt pathway (**Figure 2**).

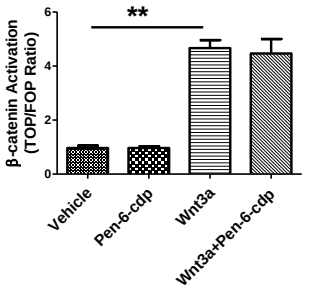


**Figure 2. Disruptor peptide does not affect the canonical Wnt pathway.** n = 4. **, $p < 0.01$, compared with vehicle control.

### c. Disruptor peptide has no cytotoxicity

Different concentrations (1 - 100 µM) of Pen-6-cdp were incubated with ATDC5 cells for 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was then added to each well at a final concentration of 500 µg/ml and incubated for 1 h at 37°C in a 5% CO2 atmosphere. The liquid in the wells was subsequently removed. DMSO was then added to each well, and the absorbance was measured at 570 nm. Data in **Figure 3** showed that Pen-6-cdp did not cause any cytotoxicity in ATDC5 cells.

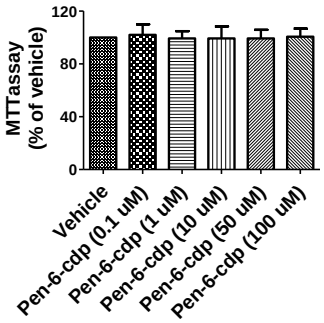


**Figure 3. Disruptor peptide does not have cytotoxicity in ATDC5 cells.**

### d. Disruptor peptide inhibits of the interaction of PTHR with β-catenin in ATDC5 cells.

The experiments were performed by immunoprecipitation assay. The ATDC5 cells were treated in the presence or absence of different concentrations of Pen-6-cdp (0.1 – 50 µM). The cells were lysed and solubilized materials were incubated with β-catenin monoclonal antibody for 1 h at 4°C, and then protein G-Sepharose 4B conjugate was added and incubated overnight at 4°C. Total lysates and immunoprecipitated proteins were analyzed by SDS-polyacrylamide gels and the band intensity for β-catenin and PTHR was quantified using the Licor Odyssey system. Data in **Figure 4** showed that Pen-6-cdp concentration-dependently inhibited the interaction of PTHR interaction with β-catenin.

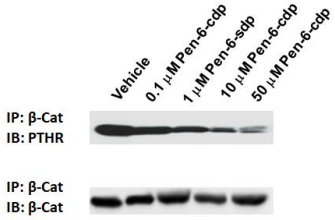


**Figure 4. Pen-6-cdp concentration-dependently inhibits the interaction of PTHR with β-catenin (β-Cat) in ATDC5 cells. The representative immunoblots are shown. IP: immunoprecipitation; IB: immunoblot.**

### e. Knockout of β-catenin expression in cells eliminates disruptor peptide effect on PTHR signaling.

Endogenous β-catenin expression in mouse ATDC5 cells (chondrogenic cell line) was knocked out using the CRISPR/Cas9 genome-editing technique. **Table 1** lists the single-stranded oligonucleotides encoding a CRISPR targeting RNA (crRNA) of β-catenin that allows sequence-specific targeting of the Cas9 nuclease. The double-stranded oligonucleotides were cloned into linearized GeneArt CRISPR nuclease vectors with an OFP reporter following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Three single colonies were identified for knockout of β-catenin expression in ATDC5 cells by western blotting (**Figure 5**). No β-catenin expression change was observed in cells transfected with control plasmid.

**Table 1. CRISPR sequences for knockout of mouse β-catenin**

| Single stranded Cloning Oligo | Sequences | | Accession number |
|---|---|---|---|
| β-catenin | Forward: | 5'- CGGGCAGTATGCAATGACTAGTTTT | NM_007614.3 |
| | Reverse: | 5'- TAGTCATTGCATACTGCCCGCGGTG | |
| Control | Forward: | 5'- CATTTCTCAGTGCTATAGAGTTTT | |
| | Reverse: | 5'- TCTATAGCACTGAGAAATGCGGTG | |

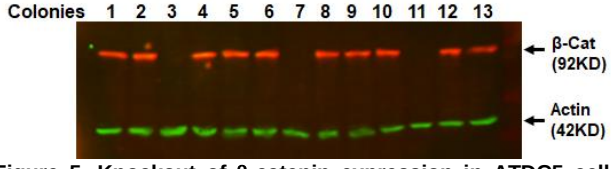


**Figure 5. Knockout of β-catenin expression in ATDC5 cells.** Actin was used for loading control.

To test whether the specificity of Pen-6-cdp effect on PTHrP-induced cAMP production and intracellular $Ca^{2+}$ mobilization is due to separation of β-catenin from PTHR, we used wild-type (WT Ctr) or β-catenin knockout (β-Cat KO) ATDC5 cells. We demonstrated that Pen-6-cdp failed to enhance PTHrP-induced cAMP formation (**Figure 6A**) and reduce intracellular $Ca^{2+}$ mobilization (**Figure 6B**) in β-catenin knockout cells compared to wild-type ATDC5 cells.

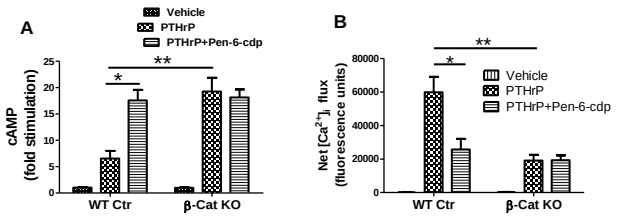


**Figure 6. Disruptor peptide fails to affect PTH-induced PTHR signaling in β-catenin knockout cells.** *, $p< 0.05$, **, $p< 0.01$, compared with WT Ctr cells treated with PTH.

## f. Effect of disruptor peptide on inhibition of ATDC5 cell differentiation *in vitro*.

Mouse ATDC5 cells undergo an orderly series of chondrogenic differentiation. After ATDC5 cell confluence, We used chondrogenic medium containing ITS (10 μg/ml insulin, 10 μg/ml transferrin, and 10 ng/ml sodium selenite) to induce ATDC5 cell differentiation in the presence or absence of PTHrP, disruptor peptide and control peptide conjugated with penetratin. Mouse primer sequences are listed in **Table 2**. The mRNA expression of type II collagen a1 (Col2a1) and Col10a1 was measured dynamically by quantitative real-time PCR using QuantiTect SYBER Green PCR Kit (Qiagen) and normalized to β-actin mRNA. Vehicle, PTHrP (10 nM) with or without Pen-6-cdp (10 μM) were added to the cultures for the first 6 h of each 48 h cycle. The culture medium was changed to fresh medium every other day. Our results show that PTHrP significantly increased *Col2a1* mRNA expression (**Figure 7**) and inhibited *Col10a1* mRNA expression (**Figure 8**). These effects of PTHrP was further enhanced by the peptide.

**Table 2. Mouse primer sequences for real-time PCR**

| Gene | Sequence | Accession number |
|---|---|---|
| **Col2a1** | Forward: 5'- agg cag aca gta cct tga ga<br>Reverse: 5'- ttg gga tca atc cag tag tc | NM_001113515 |
| **Col10a1** | Forward: 5'- ctc aaa tac cct ttc tgc tg<br>Reverse: 5'- cct ctt act gga atc cct tt | NM_009925 |
| **β-actin** | Forward: 5'- aac acc cca gcc atg tac gta g<br>Reverse: 5'- gaa ccg ctc att gcc gat agt | AF122902.1 |

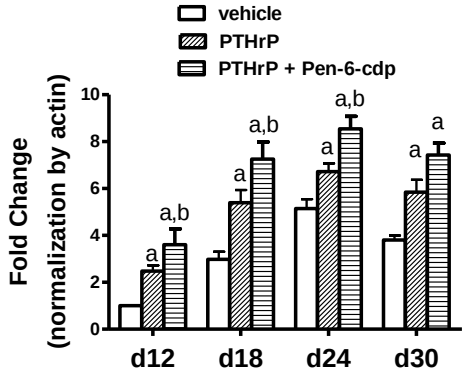


**Figure 7. Disruptor peptide enhances PTHrP-induced Col2a1 mRNA expressions in ATDC5 cells.** a, $p<0.05$, compared with vehicle control; b, $p<0.05$, compared with PTHrP.

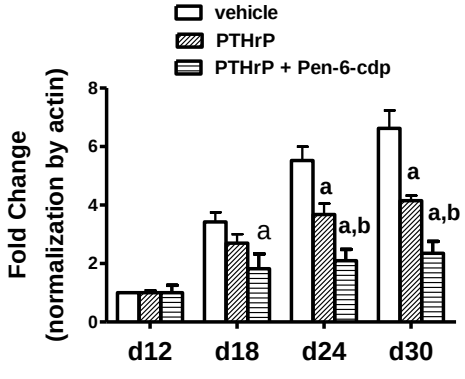


**Figure 8. Disruptor peptide promotes PTHrP-inhibition of Col10a1 mRNA expressions in ATDC5 cells.** a, $p<0.05$, compared with vehicle control; b, $p<0.05$, compared with PTHrP.

## g. Effect of disruptor peptide on PTHrP stimulation of cartilage nodule formation.

Chondrocytes derived from 11.5 days of embryo (E11.5) limb bud cells in high density micromass cultures produce extracellular matrix that contains proteoglycans and is positive to Alcian blue staining. The morphology of cartilage nodule formation was visualized dynamically for up to 6

days using EVOS FL Auto Cell Imaging System. The cartilage nodules appeared by day 2 and increased gradually over a 6-day time period (**Figure 9A**). To quantify the Alcian blue staining intensity, the wells in the stained culture plate were extracted with 6 M guanidine HCl for 10 h at room temperature. The absorbance of the extracted dye was measured at 650 nm. Limb bud mesenchymal cells in micromass cultures express PTH receptor. We assessed the effect of disruptor peptide on cartilage nodule formation induced by PTHrP. PTHrP (10 nM) was added to the culture for the first 6 h of each 48 h cycle for 4 days in the presence or absence of Pen-6cdp (10 μM). The culture medium was changed to fresh medium every other day. Our results show that PTHrP significantly increased cartilage nodule formation compared with vehicle. The disruptor peptide promoted PTHrP stimulation of cartilage nodule formation (**Figure 9B**).

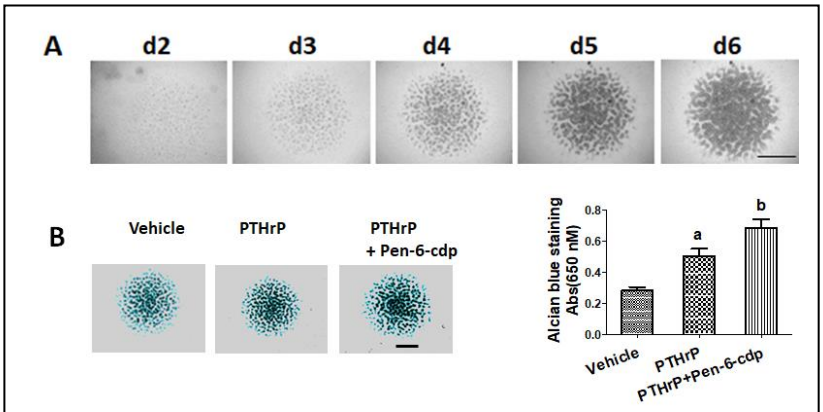


**Figure 9. Pen-6-cdp enhances PTHrP-stimulated cartilage nodule formation**. **A**. Cartilage nodule formation for d2 to d6. **B**. Effects of PTHrP in the presence or absence of Pen-6-cdp for 4d on cartilage nodule formation. a, $p<0.01$, compared with vehicle control; b, $p<0.05$, compared with PTHrP.

### h. Effect of disruptor peptide on PTHrP inhibition of chondrocyte hypertrophy

Chondrocytes derived from limb bud mesenchymal cells secrete abundant matrix. The cartilaginous tissue was manually removed for histologic evaluation. The expression of Col X level, a chondrocyte hypertrophic marker, was not detected by immunostaining on day 7, but increased on day 21 (**Figure 10**). Treatment with PTHrP in the presence or absence of Pen-6-cdp was the same as before. PTHrP inhibited Col X expression. Consistent with the effect of the disruptor peptide on cartilage nodule formation, Pen-6-cdp promoted PTHrP inhibition of chondrocyte hypertrophy.

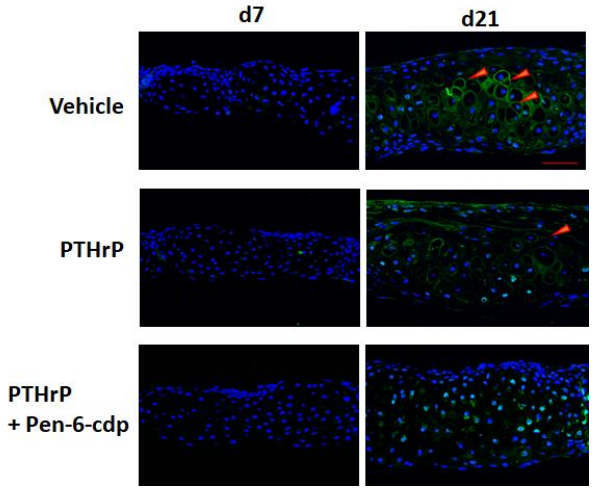


**Figure 10. Pen-6-cdp promotes PTHrP inhibition of chondrocyte hypertrophy by reducing Col X expression.**

### 3.4. Other achievements

None

### What opportunities for training and professional development has the project provided?

Nothing to Report

### How were the results disseminated to communities of interest?

Nothing to Report

**What do you plan to do during the next reporting period to accomplish the goals?**
**Effect of disruptor peptide on protection of cartilage damage in a mouse OA model.**

After termination of treatments of PTHrP in the presence or absence of Pen-6-cdp, whole right and left knee joints from half of the mice in each group will be harvested separately. Histological changes and analyses of disease severity will be examined by hematoxylin & eosin (H&E) and Safranin O staining using standard protocols. The knee cartilage lesions will be analyzed. The markers of cartilage hypertrophy, such as, Col10a1 and MMP13, will be determined by immunohistochemical analysis. MicroCT scanning will be conducted by analyzing the microarchitecture alteration of subchondral bone. Bone volume in subchondral bone will be analyzed. All the data will be analyzed and compared for protective effects of disruptor peptide on cartilage damage.

## *4.* IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report

**What was the impact on other disciplines?**

Nothing to Report

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Nothing to Report

## 5. CHANGES/PROBLEMS:

Nothing to Report

## 6. PRODUCTS:

Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Bin Wang, Ph.D., Principal Investigator (4.8 calendar months), Assistant Professor of Medicine, Center for Translational Medicine, Department of Medicine. As PI, Dr. Wang is responsible for all experiments outlined in the specific aims of the project including the design, performance, analysis, interpretation of experimental studies, preparation of manuscripts, and reporting results of the work.

Irving M Shapiro, BDS, PhD, Other Significant Contributor (no effort requested). Dr. Shapiro is an internationally-recognized cartilage biologist and Anthony and Gertrude DePalma Professor of Orthopaedic Surgery, Director, Division of Orthopaedic Research, Department of Orthopaedic

Surgery. He discusses with Dr. Wang about data interpretation, and any design/troubleshooting issues.

Research Technician, Yanmei Yang (12 calendar months), is responsible for routine cell culture, preparing media and buffers for general experiment usage. She performs biochemical experiments, including the cAMP assay, Western blotting and real time PCR, and assist in all other experiments performed by Dr. Wang, including animal studies.

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

**What other organizations were involved as partners?**

Nothing to Report

## 8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

## 9. APPENDICES:

Nothing to Report