Deletion of the PH-domain and Leucine-rich Repeat Protein Phosphatase 1 (Phlpp1) Increases Fibroblast Growth Factor (Fgf) 18 Expression and Promotes Chondrocyte Proliferation*

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Background: Phlpp1 is a tumor suppressor that represses Akt2 and other signaling pathways.

Results: Chondrocyte proliferation, matrix production, Akt2 phosphorylation, and Fgf18/Erk1/2 signaling were increased in Phlpp1−/− mice, but levels of the transcription factor FoxO1 were reduced.

Conclusion: Phlpp1 deficiency increases Akt2 activity, which diminishes FoxO1 levels and induces Fgf18 expression to stimulate Erk1/2 activity and chondrocyte proliferation.

Significance: Phlpp1 inhibition may promote cartilage regeneration.

Endochondral ossification orchestrates formation of the vertebrate skeleton and is often induced during disease and repair processes of the musculoskeletal system. Here we show that the protein phosphatase Phlpp1 regulates endochondral ossification. Phlpp1 null mice exhibit decreased bone mass and notable changes in the growth plate, including increased BrdU incorporation and matrix production. Phosphorylation of known Phlpp1 substrates, Akt2, PKC, and p70 S6 kinase, were enhanced in ex vivo cultured Phlpp1−/− chondrocytes. Furthermore, Phlpp1 deficiency diminished FoxO1 levels leading to increased expression of Fgf18, Mek/Erk activity, and chondrocyte metabolic activity. Phlpp inhibitors also increased matrix content, Fgf18 production and Erk1/2 phosphorylation. Chemical inhibition of Fgfr-signaling abrogated elevated Erk1/2 phosphorylation and metabolic activity in Phlpp1-null cultures. These results demonstrate that Phlpp1 controls chondrogenesis via multiple mechanisms and that Phlpp1 inhibition could be a strategy to promote cartilage regeneration and repair.

Skeletal formation is accomplished in part through the process of endochondral ossification. In this developmental program, a cartilage intermediate first arises to serve as a template for de novo bone formation (reviewed in Ref. 1). Strict control of chondrocyte maturation during the cartilaginous phase and subsequent coupling to bone formation ensures acquisition of optimal bone length, density, and shape. Disruption of this process during embryogenesis causes chondro dysplasias and growth plate injuries during adolescence can cause limb shortening (2, 3). Cartilage-specific genetic deficiency of anabolic factors can also result in decreased bone density, highlighting the importance of growth plate cartilage coupling to bone formation (4–6). The endochondral ossification process is reinitiated in adults during fracture healing, heterotopic ossification, and osteoarthritic progression (7).

A number of growth factors and cytokines (e.g. BMPs, Fgfs, Igf1, Hhs, Tgfβ) contribute to the formation and maturation of the cartilaginous phase of endochondral ossification (1). Many of these morphogens directly or indirectly activate Akt (protein kinase B) isoforms that are essential for chondrocyte differentiation (8–16). Akt activity is controlled by phosphorylation and substrate interactions via scaffolding proteins. A number of phosphatases, including PH-domain leucine-rich repeat protein phosphatases (Phlpp)2 1/2, dampen Akt activity to promote apoptosis and inhibit cell proliferation (17–19). Genetic deletion of Phlpp1 (also known as Phlpp, Scop, Plekhe1) slightly reduced snout-to-tail body lengths, but the effects of Phlpp1 on chondrocytes or growth plate structure were not studied (20).

We previously demonstrated that Hdac3 represses Phlpp1 transcription and that Phlpp1 is expressed at higher levels in Hdac3 suppressed chondrocytes (21). Here we examined the effects of Phlpp1 deficiency on skeletal development. Phlpp1−/− mice show increased chondrocyte proliferation and matrix production. Molecular analyses demonstrated Phlpp1 deficiency increased phosphorylation of Akt2, Fgfr3, Mek1/2, and Erk1/2. The elevation in Erk1/2 activity was attributed to increased production of Fgf18, as blocking Fg receptor (Fgfr)-mediated signaling decreased Erk1/2 phosphorylation. Furthermore, Fgfr inhibition suppressed chondrocyte proliferation induced by Phlpp1 deficiency. We conclude that Phlpp1 deficiency promotes chondrocyte differentiation via multiple pathways.

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2The abbreviations used are: Phlpp, PH-domain leucine-rich repeat protein phosphatase; BMP, bone morphogenetic protein; Erk, extracellular signal-regulated kinase; Fgf, fibroblast growth factor; Fgfr, fibroblast growth factor receptor; FoxO, forkhead box O; Hdac3, histone deacetylases; Hhs, hedgehogs; HRP, horseradish peroxidase; Igf1, insulin-like growth factor 1; PH, pleckstrin homology; PKC, protein kinase C; Plekhe, PH-domain-containing family E member; Scop, suprachiasmatic nucleus circadian oscillatory protein; Tgfβ, transforming growth factor β.

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Phlpp1-deficient Mice—Phlpp1−/− (20) and wild type (WT) littermates were maintained in an accredited facility with 12-hour light/dark cycles and supplied water and food (Pico-Lab® Rodent Diet 20, LabDiet) ad libitum. All animal research was conducted according to National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council guidelines. The Mayo Clinic Institutional Animal Care and Use Committee preapproved all animal studies. For BrdU labeling studies, mice (n = 5) were injected when they were 5 days old (P5) with 50 μg of 5-bromo-2′-deoxyuridine (BrdU) labeling reagent (Invitrogen) per kg body weight 2 h prior to euthanasia.

Microcomputed Tomography—Bone volume density in the secondary spongiosa of female distal femurs was measured with ex vivo micro-computed tomography (μCT) imaging (μCT35; Scanco Medical AG, Basserdorf, Switzerland). Trabecular bone scans were performed at 7 μm voxels with an energy setting of 70 kVp and a 300 ms integration time on 8-week-old Phlpp1−/− (n = 4) or WT (n = 3) mice.

Immunohistochemical Staining—Tibias from P5 Phlpp1−/− (n = 5) or WT (n = 5) littermates were fixed in 10% neutral buffered formalin, decalcified in 15% EDTA for 7 days, and paraffin embedded. Immunohistochemical (IHC) staining was performed with antibodies directed to BrdU, Ki67 (Cell Signaling Technologies), or IgG isotype control. Detection was accomplished using the Mouse and Rabbit Specific DAB Detection IHC Kit (AbCam, Cambridge, MA) using the substrate 3,3′-diaminobenzidine (Sigma Aldrich). Sections were counterstained with Alcian blue (1% Alcian blue, pH 6.8) or polystyrene. All animal research was conducted according to Bio-Rad D6 assay (Bio-Rad). Proteins (20 μg) were then resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blotting was performed with antibodies (1:2000 dilution) for pSer371 p70 S6K, pThr202/Tyr204-Erk1/2, Erk1/2, pSer217/221 Mek1/2, Mek1/2, pSer473-Akt1, pSer473-Akt2, PAN pSer473 Akt, Akt, pSer256 FoxO1, FoxO1, pSer660-PKC, (Cell Signaling Technologies), pTyr724 Fgf3, Fgf3 (Abcam, Cambridge, MA), PKCa (BD Biosciences, San Jose, CA), HA (Covance, Princeton, NJ), Flag (Sigma Aldrich), and tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA) and corresponding secondary antibodies conjugated to hors eradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was detected with the Supersignal West Femto Chemiluminescent Substrate (Pierce). Blots were stripped using Restore Western blot Stripping Buffer (Pierce) and reprobed when needed. Each experiment was repeated at least three times, and data from a representative experiment are shown.

RNA Isolation and Real-time PCR—Total RNA was extracted from chondrocyte cultures using TRIzol (Invitrogen) and chloroform. Messenger RNA (2 μg) was reverse transcribed using the SuperScript III first-strand synthesis system (Invitrogen). The resulting cDNAs were used to assay gene expression via real-time PCR using the following gene-specific primers: Aggrekan (5′-CCGCTTGCAGGGGGGAGTCTG-3′, 5′-GATGATGGCGGACGCGCTGA-3′), Bmp3 (5′-GACCCGGCAGAGGCTTAG-3′, 5′-CGGCTCTGAGCTGGAGGC-3′), Bmp4 (5′-AACATCCAGAGGACCAGTGA-3′, 5′-GGATGCTGTCGAGGGGAG-3′), Bmp6 (5′-TAGCAATCTGTGGGTTGGGTAGC-3′, Col2a1 (5′-ACTGTTAAGTGGGCGAAC-3′, 5′-CACCACAAATTCCTGTTCA-3′), Gapdh (5′-ACAGCCACTTGCGGCAGAGC-3′, 5′-CTGGCCCTCGCATCCAC-3′), Ihh (5′-GCTTTCCTGCCGGAGCC-3′), PTHR (5′-CGAGTGGGTTGGTGAAGAGC-3′, 5′-CCACACAAATTCCTGTTCA-3′), PTHR (5′-ACATCTGTGGGTTGGGTAGC-3′, Col2a1 (5′-ACTGTTAAGTGGGCGAAC-3′, 5′-CACCACAAATTCCTGTTCA-3′), GAPDH (5′-GGAAGGCCATCACACTTCT-3′, 5′-GGCTCACCCTTGAAGAA-3′), Fgf1 (5′-TTCACTGTCAGGACACCCACTCTG-3′, 5′-TGGTAGCTCACTGACATGCAAGA-3′), Fgf2 (5′-GGCTGCTGGTTCCAATGTC-3′, 5′-TTGAGCTCACTGACATGCAAGA-3′). Fgf1 (5′-TTCACTGTCAGGACACCCACTCTG-3′, 5′-TGGTAGCTCACTGACATGCAAGA-3′), Fgf2 (5′-GGCTGCTGGTTCCAATGTC-3′, 5′-TTGAGCTCACTGACATGCAAGA-3′), Tgfα (5′-TTGAGCTCACTGACATGCAAGA-3′), Fgf1 (5′-TTCACTGTCAGGACACCCACTCTG-3′, 5′-TGGTAGCTCACTGACATGCAAGA-3′), Fgf2 (5′-GGCTGCTGGTTCCAATGTC-3′, 5′-TTGAGCTCACTGACATGCAAGA-3′), Tgfα (5′-TTGAGCTCACTGACATGCAAGA-3′), Tgfβ2 (5′-GATAATGTCCTGGCTGGCCAG-3′, 5′-GGCTGAGGACCTTTGTTGGTGT-3′), Tgfβ3 (5′-GATCAACACACCACAACACACC-3′, 5′-CCAGGTTCGCGAGCAGTAA-3′), Type 2α1
collagen (5’-ACTGGTAAGTGGGCAAGAC-3’, 5’-CCACCACCAATCTCCTGTTCA-3’), Wnt2b (5’-TCCTGGTGTTAGCATAGGGGC-3’, 5’-GAGCGCATGATGTCTGGGTA-3’), Wnt5a (5’-CCACGCTAAGGGTTCCTATGAG-3’, 5’-ACGGCCTGCTTCAATTGTTGTA-3’) (24). Fold changes in gene expression for each sample were calculated using the 2^{ΔΔCt} method relative to control after normalization of gene-specific Ct values to Gapdh Ct values. Each experiment was performed in triplicate and repeated at least three times. The average of these experiments is reported.

Image Quantitation—Images were digitally scanned or collected using phase contrast microscopy. Densitometric values were obtained after converting each image to gray scale and measuring the mean gray values of each condition using Image J software.

Statistical Analysis—Data obtained are the means ± S.E. p values were determined with the Student’s t test.

Results

Phlpp1 Deficiency Reduces Bone Length and Density—Phlpp1-deficient mice were previously reported to have slightly decreased snout-to-tail lengths and decreased body weight compared with their WT littermates (20). We confirmed shorter femur lengths (Fig. 1A) and further found a decrease in bone mineral density. Micro-CT analyses revealed that Phlpp1 deficiency decreased bone volume density by 23% at 8 weeks of age (Fig. 1B, G, H). This was associated with a decrease in trabecular number (Tb.N) (Fig. 1C). Decreased bone mineral density was still apparent in mice at 12 weeks of age (data not shown). Trabecular thickness and trabecular spacing were not altered (Fig. 1D and E). A trend toward
decreased connective density was noted (Fig. 1F). Thus, Phlpp1 deficiency prevents bone lengthening and bone mass accrual.

Phlpp1 Suppresses Chondrocyte Proliferation and Matrix Production—To better understand how Phlpp1 deficiency might stunt bone lengthening and endochondral bone formation, we examined the growth plates of young mice. Tibial growth plates from Phlpp1/H11002/H11002 mice had 50% more cells per proliferative zone area as well as more intense Alcian blue staining (Fig. 2, A and B). BrdU labeling experiments revealed an increase in the number of BrdU-positive cells in Phlpp1-null mice (Fig. 2C). The number of Ki67-positive cells was also enhanced in Phlpp1−/− tibias (data not shown). Thus, despite shorter lengths and reduced endochondral ossification, growth plate chondrocyte proliferation, and matrix content appear to be increased in Phlpp1-deficient long bones.

To confirm our in vivo observations, immature murine chondrocytes were cultured ex vivo in micromasses as previously described (21, 22). After 3 days, the chondrocyte micromasses derived from Phlpp1−/− mice stained more intensely with Alcian blue (Fig. 3A). Phlpp1−/− chondrocytes also produced more transcripts for two cartilage matrix genes, type 2a1 collagen (Col2) and aggrecan (Fig. 3, B and C). PTHR expression was slightly increased, but expression of Ihh and PTHrP was unchanged (Fig. 3, D–F). Consistent with data from other cell types (25), the phosphorylation of Akt2, but not Akt1, was increased in Phlpp1 deficient cultures (Fig. 3G). PKC phosphorylation also increased and PKCα was more abundant and stable, as previously observed (26). FoxO1 stability is negatively regulated by Akts, including Akt2 (27–30). In accordance with increased Akt2 activity, phosphorylated and inactive FoxO1 levels increased by 42%, and total levels were reduced by 20% in Phlpp1 KO chondrocytes (Fig. 3G).

Phlpp1 Deficiency Increases Fgf18 Production and Activates Fgfr3, Mek, and Erk1/2 Signaling—To determine how Phlpp1 affects chondrocytes, we surveyed a panel of morphogens known to regulate cartilage development (Fig. 4A). Several cytokines (including Tgfβ2/3 and Wnt2b) were modestly higher in Phlpp1−/− cells, but the most highly induced gene of those tested was Fgf18 (Fig. 4A). Fgf18 binds to Fgfrs. To determine if
increased phosphorylation of Mek1/2 and Erk1/2 was due to direct effects of Phlpp1 (19) or due to signaling through Fgfr3 (31–33), we examined Fgfr3 activation. Fgfr3 phosphorylation was increased in Phlpp1/H11002/H11002 chondrocytes (Fig. 3G). An Fgfr inhibitor, PD173074 (34, 35), decreased phosphorylation of Mek1/2 and Erk1/2 in Phlpp1/H11002/H11002 mice to WT levels (Fig. 4, B and C). Phlpp1/H11002 chondrocytes showed elevated MTS activity as compared with WT cells (Fig. 4D). The Fgfr inhibitor decreased MTS activity of Phlpp1/H11002 chondrocytes in a concentration-dependent fashion, but WT chondrocyte MTS activity was unaffected at any concentration (Fig. 4D). A Mek1/2 inhibitor, U0126, also decreased MTS activity of Phlpp1/H11002 chondrocytes at both concentrations tested (Fig. 4D). WT chondrocyte MTS activity was unaffected at 10 μM, but was suppressed at 50 μM. These results demonstrate that Phlpp1 suppresses chondrocyte metabolic activity by negatively impacting Fgfr/Mek1/2/Erk1/2 signaling.

FoxO1 Represses Fgf18 Expression in Chondrocytes—FoxO1 represses Fgf18 transcription (36) and therefore may link enhanced Akt2 signaling to Erk1/2 activation in Phlpp1/H11002/H11002 chondrocytes. To test this hypothesis, a Phlpp1 deletion mutant (Phlpp1/H9004C) that inhibits interactions between Akt2 and Phlpp1 was introduced in chondrogenic ATDC5 cells (18). Phlpp1/H9004C promoted Fgf18 production (Fig. 5). This induction was blocked by expression of a constitutively active form of FoxO1 (FoxO1A3, Fig. 5). Furthermore, DNA binding of FoxO1 was required to repress Fgf18 expression as a FoxO1 mutant construct lacking the DNA-binding domain (FoxO1A3HR)
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Chemical Inhibition of Phlpp Induces Fgf18 Expression and Erk1/2 Activation—We next determined if the Phlpp inhibitor, NCS 117079, promotes anabolic signaling in chondrogenic ATDC5 cells (23). This Phlpp inhibitor dose-dependently increased activation of key signaling pathways, including Akt and PKC (Fig. 6A). Erk1/2 and p70 S6 kinase phosphorylation was induced at the 25 μM concentration (Fig. 6A). The Phlpp inhibitor also increased Fgf18 expression and Alcian blue staining of micromass cultures (Fig. 6, B and C). Together these data demonstrate that Phlpp1 inhibition promotes chondrocyte proliferation and Fgf18 expression (Fig. 7).

Discussion

Because Phlpp1 deficiency causes growth retardation, we sought to determine if Phlpp1 regulates chondrocyte function during endochondral ossification. Our results show that Phlpp1 is a regulator of endochondral bone development, as chondrocyte proliferation and matrix synthesis are enhanced in Phlpp1-deficient mice. This likely occurs due to the ability of Phlpp1 to directly control the activity of anabolic signaling pathways, including Akt2, PKC, and p70 S6 kinase, that are known facilitators of proliferation and matrix production by chondrocytes (21, 37, 38). Activation of these pathways triggers subsequent events, including but not limited to, Fgf18 production and downstream activation of Fgfr and Mek/Erk signaling. Thus, Phlpp1 is active during endochondral bone formation. The effects of Phlpp1 deletion are similar to those observed in mice lacking GDF5 or HIF1α that are characterized by increased chondrocyte proliferation and decreased long bone length (39, 40). The effects of Phlpp1 deficiency on distinct chondrocytes populations, osteoblasts and/or osteoclasts in the developing bone cannot be addressed with this model. Targeted and regulated deletion of Phlpp1 in these and other cell types are needed to fully understand why germline Phlpp1 deficiency produces shorter and thinner bones despite increased BrdU labeling and matrix production.

Phlpp1 preferentially dephosphorylates Akt2, while minimally affecting Akt1 or Akt3 in non-cartilagenous tissues (25).
We likewise found that Akt2 phosphorylation was enhanced in Phlpp1−/− chondrocytes. Although little is known about the specific role of Akt2 on chondrocyte differentiation, Akt2−/− mice have reduced body size (41), suggesting a possible growth plate defect. Akt1 phosphorylation was minimally affected in Phlpp1−/− chondrocytes. The substrate preference for Akt2 may explain why both Hdac3 cKO and Phlpp1−/− mice have reduced bone mass. Although Phlpp1 was increased in Hdac3 cKO chondrocytes, the phosphorylation of both Akt1 and Akt2 were reduced (21), suggesting that additional phosphatases, besides Phlpp1, may be deregulated in Hdac3 cKO chondrocytes.

We also noted that Phlpp1 deficiency increases expression of several growth factors, including robust induction of Fgf18. We also demonstrated enhanced Fgf18 production and signaling through Fgfr leading to enhanced Mek/Erk activation and chondrocyte proliferation (Fig. 7). The effects on increased growth factor expression on matrix production, however, were not explored here. Fgf18 is naturally expressed within joints, the perichondrium and growth plates of developing bones (42, 43). Our results are inconsistent with two main observations: 1) that germline Fgf18 deficiency increases chondrocyte proliferation during embryonic development (42, 43), and 2) that Fgfr3 activation decreases chondrocyte proliferation and causes limb shortening in humans (44). However, other studies demonstrate that Fgf18 promotes chondrocyte proliferation and cartilage maintenance, particularly of articular chondrocytes (32, 43, 45–49). Moreover, gain-of-function Fgfr3 mutations expand the pool of proliferative, Sox9-positive chondrocytes and decrease chondrocyte hypertrophy (50, 51). Fgf18 also promotes chondrogenesis, chondrocyte proliferation and cartilage regeneration in surgically induced models of osteoarthritis (31, 32, 43, 52–56). In clinical trials, intra-articular injections of recombinant Fgf18 resulted in dose-dependent increases in total cartilage content, even though thickness of the central medial femorotibial compartment cartilage was unchanged (57). Thus, the effects of Fgf18/Fgfr signaling are complex and may be temporally and spatially controlled. Here we show that increased Fgf18/Fgfr signaling is responsible for some of the effects of Phlpp1 deficiency in micromass cultures. However, the role of Fgf18 within the expanded proliferative zone of the Phlpp1−/− growth plate remains to be determined. Given that Phlpp1 loss of function, either via genetic deficiency or small molecule inhibition, promotes chondrocyte maturation and Fgf18 production in vitro, Phlpp1 is an enticing target for cartilage tissue engineering.

Other groups demonstrated the importance of Akt activity in controlling Fgf18 production and Mek/Erk activation. Conditional deletion of PTEN, a phosphatase that suppresses PI3K/Akt signaling upstream of Phlpp1, in limb progenitor cells enhanced Fgf18 expression (36). The authors attributed this regulation to decreased levels of FoxO1, which represses Fgf18 promoter activity (36). We also found that disrupting the substrate binding ability of Phlpp1 enhances Fgf18 expression, and that this effect is antagonized by expression of an Akt-insensitive FoxO1 mutant.

In summary, we show that germline deletion of Phlpp1 disrupts limb development, but promotes chondrogenesis and increases cartilaginous matrix production, which may be attributed to increases in cartilage anabolic signaling pathways including Akt2, PKC, and p70 S6 kinase, and relief of FoxO1 suppression of Fgf18. Elevated Fgf18 levels signal through Fgfr to activate Mek/Erk signaling and proliferation. Importantly, Phlpp1 chemical inhibition enhances signaling pathway activation, Fgf18 expression and cartilage matrix production. Thus, Phlpp1 may serve as a target to promote cartilage maintenance and/or regeneration and prevent hypertrophic ossification.

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