Deletion of the PH-domain and Leucine Rich Repeat Protein Phosphatase 1 (Phlpp1) Increases Fibroblast Growth Factor (Fgf) 18 Expression and Protects Against Surgically-Induced Osteoarthritis

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Introduction:
Osteoarthritis (OA) is a degenerative joint disease and leading cause of disability. Characterized by degradation of articular cartilage, synovitis, subchondral bone thickening, osteophytes, and other joint changes, OA is extremely painful and debilitating. Joint replacement is the only cure for OA, but implants are not available for all joints and surgery poses many risks. Current treatments (including over-the-counter pain medications and intra-articular injections of corticosteroids or hyaluronates) are palliative and do not modify the disease process. Today, there is no FDA-approved disease modifying OA drug (DMOAD). Although Fgf18 is an emerging DMOAD candidate, additional strategies are needed to promote articular cartilage repair and/or slow articular cartilage degradation.

In response to the structural and biomechanical changes during OA progression, articular chondrocytes re-initiate a development-like process where they begin to proliferate, enter hypertrophy and eventually ossify or undergo apoptosis. As this continues, chondrocytes produce fewer gene transcripts encoding for cartilage matrix proteins and increase production of cartilage degrading enzymes. We recently identified the protein phosphatase Phlpp1 as an effector of chondrocyte differentiation in vitro [1]. Phlpp1 terminates Akt2 signaling but also dephosphorylates and inactivates other substrates to arrest matrix production and induce apoptosis [2]. In this study, we hypothesized that Phlpp1 contributes to OA disease progression and inhibits proper endochondral bone formation in humans and mice.

Methods:

Phlpp1 Deficient Mice: The Mayo Clinic Institutional Animal Care and Use Committee approved all animal research.

Isolation, Culture, and Analysis of IMAC Cells: Immature mouse articular chondrocytes (IMACs) were collected from Phlpp1⁻/⁻ or WT littermates that were 5 days old as previously described [3]. Cultures were treated with 10 µM U0126 (Erk1/2 inhibitor), 0.1 µM PD173074 (FgfR inhibitor), NCS 117079 (Phlpp inhibitor) [4] or DMSO. Cell lysates were collected for Western blotting [1]. Total RNA was extracted using TRizol and chloroform. mRNA (2 µg) was reverse transcribed and the resulting cDNAs were used to assay gene expression via real-time PCR.

DMM Surgery and Histological Assessments: OA was induced by destabilizing the medial meniscus (DMM) of the right hind limb in 12 week-old, male mice (n = 7 WT, 7 Phlpp1⁻/⁻) as described [5]. Limbs were dissected 12 weeks after surgery and fixed in 10% neutral buffered formalin, decalcified in 15% EDTA, and embedded in paraffin. Sections were collected every 100 µM through the entire joint and
Safranin O/Fast Green staining was performed. Osteoarthritis Research Society International (OARSI) scores (p = 0.03) were assessed by three blinded reviewers [6].

**Immunohistochemical Staining:** Tibias from 5-day old Phlpp1−/− (n = 5) or WT (n = 5) littermates were fixed in 10% neutral buffered formalin, decalcified in 15% EDTA for 7 days, and embedded in paraffin. Surgically discarded, de-identified articular cartilage was collected from patients undergoing joint arthroplasty for OA or a femoral neck fracture (FNFx) repair. Immunohistochemical (IHC) staining was performed with Phlpp1 and BrdU antibodies.

**Statistical Analysis:** Data are shown as the mean ± standard error of the mean (SEM). p values were determined with the student’s t-test.

**Results:**

PHLPP1 was highly expressed in human articular cartilage from OA patients, but was undetectable by IHC in articular cartilage samples from patients suffering an FNFx (Fig 1A). To determine if Phlpp1 contributes to OA progression, we evaluated the effects of Phlpp1 deficiency on OA progression in mice using the DMM model [5]. Phlpp1−/− mice showed significant reductions in OARSI scores and subchondral bone thickening (Fig 1B), suggesting that loss of Phlpp1 produces a protective effect against OA progression.

**Figure 1.** (A) PHLPP1 IHC in human OA articular cartilage or control, FNFx articular cartilage. (B) OARSI scores from DMM surgeries performed on WT or Phlpp1−/− mice. (C) Fgf18 expression in Phlpp1−/− cells. (D) Fgf18 expression and Alcian blue staining of micromasses treated with the Phlpp1 inhibitor NCS117079.

To elucidate the mechanism of this protective effect, we examined the effects of Phlpp1 deficiency during bone development. Phlpp1 null mice exhibit reduced bone mass and growth plate abnormalities, including increased chondrocyte proliferation and matrix production. Furthermore, Phlpp1 deficiency promoted expression of Fgf18 (Fig. 1C), a growth factor that facilitates chondrogenesis, chondrocyte proliferation and cartilage regeneration in surgically-induced models of osteoarthritis. Erk1/2 activity and chondrocyte proliferation were also elevated. Chemical inhibition of the Fgf18 receptor, Fgfr3, abrogated increased Erk1/2 phosphorylation observed in Phlpp1 null chondrocytes. Furthermore, blocking the function of Fgfr3 or Erk1/2 suppressed proliferation induced by Phlpp1 deficiency.
Increased production of Fgf18 was attributed to diminished FoxO1 levels in Phlpp1−/− cells. Phlpp inhibitors (Fig 1D&E) also augmented Erk1/2 phosphorylation, matrix content and Fgf18 production.

**Discussion:**
In this study we show that PHLPP1 is highly expressed in articular cartilage from osteoarthritis (OA) patients but not in healthy articular cartilage. Preliminary data suggest that this increased PHLPP1 expression in OA human articular cartilage is due to CpG island demethylation of the Phlpp1 promoter. Importantly, Phlpp1 genetic deficiency protected against OA progression in a surgically induced mouse model. We found that chondrocyte proliferation and cartilaginous matrix production are enhanced in Phlpp1−/− mice due increased production of Fgf18 and consequent activation of Erk1/2 activity. Together, these results demonstrate that suppression of Phlpp1 expression and/or activity is protective against cartilage degeneration in OA.

**Significance:**
Phlpp1 contributes to cartilage development and OA progression and may be a therapeutic target for OA treatment.

ORS 2015 Annual Meeting
Poster No: 0400