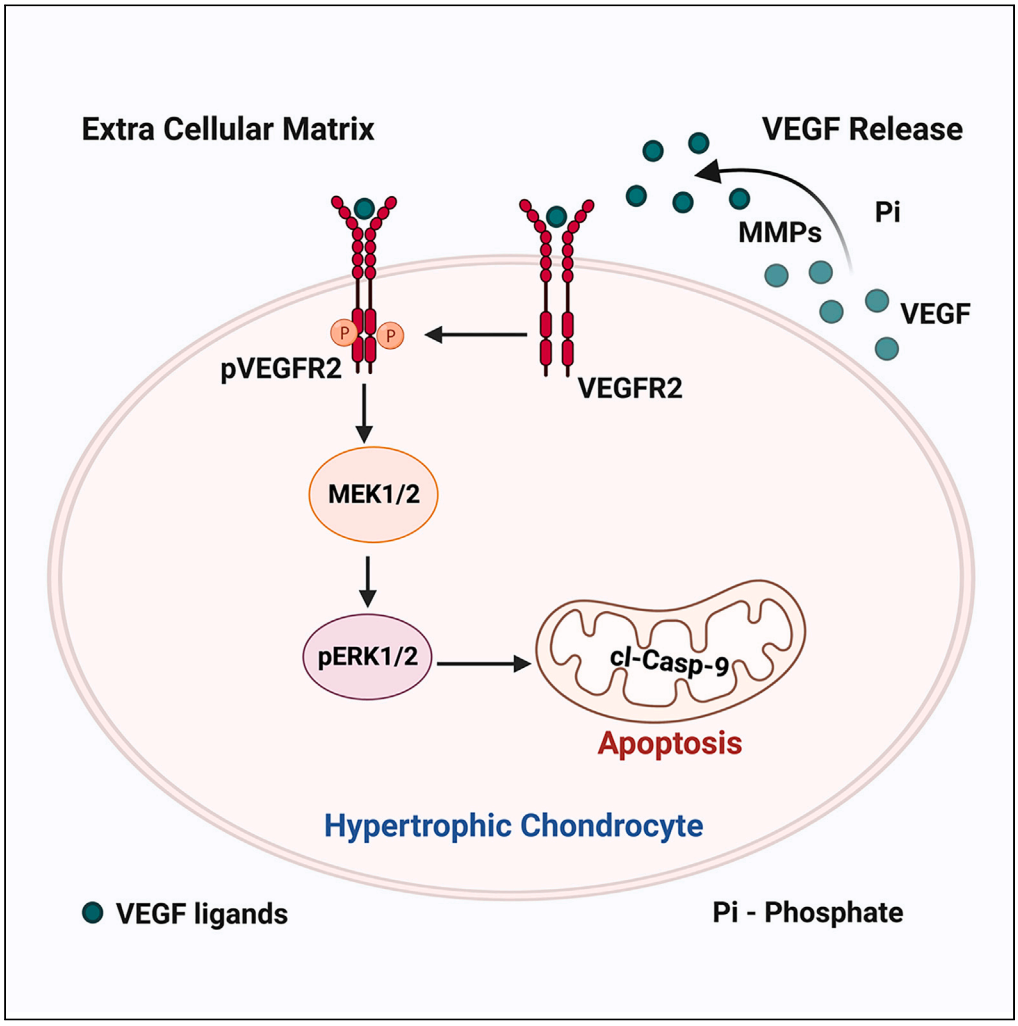
iScience

CellPress
OPEN ACCESS

Article

Phosphate-induced activation of VEGFR2 leads to caspase-9-mediated apoptosis of hypertrophic chondrocytes



Prem Swaroop Yadav, Garyfallia Papaioannou, Margaret M. Kobelski, Marie B. Demay

demay@helix.mgh.harvard.edu

Highlights

VEGFR2 mediates the actions of Pi on hypertrophic chondrocyte apoptosis

VEGFR2 ligands activate hypertrophic chondrocyte apoptosis

Pi increases the bioavailability of VEGF ligands in cultured hypertrophic chondrocytes

Dietary phosphate restriction exacerbates the growth plate defects in VEGFR2 KO mice

Yadav et al., iScience 26, 107548
September 15, 2023
https://doi.org/10.1016/j.isci.2023.107548

iScience

CellPress
OPEN ACCESS

Article

Phosphate-induced activation of VEGFR2 leads to caspase-9-mediated apoptosis of hypertrophic chondrocytes

Prem Swaroop Yadav,[1,2] Garyfallia Papaioannou,[1,2] Margaret M. Kobelski,[1] and Marie B. Demay[1,2,3,*]

SUMMARY

Low circulating phosphate (Pi) leads to rickets, characterized by expansion of the hypertrophic chondrocytes (HCs) in the growth plate due to impaired HC apoptosis. Studies in HCs demonstrate that Pi activates the Raf/MEK/ERK1/2 and mitochondrial apoptotic pathways. To determine how Pi activates these pathways, a small-molecule screen was undertaken to identify inhibitors of Pi-induced ERK1/2 phosphorylation in HCs. Vascular endothelial growth factor receptor 2 (VEGFR2) was identified as a target. *In vitro* studies in HCs demonstrate that VEGFR2 inhibitors block Pi-induced pERK1/2 and caspase-9 cleavage. Like Pi, rhVEGF activates ERK1/2 and caspase-9 in HCs and induces phosphorylation of VEGFR2, confirming that Pi activates this signaling pathway in HCs. Chondrocyte-specific depletion of VEGFR2 leads to an increase in HCs, impaired vascular invasion, and a decrease in HC apoptosis. Thus, these studies define a role for VEGFR2 in transducing Pi signals and mediating its effects on growth plate maturation.

INTRODUCTION

One of the hallmark features of endochondral bone formation is the hypertrophic differentiation of chondrocytes, a key event required for the longitudinal growth of developing long bones. These hypertrophic chondrocytes (HCs) undergo apoptosis and are replaced by bone. Hypophosphatemia, due to genetic mutations or dietary phosphate restriction, impairs hypertrophic chondrocyte apoptosis, leading to rickets. Thus, circulating phosphate is a key determinant of hypertrophic chondrocyte apoptosis *in vivo*.[1,2]

The extracellular signal-regulated kinases, ERK1 and ERK2, regulate chondrocyte differentiation during endochondral bone formation.[3] ERK1/2 signaling is essential for the terminal differentiation of hypertrophic chondrocytes. Ablation of ERK1/2 in limb mesenchyme, or chondrocytes causes expansion of the hypertrophic zone in the growth plate,[3,4] consistent with the role of ERK1/2 in hypertrophic chondrocyte terminal differentiation and apoptosis.

ERK1/2 are activated upon phosphorylation by mitogen-activated protein kinase 1/2 (MEK1/2), which in turn are activated by upstream rapidly accelerated fibrosarcoma (RAF) kinases. There are three Raf kinases in mammals: A-Raf, B-Raf, and C-Raf,[5] which play a redundant role in the growth plate. Mice lacking all three Raf isoforms in chondrocytes die neonatally and exhibit a significant expansion of the hypertrophic chondrocyte layer of the growth plate, accompanied by decreased hypertrophic chondrocyte apoptosis.[6] Mice with chondrocyte-specific ablation of A-Raf and B-Raf have normal long bone development[7] whereas chondrocyte-specific deletion of C-Raf results in expansion of hypertrophic chondrocytes due to increased ubiquitin-dependent vascular endothelial growth factor (VEGF) degradation and impaired vascular invasion.[7,8] The PTH/PTHrP signaling pathway inhibits ERK1/2 activation,[9] and delays chondrocyte maturation, thereby maintaining the precise balance between proliferation and differentiation.[7,10,11]

While activation of mitochondrial ERK1/2 signaling is required for cleavage of caspase-9 and induction of hypertrophic chondrocyte apoptosis *in vivo* and *in vitro*, the mechanism by which phosphate (Pi) activates this signaling cascade has not been identified. Thus, an unbiased small-molecule screen of kinase inhibitors was undertaken to identify inhibitors of phosphate-induced ERK1/2 activation in HC. These studies demonstrated that the vascular endothelial growth factor receptor 2 (VEGFR2) signaling pathway not only senses extracellular Pi but also mediates the biological effects of extracellular Pi on growth plate maturation.

[1]Endocrine Unit, Massachusetts General Hospital, Boston, MA, USA

[2]Harvard Medical School, Boston, MA, USA

[3]Lead contact

*Correspondence: demay@helix.mgh.harvard.edu

https://doi.org/10.1016/j.isci.2023.107548

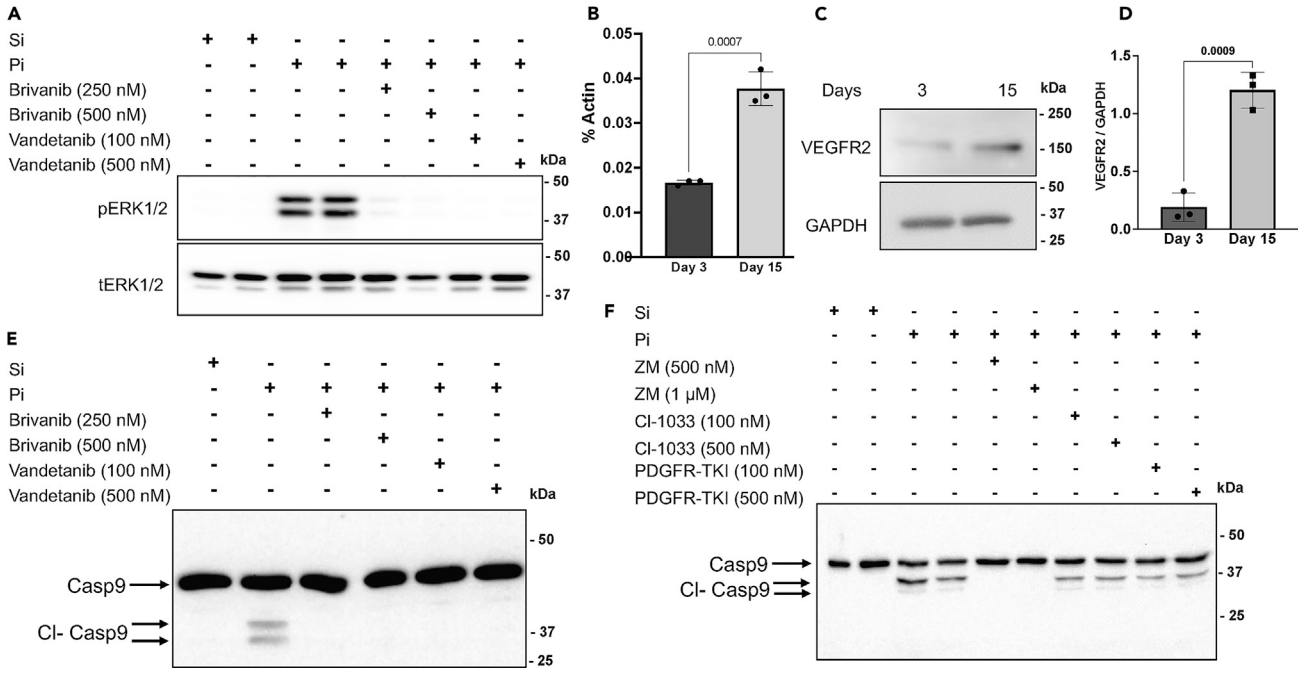


Figure 1. Pi-induced activation of VEGFR2 is required for Pi-induced ERK1/2 phosphorylation and caspase-9 cleavage

(A) HCs were pre-treated for 2 h with the VEGFR2 inhibitors brivanib and vandetanib followed by a 10 min treatment with 7 mM Si or 7 mM Pi. Western analyses were performed using anti-pERK1/2 and anti-tERK1/2 antibodies.

(B) VEGFR2 mRNA expression day 3 (proliferative) and day 15 (hypertrophic) chondrocytes was evaluated by rt-qPCR and normalized for actin in the same sample.

(C) Protein lysates from similar samples were subjected to Western analyses using anti-VEGFR2 and anti-GAPDH antibodies.

(D) Densitometric quantification of western analyses was performed using blots obtained from three independent chondrocyte preparations. Data represent mean +/− the SD; p value is indicated above the line.

(E) HCs were pre-treated for 2 h with brivanib or vandetanib, followed by 18 h of treatment with 7 mM Si or 7 mM Pi. Western analyses were performed using anti-caspase-9 antibody. All data are representative of that obtained from at least three independent chondrocyte preparations.

(F) HCs were pre-treated for 2 h with ZM 323881 HCl (VEGFR2 inhibitor), Cl-1033 (EGFR inhibitor), or PDGFR-TKI (PDGFR Tyrosine Kinase Inhibitor IV), followed by 18 h of treatment with 7 mM Si or 7 mM Pi. Western analyses were performed using anti-caspase-9 antibody. All data are representative of that obtained from at least three independent chondrocyte preparations.

RESULTS

Small-molecule kinase inhibitor screening

Activation of Erk1/2 is required for phosphate-induced hypertrophic chondrocyte apoptosis. To identify the upstream mediator of phosphate-induced ERK1/2 activation in hypertrophic chondrocytes, an unbiased small-molecule kinase inhibitor screen was undertaken using the AlphaScreen SureFire *p*-ERK1/2 Assay. A total of 1438 unique small-molecule inhibitors were screened, and 28 compounds were identified that inhibited Erk1/2 phosphorylation by more than 80% at a concentration of 11 µM (Table S1). Dose-response studies demonstrated that, unlike the concentrations of Jak, ALK, EGFR, PDK1, and aurora kinase inhibitors required to inhibit phosphate-induced pERK1/2 (1–2 µM), the VEGFR2 inhibitors axitinib and AG 13958 were able to do so at lower concentrations (100–200 nM), consistent with their reported IC50 in cell models. Therefore, VEGFR2 was selected for further studies.

To confirm that VEGFR2 signaling is required for Pi-induced ERK1/2 activation, hypertrophic chondrocytes were treated with the VEGFR2 inhibitors, brivanib and vandetanib for 2 h prior to adding Pi or sulfate (Si) as a control anion (Figure 1A). Western analyses demonstrating inhibition of ERK1/2 phosphorylation by these VEGFR2 inhibitors confirm the small-molecule screen results, identifying VEGFR2 as an upstream mediator of Pi-induced ERK1/2 phosphorylation in hypertrophic chondrocytes.

VEGFR2 expression increases during hypertrophic differentiation of chondrocytes

Because phosphate-induced apoptosis of chondrocytes is differentiation dependent,[1] expression of VEGFR2 was examined in primary costal chondrocytes cultured for three (proliferative chondrocytes)

and 15 days (hypertrophic chondrocytes). RT-qPCR analysis demonstrated that VEGFR2 mRNA expression was upregulated 2.7 ± 0.6-fold in hypertrophic vs. proliferative chondrocytes (Figure 1B). Consistent with this, an increase in VEGFR2 protein expression was also observed during the hypertrophic differentiation of primary chondrocytes (Figures 1C and 1D).

VEGFR2 inhibition impairs Pi-induced caspase-9 activation in HC

To determine if Pi-induced activation of VEGFR2 is required for HC apoptosis, HCs were pre-treated with brivanib (250 and 500 nM) and vandetanib (100 and 500 nM) for 2 h prior to the addition of Pi or Si. As shown in previous studies, Pi-induced caspase-9 cleavage,[1,2] however, 2 h of pretreatment with the VEGFR2 inhibitors blocked Pi-induced activation of caspase-9 (Figure 1E). Pretreatment of HCs with ZM 323881 HCl, another highly selective VEGFR2 inhibitor (500 nM and 1 μM), also blocked Pi-induced activation of caspase-9 (Figure 1F), whereas pretreatment of HCs with selective inhibitors of EGFR (CI-1033), and PDGFR (PDGFR Tyrosine Kinase Inhibitor IV) signaling failed to block Pi-induced activation of caspase-9 (Figure 1F). Thus, VEGFR2 signaling is required for phosphate-induced ERK1/2 phosphorylation and caspase-9 activation, key events in phosphate-mediated hypertrophic chondrocyte apoptosis.

Pi induces phosphorylation of VEGFR2 in HCs

To evaluate whether phosphate directly activates VEGFR2, phosphorylation of VEGFR2 was examined 10 min after treatment of HCs with Pi, using two recombinant VEGFA splice variant proteins, VEGF-121 and VEGF-165, as positive controls. Both Pi and rhVEGF induced phosphorylation of VEGFR2 at tyrosine 996 (Y996) (Figure 2A). Previous studies have demonstrated that Pi-induced ERK1/2 phosphorylation requires MEK1/2 activity.[2] While pretreatment of hypertrophic chondrocytes with the MEK1/2 inhibitor U0126 failed to block Pi-induced VEGFR2 phosphorylation (Figure 2A), it blocked both Pi and rhVEGF-induced pERK1/2 (Figure 2B). This suggests that Pi and rhVEGF2 activate ERK1/2 by the same pathway and that phosphorylation of VEGFR2 by rhVEGF and Pi is upstream of ERK1/2 activation. Like Pi, rhVEGF induces cleavage of caspase-9 in HCs (Figure 2C) suggesting that activation of VEGFR2 signaling is the mechanism by which Pi induces HC apoptosis.

Pi increases VEGFA bioavailability

Previous studies have demonstrated that VEGF ligands can be released from the plasma membrane via a mechanism called shedding[12,13] and can also be released from the matrix in response to matrix metalloproteinase (MMP) activity.[14] To determine if Pi treatment leads to increased bioavailable VEGF, VEGFA was quantitated in the conditioned medium of HCs treated with 7 mM Pi or 7 mM Si. A significant increase in VEGFA in the cultured media was observed in response to 10 min of treatment with Pi (Figure 3A). Pretreatment of HCs with a Pan-MMP inhibitor (MMP-V) significantly inhibited the Pi-induced increase in VEGF in the conditioned medium of HCs. Western blot analysis of lysates from the same chondrocytes revealed that MMP inhibition attenuated Pi-induced ERK1/2 phosphorylation (Figures 3B and 3C). Thus, inhibiting matrix metalloprotease activity attenuates phosphate-induced increases in conditioned media VEGFA and pERK1/2 activation.

Chondrocyte-specific deletion of VEGFR2 impairs growth plate development *in vivo*

Since inhibiting VEGFR2 impairs Pi-induced ERK1/2 phosphorylation and hypertrophic chondrocyte apoptosis *in vitro*, investigations were undertaken to define a role for chondrocyte VEGFR2 signaling during long bone development *in vivo*. Mice lacking VEGFR2 in chondrocytes were generated by crossing mice expressing the Cre recombinase under the control of collagen type II regulatory elements (ColII-Cre) with VEGFR2$^{f/KDR}$ heterozygous mice,[15] where one VEGFR2 allele is deleted in the germline (KDR) and the other floxed (f) allele is recombined in chondrocytes. Despite this strategy, VEGFR2 mRNA expression in the hypertrophic chondrocytes of the ColII-Cre+; VEGFR2$^{f/KDR}$ remained at 66% that of VEGFR2$^{f/f}$ mice (Figure 4A) and VEGFR2 protein expression was 33.3% that of VEGFR2$^{f/f}$ mice (Figures 4B and 4C).

The ColII-Cre+; VEGFR2$^{f/KDR}$ mice were born at the normal Mendelian frequency and were visually indistinguishable from their control littermates (ColII-Cre-; VEGFR2$^{f/f}$) at birth. Their long bone phenotype was examined at e15.5, the time of the vascular invasion as well as postnatally. H&E staining of humerus sections demonstrated a decrease in vascular invasion at e15.5, P0, and P8, the latter time point exhibiting a delay in the appearance of the secondary ossification center in ColII-Cre+; VEGFR2$^{f/KDR}$ embryos compared to VEGFR2$^{f/f}$ control littermates (Figures 5A–5C). Consistent with this, decreased immunoreactivity of the endothelial cell marker, Endomucin

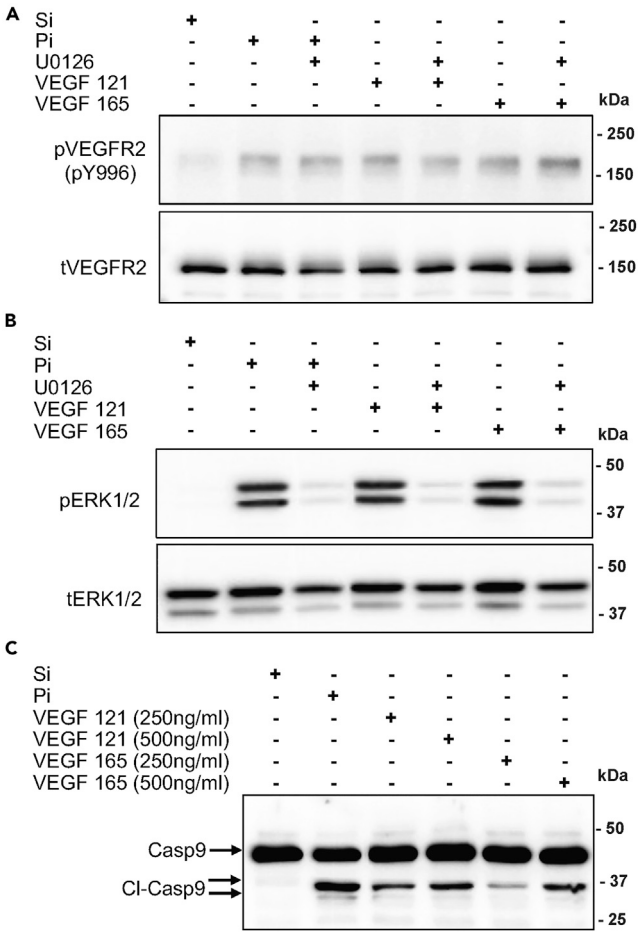


Figure 2. Pi and VEGF ligands induce VEGFR2 tyrosine 996 phosphorylation, ERK1/2 phosphorylation, and caspase-9 cleavage

(A) HCs were pretreated with the MEK inhibitor U0126 (30 μM) for 1 h, followed by treatment with 7 mM Si, 7 mM Pi, rhVEGFA 121, or rhVEGFA 165 (50 ng/mL) for 10 min. Western analyses were performed using anti-p(Y996) VEGFR2 and anti-total VEGFR2 antibodies.

(B) HCs were pretreated with the MEK inhibitor U0126 (30 μM) for 1 h, followed by treatment with 7 mM Si, 7 mM Pi, rhVEGFA 121, or rhVEGFA 165 (50 ng/mL) for 10 min. Western analyses were performed using anti-pERK1/2 and anti-tERK1/2 antibodies.

(C) HCs were treated with 7 mM Si, 7 mM Pi, rhVEGFA 121 or rhVEGFA 165 for 18 h. Western analyses were performed using anti-caspase-9 antibody. Data are representative of that obtained from at least three independent chondrocyte preparations. Cl-Casp9, Cleaved caspase-9.

was observed. This was associated with expansion of the Col X-expressing hypertrophic chondrocyte zone of the ColII-Cre+; VEGFR2$^{f/KDR}$ mice at e15.5, P0, and P8 (Figures 5A–5C). At P0, there was a 1.8-fold increase in the number of hypertrophic chondrocytes per column in the ColII-Cre+; VEGFR2$^{f/KDR}$ mice versus control mice (Figures 5A and 5B), whereas at d8, the increase was 1.3-fold. This was accompanied by a decrease in the number of TUNEL-positive apoptotic hypertrophic chondrocytes (Figures 5A–5C).

Dietary phosphate restriction exacerbates the growth plate phenotype of ColII-Cre+; VEGFR2$^{f/KDR}$ mice

Hypophosphatemia resulting from dietary phosphate restriction leads to expansion of the HC layer of the growth plate in wild-type mice, due to impaired apoptosis of these cells.[1] To evaluate whether diet-induced hypophosphatemia worsens the phenotype of the ColII-Cre+; VEGFR2$^{f/KDR}$ growth plates, VEGFR2$^{f/f}$ and ColII-Cre+; VEGFR2$^{f/KDR}$ mice were weaned onto a low-phosphate diet P18 and sacrificed at P24.

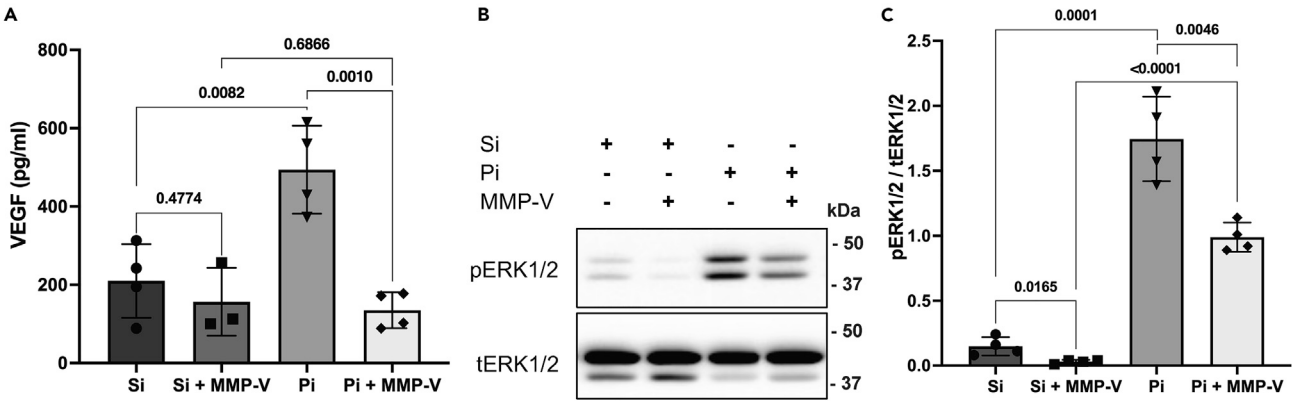


Figure 3. Pi increases VEGFA bioavailability

(A) HCs were pretreated for 2 h with the pan-MMP inhibitor, MMP-V (10 μM) followed by treatment with Si (7 mM) or Pi (7 mM) for 10 min. ELISA was performed to quantitate VEGFA in the conditioned media. Data represent the mean ± SD; of that obtained from three to four independent chondrocyte preparations; p values are indicated above the line.

(B) Western analyses of the treated HCs using anti-pERK1/2 & tERK1/2 antibodies. (C) Densitometric quantitation of western data in (B). Densitometric data represent mean ± SD; of that obtained from three to four independent chondrocyte preparations; p values are indicated above the line.

Phosphate restriction led to a significant decrease in serum Pi levels in mice of both genotypes; however, there was no significant difference in the degree of hypophosphatemia between the two genotypes (6.44 mg/dL in VEGFR2$^{f/f}$ vs. 6.02 mg/dL in ColII-Cre+; VEGFR2$^{f/KDR}$ mice, (p = 0.231). While diet-induced hypophosphatemia led to the expansion of the HC layer of the growth plate, accompanied by decreased apoptosis in mice of both genotypes, the number of HC in the growth plate of the phosphate-restricted ColII-Cre+; VEGFR2$^{f/KDR}$ animals was further increased by 1.56-fold relative to that of the VEGFR2$^{f/f}$ mice fed the same low-phosphate diet (p < 0.0001) (Figure 6). This expansion of the growth plate was accompanied by a significant reduction in the number of TUNEL-positive hypertrophic chondrocytes (Figure 6).

DISCUSSION

Hypertrophic chondrocytes secrete angiogenic factors, including VEGF ligands which promote vascular invasion, a crucial step in growth plate maturation.[16,17] Studies defining a role for the VEGF signaling pathway in skeletal development and maturation have been hampered by the embryonic lethality of knockout mouse models. Heterozygous germline loss of VEGF is embryonic lethal due to severe vascular defects.[18] Similarly, germline deletion of VEGFR1 or VEGFR2 results in embryonic lethality at e8.5 and e9.5 due to defects in the development of blood vessels, hematopoietic, and endothelial cells.[15,19] While conditional deletion of VEGF in ColII-Cre-expressing cells leads to embryonic lethality at e10.5 in most affected offspring, the few fetuses that survive until e17.5 exhibit aberrant vascular invasion and expansion of the hypertrophic zone of the growth plate in the developing tibia and humerus,[20,21] suggesting a role for chondrocyte-derived VEGF in growth plate development.

Inhibiting VEGF signaling postnatally using a VEGF decoy receptor or a VEGF-blocking antibody causes expansion of the hypertrophic chondrocyte layer of the growth plate in growing mice and primates, respectively.[17,22] Similarly, blocking VEGFR2 signaling using the small-molecule tyrosine kinase inhibitor vandetanib causes a dose-dependent expansion of the hypertrophic chondrocyte layer in growing rodents.[23] These studies establish a role for VEGF/VEGFR2 signaling in growth plate maturation, however, do not identify the mechanism nor the cell type in which signaling is required.

VEGF secreted by HCs is sequestered in the extracellular matrix.[24] MMP9 activity is required to release this VEGF and render it bio-available. Consistent with this, *Mmp9* knockout mice, like mice lacking VEGF, exhibit expansion of the hypertrophic chondrocyte zone of the growth plate, with impaired vascular invasion.[14,17,20,24] While these studies support a role for the VEGF signaling pathway in growth plate development and maturation, they do not address whether the effects of VEGF are paracrine or autocrine. However, our studies have addressed this, by demonstrating that inhibiting VEGFR2 signaling in hypertrophic chondrocytes impairs phosphate-induced apoptosis of these cells *in vitro* and *in vivo*, resulting in impaired

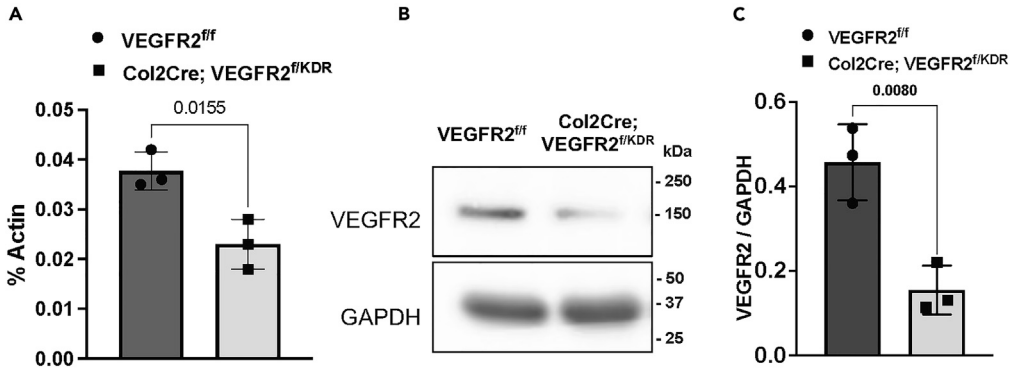


Figure 4. Chondrocyte-specific knockdown of VEGFR2 *in vivo*

(A) rt-qPCR analysis of RNA from cultured hypertrophic chondrocytes isolated from VEGFR2$^{f/f}$ and Col2Cre; VEGFR2$^{f/KDR}$ mice.

(B) Western analyses of cultured hypertrophic chondrocytes isolated from VEGFR2$^{f/f}$ and Col2Cre; VEGFR2$^{f/KDR}$ mice were performed using anti-VEGFR2 and anti-GAPDH antibodies.

(C) Densitometric quantitation of western analyses was performed using blots from three independent chondrocyte preparations. Data represent the mean ± SD; of that obtained from three to four experiments; the p value is indicated above the line.

growth plate maturation *in vivo*. They also demonstrated that VEGFR2 ligands are able to induce ERK1/2 phosphorylation and caspase-9-mediated apoptosis in hypertrophic chondrocytes. Thus, these investigations define a novel non-canonical role for VEGFR2 in transducing Pi signals and mediating its effects on HC apoptosis and growth plate maturation.

Vitamin D is critical for normal growth plate maturation. Studies in mice lacking the vitamin D receptor demonstrate that normalizing mineral ion levels, notably, phosphate, prevents the development of rickets,[1,25] because phosphate induces ERK1/2 phosphorylation and activates the mitochondrial apoptotic pathway in hypertrophic chondrocytes.[2] Interestingly, the active metabolite of vitamin D, 1,25-dihydroxyvitamin D has also been shown to activate pERK1/2, thus, can in part compensate for the effects of low phosphate.[26] Since ERK1/2 activation by 1,25-dihydroxyvitamin D requires several hours,[26] and 1,25-dihydroxyvitamin D induces the expression of VEGF in chondrocytes,[27,28] it is possible that the actions of 1,25-dihydroxyvitamin D on pERK1/2 are due to increases in VEGF which then activates VEGFR2 in chondrocytes. Consistent with this, chondrocyte-specific inactivation of the vitamin D receptor or the enzyme required for vitamin D activation (Cyp27b1) results in impaired vascular invasion and reduced VEGF expression.[29,30]

There has been increasing interest in the cell fate of growth plate chondrocytes. While our studies clearly demonstrate that extracellular phosphate activates VEGFR2 signaling and apoptosis in hypertrophic chondrocytes, lineage-tracing studies demonstrate that cells labeled by chondrocyte-specific Cre drivers, including Col II, Aggrecan, and Col10a-Cre, can give rise to skeletal stem cells, adipocytes, osteoblasts, and osteocytes.[31–35] While similar findings using three different Cre drivers diminish the likelihood that this is due to the "leakiness" of the Cre driver, it has been challenging to determine the percentage of hypertrophic chondrocytes that undergo apoptosis versus *trans*-differentiation. It has also been challenging to determine the percentage of osteoblasts that derive from these labeled chondrocytes since quantitation has relied primarily on histological imaging and immunohistochemistry of matrix proteins expressed by osteoblasts, some of which are also expressed by late hypertrophic chondrocytes.[36–38] The reported percentage of osteoblasts derived from chondrocytes has varied from 15% to 83%.[32–34,39] Studies performed to address the potential contribution of these cells to the mature skeleton have revealed that, while chondrocyte-specific Runx2 deletion impairs *trans*-differentiation of chondrocytes to osteoblasts,[39] this has no impact on bone structure, volume, biomechanical, or static/dynamic histomorphometric parameters in six-week-old mice.[39]

Prevention and treatment of hypophosphatemic rickets has been challenging due to the numerous daily doses of phosphate required and the rapid renal clearance of ingested phosphate. In addition, phosphate can induce the expression of parathyroid hormone, which, in turn, increases renal phosphate losses. Studies have demonstrated that 1,25-dihydroxyvitamin D can prevent growth plate expansion and impaired hypertrophic chondrocyte apoptosis seen in hypophosphatemic states.[26,40,41] Our studies provide molecular insight into this effect, in

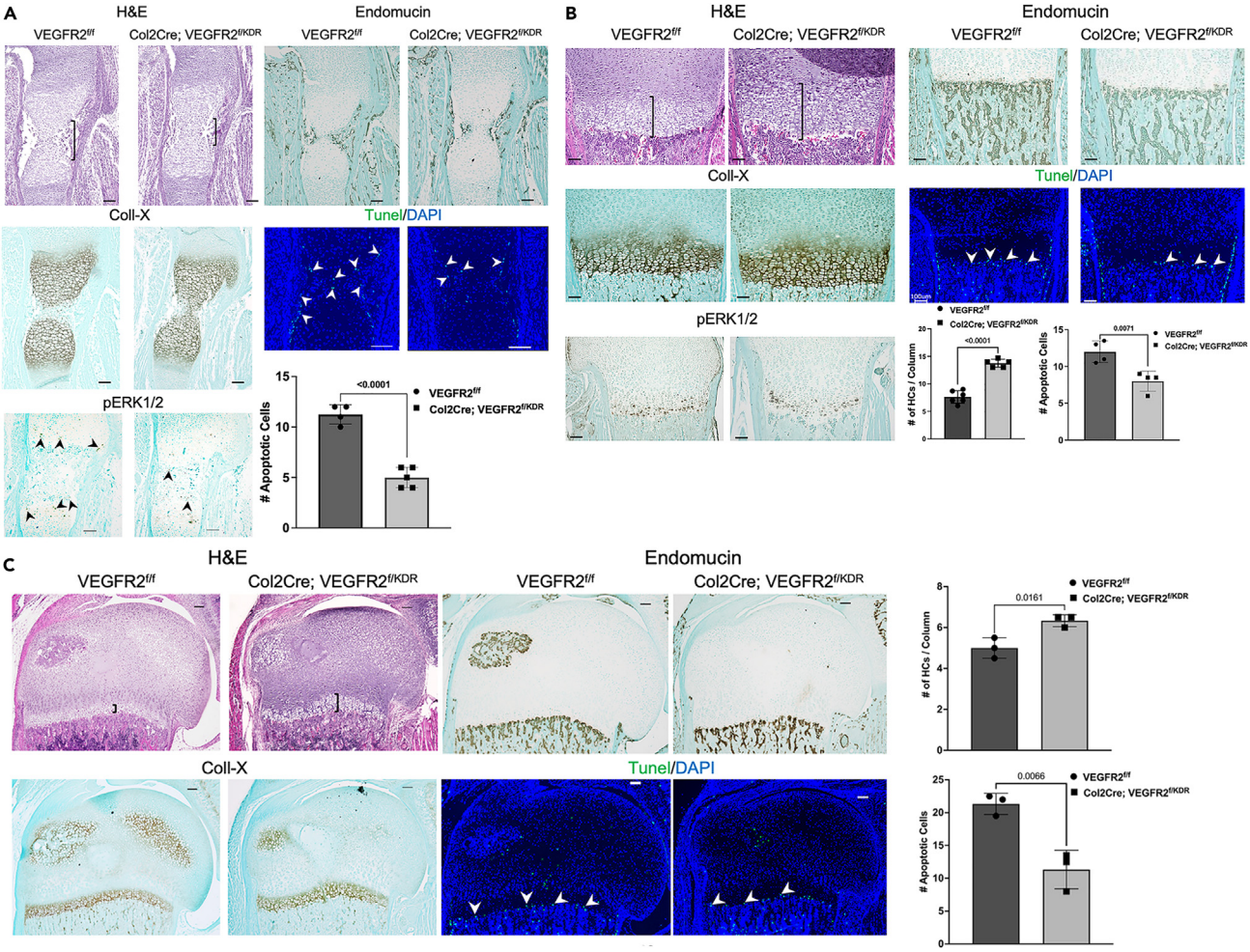


Figure 5. Chondrocyte-specific knockdown of VEGFR2 impairs growth plate development *in vivo*

(A–C) H&E staining was performed on humeral sections of e15.5 (5A), P0 (5B), and P8 (5C) mice. Brackets on H&E stained images at e15.5 indicate the region of vascular invasion. Brackets on H&E stained images at P0 and P8 indicate the hypertrophic chondrocyte layer. Immunohistochemistry (IHC) for Endomucin, an endothelial cell marker, demonstrated reduced vascular invasion in Col2Cre; VEGFR2$^{f/KDR}$ embryos. IHC for Collagen type X (Coll-X), a marker of hypertrophic chondrocytes, demonstrated expansion of the HC layer in these embryos. A reduction in the number of pERK1/2-positive HCs in Col2Cre; VEGFR2$^{f/KDR}$ embryos was observed at e15 and P0 (5A and 5B). TUNEL labeling (arrows) revealed a decrease in the number of apoptotic late HCs in Col2Cre; VEGFR2$^{f/KDR}$ mice. Nuclei are stained blue with DAPI. TUNEL-positive cells were quantitated in the distal two rows of the HC layer at e15.5 (5A), P0 (5B), and P8 (5C). Data represent the mean ± SD of that obtained from four mice per group. The p value is indicated above the line. The scale bar represents 100 μM.

that 1,25-dihydroxyvitamin D has been shown to increase mitochondrial pERK1/2[26] as well as induce expression of VEGFR2 ligands in HCs,[27,30] thus compensating for the decreased VEGFR2 activation seen in hypophosphatemia. These studies also have therapeutic implications in that targeting VEGFR2 ligands to the maturing growth plate would be expected to increase the therapeutic benefit of vitamin D metabolites. Our previous investigations[1] have demonstrated that phosphate activates this signaling pathway in HC but not in proliferative chondrocytes or NIH 3T3 fibroblastic cells; thus, the role of VEGFR2 signaling in phosphate-mediated vascular mineralization remains unclear.

Limitations of the study

The current studies are limited by the modest knockdown in chondrocyte VEGFR2 expression *in vivo*, despite the use of both a haploinsufficient and a floxed allele. However, despite this limited knockdown, they establish a role for VEGFR2 signaling in normal growth plate development and maturation, as well as in the prevention of rickets in hypophosphatemic states. They demonstrate that phosphate activates MMP-dependent release of VEGFA from the surrounding matrix, which then activates VEGFR2 to induce

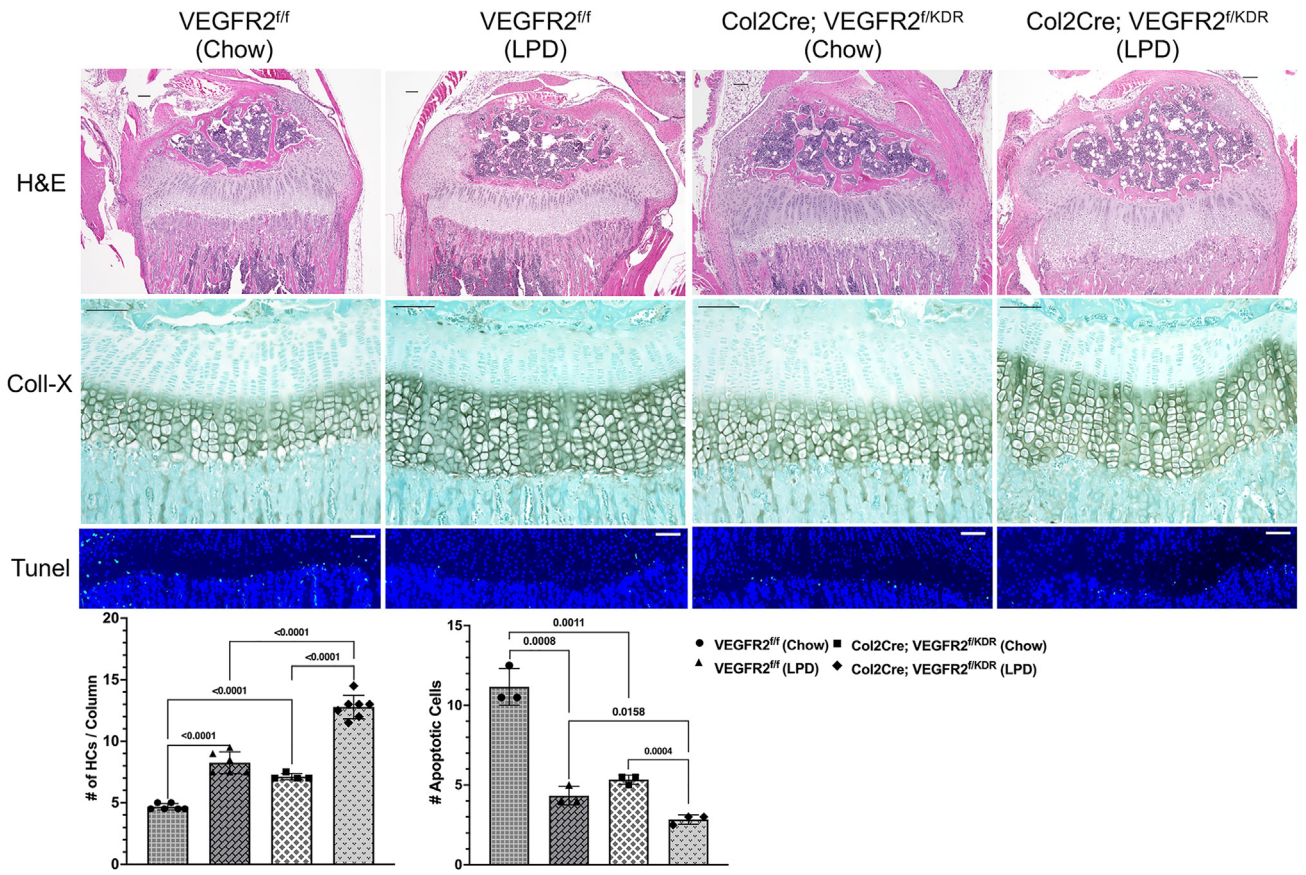


Figure 6. Dietary phosphate restriction exacerbates the growth plate phenotype of ColII-Cre+; VEGFR2$^{f/KDR}$ mice

H&E staining was performed on tibial sections of P24 mice weaned onto a phosphate-restricted diet on d18. Immunohistochemistry for Collagen type X (Coll-X) showed expansion of the HC layer in Col2Cre; VEGFR2$^{f/KDR}$ mice. Quantification of the number of HCs per column in the growth plates is shown. TUNEL labeling (arrows) revealed a reduction in the number of apoptotic HCs in Col2Cre; VEGFR2$^{f/KDR}$ mice. Nuclei are stained blue with DAPI. TUNEL-positive cells were quantitated in the distal two rows of the HC layer. Data represent the mean ± SD of that obtained from four mice per group. The p value is indicated above the line. The scale bar represents 100 μM. Chow is regular diet and LPD represents low-phosphate diet.

the mitochondrial apoptotic pathway. These studies suggest that modulation of the VEGF/VEGFR2 signaling pathway may be beneficial in the prevention of hypophosphatemic rickets.

The limitations of the *in vitro* studies are the need to use small-molecule inhibitors to impair VEGFR2 signaling. While highly selective inhibitors were used at doses consistent with their IC50 in cells, there are off-target effects of these inhibitors at high doses. However, highly selective inhibitors for pathways affected by these off-target effects (EGFR and PDGFR) do not impair caspase-9 activation by Pi, ruling out these signaling pathways. Due to the robust matrix synthesized by chondrocytes during the 14 days differentiation process *in vitro*, efforts to silence VEGFR2 in hypertrophic chondrocytes were unsuccessful, and silencing in early proliferative chondrocytes resulted in poor knockdown, presumably due to overgrowth of the uninfected cells.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107548.

ACKNOWLEDGMENTS

We are thankful to the Histology Core Facility at the Center for Skeletal Research, Massachusetts General Hospital, Boston, and to the staff at the ICCB-Longwood Screening Facility at Harvard Medical School, Boston, USA. Graphical abstract was created with Biorender.com.

Funding: This work was funded by NIH R01-AR072650 and supported by the MGH Center for Skeletal Research (NIH P30-AR075042).

AUTHOR CONTRIBUTIONS

P.S.Y. designed and performed the studies, interpreted data, wrote the manuscript, addressed the revision, and revised the manuscript. G.P. designed and performed the studies and interpreted data. M.M.K. performed studies. M.B.D. designed the studies, interpreted the data, and participated in writing and revising the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: March 3, 2023
Revised: June 21, 2023
Accepted: August 2, 2023
Published: August 7, 2023

REFERENCES

1. Sabbagh, Y., Carpenter, T.O., and Demay, M.B. (2005). Hypophosphatemia leads to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes. Proc. Natl. Acad. Sci. USA *102*, 9637–9642. https://doi.org/10.1073/pnas.0502249102.

2. Miedlich, S.U., Zalutskaya, A., Zhu, E.D., and Demay, M.B. (2010). Phosphate-induced apoptosis of hypertrophic chondrocytes is associated with a decrease in mitochondrial membrane potential and is dependent upon Erk1/2 phosphorylation. J. Biol. Chem. *285*, 18270–18275. https://doi.org/10.1074/jbc.M109.098616.

3. Chen, Z., Yue, S.X., Zhou, G., Greenfield, E.M., and Murakami, S. (2015). ERK1 and ERK2 regulate chondrocyte terminal differentiation during endochondral bone formation. J. Bone Miner. Res. *30*, 765–774. https://doi.org/10.1002/jbmr.2409.

4. Matsushita, T., Chan, Y.Y., Kawanami, A., Balmes, G., Landreth, G.E., and Murakami, S. (2009). Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. Mol. Cell Biol. *29*, 5843–5857.

5. Peyssonnaux, C., and Eychène, A. (2001). The Raf/MEK/ERK pathway: new concepts of activation. Biol. Cell *93*, 53–62. https://doi.org/10.1016/s0248-4900(01)01125-x.

6. Papaioannou, G., Petit, E.T., Liu, E.S., Baccarini, M., Pritchard, C., and Demay, M.B. (2017). Raf Kinases Are Essential for Phosphate Induction of ERK1/2 Phosphorylation in Hypertrophic Chondrocytes and Normal Endochondral Bone Development. J. Biol. Chem. *292*, 3164–3171. https://doi.org/10.1074/jbc.M116.763342.

7. Provot, S., Nachtrab, G., Paruch, J., Chen, A.P., Silva, A., and Kronenberg, H.M. (2008). A-raf and B-raf are dispensable for normal endochondral bone development, and

parathyroid hormone-related peptide suppresses extracellular signal-regulated kinase activation in hypertrophic chondrocytes. Mol. Cell Biol. *28*, 344–357.

8. Liu, E.S., Raimann, A., Chae, B.T., Martins, J.S., Baccarini, M., and Demay, M.B. (2016). c-Raf promotes angiogenesis during normal growth plate maturation. Development *143*, 348–355. https://doi.org/10.1242/dev.127142.

9. Liu, E.S., Zalutskaya, A., Chae, B.T., Zhu, E.D., Gori, F., and Demay, M.B. (2014). Phosphate interacts with PTHrP to regulate endochondral bone formation. Endocrinology *155*, 3750–3756. https://doi.org/10.1210/en.2014-1315.

10. Guo, J., Chung, U.I., Kondo, H., Bringhurst, F.R., and Kronenberg, H.M. (2002). The PTH/PTHrP receptor can delay chondrocyte hypertrophy in vivo without activating phospholipase C. Dev. Cell *3*, 183–194. https://doi.org/10.1016/s1534-5807(02)00218-6.

11. Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M., and Tabin, C.J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science *273*, 613–622. https://doi.org/10.1126/science.273.5275.613.

12. Guzmán-Hernández, M.L., Potter, G., Egervári, K., Kiss, J.Z., and Balla, T. (2014). Secretion of VEGF-165 has unique characteristics, including shedding from the plasma membrane. Mol. Biol. Cell *25*, 1061–1072. https://doi.org/10.1091/mbc.E13-07-0418.

13. Taraboletti, G., D'Ascenzo, S., Giusti, I., Marchetti, D., Borsotti, P., Millimaggi, D., Giavazzi, R., Pavan, A., and Dolo, V. (2006). Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. Neoplasia *8*, 96–103. https://doi.org/10.1593/neo.05583.

14. Vu, T.H., Shipley, J.M., Bergers, G., Berger, J.E., Helms, J.A., Hanahan, D., Shapiro, S.D., Senior, R.M., and Werb, Z. (1998). MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell *93*, 411–422. https://doi.org/10.1016/s0092-8674(00)81169-1.

15. Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62–66. https://doi.org/10.1038/376062a0.

16. Bluteau, G., Julien, M., Magne, D., Mallein-Gerin, F., Weiss, P., Daculsi, G., and Guicheux, J. (2007). VEGF and VEGF receptors are differentially expressed in chondrocytes. Bone *40*, 568–576. https://doi.org/10.1016/j.bone.2006.09.024.

17. Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., and Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. *5*, 623–628. https://doi.org/10.1038/9467.

18. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439–442. https://doi.org/10.1038/380439a0.

19. Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66–70. https://doi.org/10.1038/376066a0.

20. Haigh, J.J., Gerber, H.P., Ferrara, N., and Wagner, E.F. (2000). Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. Development *127*, 1445–1453.

21. Zelzer, E., McLean, W., Ng, Y.S., Fukai, N., Reginato, A.M., Lovejoy, S., D'Amore, P.A., and Olsen, B.R. (2002). Skeletal defects in VEGF(120/120) mice reveal multiple roles for VEGF in skeletogenesis. Development *129*, 1893–1904.

22. Ryan, A.M., Eppler, D.B., Hagler, K.E., Bruner, R.H., Thomford, P.J., Hall, R.L., Shopp, G.M., and O'Neill, C.A. (1999). Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol. Pathol. *27*, 78–86. https://doi.org/10.1177/019262339902700115.

23. Wedge, S.R., Ogilvie, D.J., Dukes, M., Kendrew, J., Chester, R., Jackson, J.A., Boffey, S.J., Valentine, P.J., Curwen, J.O., Musgrove, H.L., et al. (2002). ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res. *62*, 4645–4655.

24. Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J. Biol. Chem. *267*, 26031–26037.

25. Li, Y.C., Pirro, A.E., Amling, M., Delling, G., Baron, R., Bronson, R., and Demay, M.B. (1997). Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc. Natl. Acad. Sci. USA *94*, 9831–9835. https://doi.org/10.1073/pnas.94.18.9831.

26. Liu, E.S., Martins, J.S., Raimann, A., Chae, B.T., Brooks, D.J., Jorgetti, V., Bouxsein, M.L., and Demay, M.B. (2016). 1,25-Dihydroxyvitamin D Alone Improves Skeletal Growth, Microarchitecture, and Strength in a Murine Model of XLH, Despite Enhanced FGF23 Expression. J. Bone Miner. Res. *31*, 929–939. https://doi.org/10.1002/jbmr.2783.

27. Lin, R., Amizuka, N., Sasaki, T., Aarts, M.M., Ozawa, H., Goltzman, D., Henderson, J.E., and White, J.H. (2002). 1Alpha,25-dihydroxyvitamin D3 promotes vascularization of the chondro-osseous junction by stimulating expression of vascular endothelial growth factor and matrix metalloproteinase 9. J. Bone Miner. Res. *17*, 1604–1612.

28. Cardus, A., Panizo, S., Encinas, M., Dolcet, X., Gallego, C., Aldea, M., Fernandez, E., and Valdivielso, J.M. (2009). 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. Atherosclerosis *204*, 85–89. https://doi.org/10.1016/j.atherosclerosis.2008.08.020.

29. Masuyama, R., Stockmans, I., Torrekens, S., Van Looveren, R., Maes, C., Carmeliet, P., Bouillon, R., and Carmeliet, G. (2006). Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. J. Clin. Invest. *116*, 3150–3159. https://doi.org/10.1172/JCI29463.

30. Naja, R.P., Dardenne, O., Arabian, A., and St Arnaud, R. (2009). Chondrocyte-specific modulation of Cyp27b1 expression supports a role for local synthesis of 1,25-dihydroxyvitamin D3 in growth plate development. Endocrinology *150*, 4024–4032. https://doi.org/10.1210/en.2008-1410.

31. Yang, L., Tsang, K.Y., Tang, H.C., Chan, D., and Cheah, K.S.E. (2014). Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proc. Natl. Acad. Sci. USA *111*, 12097–12102. https://doi.org/10.1073/pnas.1302703111.

32. Yang, G., Zhu, L., Hou, N., Lan, Y., Wu, X.M., Zhou, B., Teng, Y., and Yang, X. (2014). Osteogenic fate of hypertrophic chondrocytes. Cell Res. *24*, 1266–1269. https://doi.org/10.1038/cr.2014.111.

33. Zhou, X., von der Mark, K., Henry, S., Norton, W., Adams, H., and de Crombrugghe, B. (2014). Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. PLoS Genet. *10*, e1004820. https://doi.org/10.1371/journal.pgen.1004820.

34. Long, J.T., Leinroth, A., Liao, Y., Ren, Y., Mirando, A.J., Nguyen, T., Guo, W., Sharma, D., Rouse, D., Wu, C., et al. (2022). Hypertrophic chondrocytes serve as a reservoir for marrow-associated skeletal stem and progenitor cells, osteoblasts, and adipocytes during skeletal development. Elife *11*, e76932. https://doi.org/10.7554/eLife.76932.

35. Park, J., Gebhardt, M., Golovchenko, S., Perez-Branguli, F., Hattori, T., Hartmann, C., Zhou, X., deCrombrugghe, B., Stock, M., Schneider, H., and von der Mark, K. (2015). Dual pathways to endochondral osteoblasts: a novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage. Biol. Open *4*, 608–621. https://doi.org/10.1242/bio.201411031.

36. Neugebauer, B.M., Moore, M.A., Broess, M., Gerstenfeld, L.C., and Hauschka, P.V. (1995). Characterization of structural sequences in the chicken osteocalcin gene: expression of osteocalcin by maturing osteoblasts and by hypertrophic chondrocytes in vitro. J. Bone

Miner. Res. *10*, 157–163. https://doi.org/10.1002/jbmr.5650100122.

37. Gerstenfeld, L.C., and Shapiro, F.D. (1996). Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. J. Cell. Biochem. *62*, 1–9. https://doi.org/10.1002/(SICI)1097-4644(199607)62:1%3C1::AID-JCB1%3E3.0.CO;2-X.

38. Riminucci, M., Bradbeer, J.N., Corsi, A., Gentili, C., Descalzi, F., Cancedda, R., and Bianco, P. (1998). Vis-a-vis cells and the priming of bone formation. J. Bone Miner. Res. *13*, 1852–1861. https://doi.org/10.1359/jbmr.1998.13.12.1852.

39. Qin, X., Jiang, Q., Nagano, K., Moriishi, T., Miyazaki, T., Komori, H., Ito, K., Mark, K.v.d., Sakane, C., Kaneko, H., and Komori, T. (2020). Runx2 is essential for the transdifferentiation of chondrocytes into osteoblasts. PLoS Genet. *16*, e1009169. https://doi.org/10.1371/journal.pgen.1009169.

40. Miedlich, S.U., Zhu, E.D., Sabbagh, Y., and Demay, M.B. (2010). The receptor-dependent actions of 1,25-dihydroxyvitamin D are required for normal growth plate maturation in NPt2a knockout mice. Endocrinology *151*, 4607–4612. https://doi.org/10.1210/en.2010-0354.

41. Kaneko, I., Segawa, H., Ikuta, K., Hanazaki, A., Fujii, T., Tatsumi, S., Kido, S., Hasegawa, T., Amizuka, N., Saito, H., and Miyamoto, K.I. (2018). Eldecalcitol Causes FGF23 Resistance for Pi Reabsorption and Improves Rachitic Bone Phenotypes in the Male Hyp Mouse. Endocrinology *159*, 2741–2758. https://doi.org/10.1210/en.2018-00109.

42. Lefebvre, V., Garofalo, S., Zhou, G., Metsäranta, M., Vuorio, E., and De Crombrugghe, B. (1994). Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. Matrix Biol. *14*, 329–335. https://doi.org/10.1016/0945-053x(94)90199-6.

43. Peterson, T. (2010). Densitometric analysis using NIH Image. J. NAVBO eNewsletter *16*.

44. Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. *3*, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

45. Ovchinnikov, D.A., Deng, J.M., Ogunrinu, G., and Behringer, R.R. (2000). Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. Genesis *26*, 145–146.

46. Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K., et al. (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell Stem Cell *4*, 263–274. https://doi.org/10.1016/j.stem.2009.01.006.

47. Ko, F.C., Kobelski, M.M., Zhang, W., Grenga, G.M., Martins, J.S., and Demay, M.B. (2021). Phosphate restriction impairs mTORC1 signaling leading to increased bone marrow adipose tissue and decreased bone in growing mice. J. Bone Miner. Res. *36*, 1510–1520. https://doi.org/10.1002/jbmr.4312.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-pErk1/2	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #9101; RRID: AB_331646
Anti-Erk1/2	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #9102; RRID:AB_330744
Anti-Caspase 9	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #9504; RRID:AB_2275591
Anti-pVEGFR2 (Tyr996)	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #2474; RRID:AB_331023
Anti-VEGFR2	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #2479; RRID:AB_2212507
Anti-VEGFR2	Bioss, Woburn, Massachusetts, USA	Cat. #bs-10412; RRID AB_2942075
Anti-GAPDH	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #2118; RRID:AB_561053
Anti-rabbit IgG HRP	Cell Signaling Technologies, Danvers, Massachusetts, USA	Cat. #7074; RRID:AB_2099233
Anti-Endomucin	Abcam, Waltham, Massachusetts, USA	Cat. #ab106100; RRID:AB_10859306
Anti-Collagen type X	ABclonal, USA	Cat. #A6889; AB_2767448
Chemicals, enzymes, and recombinant proteins		
Collagenase type -II	Worthington, New Jersey, USA	Cat. #LS004177
Dulbecco's Modified Eagle Medium (DMEM)	Corning, USA	Cat. #10-013-CV
Fetal bovine Serum	HyClone, Utah, USA	Cat. #AMF-16453
Penicillin/Streptomycin	Gibco, USA	Cat. #10378016
Ascorbic acid	Sigma, St. Louis, USA	Cat. #A-2174
Brivanib	Selleckchem, Texas, USA	Cat. #S1084
Vandetanib	Selleckchem, Texas, USA	Cat. #S1046
ZM 323881 HCl	Selleckchem, Texas, USA	Cat. #S2896
MMP Inhibitor-V	CAS 223472-31-9; Calbiochem, USA	Cat. # 444290)
EGFR inhibitor, CI-1033	Sigma USA	Cat. #C7249
PDGFR Tyrosine Kinase Inhibitor IV	Sigma USA	Cat. #521233
U0126	Cell Signaling Technologies, Danvers, Massachusetts, USA,	Cat. #9903
BCA protein assay	Pierce, Thermo Scientific, USA	Cat. #23223
Fluoroshield™ mounting media with DAPI	Millipore Sigma, USA	Cat. #F6057
West Dura ECL	Thermo Scientific, USA	Cat. #34075
Non-Fat dried milk	BioRad, USA	Cat. #1706404
Bovine Serum Albumin (BSA)	Sigma Aldrich, St. Louis, USA	Cat. #A9418
SuperScript Reverse Transcriptase II	Invitrogen, Carlsbad, CA, USA	Cat. #18064022
Phosphate restricted mouse diet	Envigo, Indianapolis, USA	Cat. #TD03486

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
rhVEGF (165)	Abcam, Waltham, Massachusetts, USA	Cat. #ab259412
rhVEGF (121)	BioLegend, San Diego, CA, USA	Cat. #583204
Critical commercial assays		
SureFire ERK Assay Kit	Perkin Elmer, Waltham, Massachusetts, USA	Cat. #TGRES10K
VEGF ELISA Kit	Bioss, Woburn, Massachusetts, USA	Cat. #BSKM1018
In Situ Cell Death Detection Kit	Roche, Sigma Germany	Cat. #11684795910
Experimental models: Organisms/strains		
Mouse: WT (C57BL/6J)	The Jackson laboratory	Strain #000664
Mouse: $VEGFR2^{F/KDR}$	The Jackson laboratory	Strain #018977 (Ref. #15)
Mouse: Col2-Cre	The Jackson laboratory	Strain #003554 (Ref. #45)
Oligonucleotides		
Mouse: VEGFR2	IDT	5'- FW AGCGTGATTCTGAGGAAAGG-3' 5' REV-ACTGACAGAGGCGATGAATG-3'
Mouse: Actin	IDT	5' FW- CCTCTATGCCAACACAGTGC-3' 5' REV- ACATCTGCTGGAAGGTGGAC-3'
Software and algorithms		
GraphPad Prism Version 9.2.0	GraphPad Software, San Diego, California, USA	https://www.graphpad.com
ImageJ	NIH	https://imagej.nih.gov/ij
Adobe Photoshop Version CS5.1	Adobe	https://www.adobe.com/products/photoshop

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Marie B. Demay (demay@helix.mgh.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper does not generate any RNASeq or Gene Expression Omnibus (GEO) related data.
- Any additional information required about the data reported in this paper is available from the lead contact upon request.
- This paper does not report any original code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell culture

Primary chondrocytes were isolated from the rib cages of 3-day-old WT mice using sequential digestion with collagenase type-II (Worthington, New Jersey, USA). Chondrocytes were plated onto gelatin-coated plates at a density of 3.0 $X10^5/cm^2$ in Dulbecco's Modified Eagle Medium (DMEM) (Corning, USA) supplemented with 10% fetal bovine serum (HyClone, Utah, USA), 1% penicillin/streptomycin (Gibco, USA). Once chondrocytes reached confluence, cells were differentiated for two weeks, in media containing 25 μg/mL ascorbic acid (Sigma, St. Louis, USA), at 37°C and 5% CO2 as previously described.[1,42]

METHOD DETAILS

Small molecule inhibitor screening

Chondrocytes were plated in 384 well plates at a density of 2000 cells/well and grown in ascorbic acid supplemented DMEM/10% FCS for two weeks to allow for hypertrophic differentiation. After incubation overnight in DMEM with 0.5% FCS, small molecule inhibitors were added. One hour later, cells were treated with sodium phosphate or control ion (sodium sulfate) for 15 min. A total of 1438 unique small-molecule inhibitors from 4 compound libraries (Biomol 4 FDA Approved Drug library, EMD Kinase Inhibitor 1, SYNthesis med chem Kinase Inhibitor 2, Selleck Bioactive Compound library) were screened for compounds that inhibit phosphate induced ERK1/2 phosphorylation in hypertrophic chondrocytes. The list of small molecule kinase inhibitors screened in AlphaScreen SureFire Assay is provided in Table S1. The first and second columns indicate the manufacturer and catalog number. The third column indicates the chemical name and the fourth, the target of the inhibitor. The SureFire ERK Assay (PerkinElmer, Waltham, Massachusetts, USA) was used to detect ERK1/2 phosphorylation according to the manufacturer's instructions. Screening was conducted in duplicate. Twenty-eight compounds were identified that inhibited ERK1/2 phosphorylation by more than 80%. These were further evaluated for dose-response of inhibition of phosphate-induced pERK1/2 (from 11 μM to 2.2 nM).

Western blotting

Primary chondrocytes were pre-treated with inhibitors [Brivanib (Selleckchem, Texas, USA), Vandetanib (Selleckchem, Texas, USA), MMP Inhibitor-V (CAS 223472-31-9; Calbiochem, USA), ZM 323881 HCl (Selleckchem, Texas, USA), EGFR inhibitor, CI-1033 (Sigma, USA), PDGFR Tyrosine Kinase Inhibitor IV (Sigma, USA), U0126 (Cell Signaling Technologies, Danvers, Massachusetts, USA)] prior to treatment with phosphate (Pi), rhVEGFA (Abcam, Waltham, Massachusetts, USA, (VEGF-165), BioLegend, San Diego, CA, USA, (VEGF-121) or sulfate (Si). Protein concentration was measured using the BCA protein assay (Pierce, Thermo Scientific, USA) and proteins were subjected to western analysis. Membranes were blocked with 5% non-fat dried milk (BioRad, USA) or 5% BSA (Sigma Aldrich, St. Louis, USA) prior to incubation with antibodies against pErk1/2 (1:1000; Cell Signaling Technologies), Erk1/2 (1:1000; Cell Signaling Technologies), Cleaved Caspase 9 (1:500, Cell Signaling Technologies), pVEGFR2-Tyr996 (1:1000, Cell Signaling Technologies), total VEGFR2 (1:1000, Bioss), used for evaluating VEGFR2 knockdown, and total VEGFR2 (1:1000, Cell Signaling Technologies) used for Western blot analysis.

Following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG HRP antibody; 1:3000; Cell Signaling Technologies), anti-GAPDH (1:2000, Cell Signaling Technologies) signals were detected using West Dura ECL (Thermo-Scientific).

Densitometric analysis

Densitometric analysis of western blots was performed using ImageJ (NIH) software.[43]

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from chondrocytes using the RNeasyMicro Kit (Qiagen, Waltham, MA, USA). One μg of total RNA was reverse-transcribed with SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA). Target genes expression was evaluated using a QuantStudio 3 thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA). Target gene expression was normalized for actin in the same sample,[44] using the following primer sets: mouse VEGFR2 qPCR FW AGCGTGATTCTGAGGAAAGG, mouse VEGFR2 qPCR REV ACTGACAGAGGCGATGAATG, mouse Actin qPCR FW CCTCTATGCCAACACAGTGC, and mouse Actin qPCR REV ACATCTGCTGGAAGGTGGAC.

ELISA assay

Mouse VEGFA was quantitated in the conditioned media of hypertrophic chondrocytes using a mouse VEGFA ELISA kit (Bioss, Woburn, Massachusetts, USA Cat# BSKM1018) following the manufacturer's protocol.

Animal studies

Animal studies were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. All mice were on a C57BL/6J, background, maintained in a virus- and parasite-free barrier facility, and exposed to a 12 h light/dark cycle. Mice with chondrocyte-specific ablation of VEGFR2 were generated by mating mice expressing Cre recombinase driven by the collagen type II (Col2a1) promoter[45]

with VEGFR2$^{f/f}$ mice.[46] These mice were further mated with VEGFR2 heterozygous mice,[15] to obtain mice where one VEGFR2 allele is deleted in the germline and the floxed allele is recombined in chondrocytes. The phenotype of mice with chondrocyte specific VEGFR2 knockdown [heterozygous germline VEGFR2 deletion (KDR), heterozygous for the floxed VEGFR2 allele, and heterozygous for the Col2-Cre transgene (Col2Cre; VEGFR2$^{f/KDR}$)] was compared to that of Cre-negative littermates homozygous for the VEGFR2 floxed allele (VEGFR2$^{f/f}$).

For post-natal analysis, the mice were weaned at 18 days of age onto a standard diet (Chow) or onto a phosphate restricted diet ((Envigo TD03486, Indianapolis, USA).[47] Growth plates were analyzed at 24 days of age.[1]

Histology and immunohistochemistry

Bones were fixed in 10% formalin-PBS (pH 7.4) overnight at 4°C prior to processing for paraffin sectioning. Growth plate morphology was evaluated by Hematoxylin and Eosin (H&E) staining of 5 μM paraffin sections.[2,6] Sections were also subjected to immunohistochemistry to evaluate the expression of type-X collagen (1/300, ABclonal, USA), pERK1/2 (1/500, Cell Signaling Technologies), and Endomucin (1/200, Abcam, Waltham, Massachusetts, USA) as previously reported.[2,6]

Tunel assay

Apoptosis of hypertrophic chondrocytes was evaluated using TUNEL based *in situ* cell death detection kit (Roche Diagnostics, USA) as previously reported.[1] Sections were permeabilized with 2 ug/ml proteinase K for 30 min at 37°C and incubated with TUNEL reaction mix for 2 h at 37°C, followed by washing with 1X phosphate buffered saline and mounted with antifade mounting media (Fluoroshield with DAPI Sigma, St. Louis, USA).

Serum biochemistry

Serum phosphorous was measured using the phosphate assay kit (Abcam, Cambridge, MA, USA) following the manufacturer's protocol.[47]

QUANTIFICATION AND STATISTICAL ANALYSIS

The Student's *t* test was performed (using Prism version 9.2.0, GraphPad Software, San Diego, California, USA) to evaluate statistical significance. A p value <0.05 was considered significant.