GRK2 is a novel major regulator of chondrocyte differentiation during postnatal skeletal development

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INTRODUCTION: We recently showed that the G protein-coupled receptor (GPCR) kinase 2 (GRK2) promotes articular chondrocyte hypertrophic differentiation by desensitizing GPCR during osteoarthritis. During endochondral ossification (EO), chondrocyte differentiation in the growth plate (GP) is essential for cartilage-to-bone transition. This is tightly regulated by the parathyroid hormone-related protein and parathyroid hormone receptor-1 (PTH1R), a GPCR. With GRK2 being a major regulator of GPCRs, the mechanisms by which GRK2 interacts with the molecular regulators of EO are still unknown. The ability of GRK2 to (1) desensitize PTH1R and dampen its pro-proliferation and anti-differentiation signaling, and (2) directly promote hypertrophic signaling represent possible, yet uninvestigated, regulatory mechanisms of chondrocyte differentiation during EO. Our central **hypothesis** is that GRK2 regulates postnatal EO by desensitizing PTH1R and/or by directly promoting hypertrophic signaling in GP chondrocytes to ensure their proper differentiation. Here, we utilized mice with inducible chondrocyte specific GRK2 and/or PTH1R knockout (GRK2-icKO) and primary chondrocyte cultures to determine the role of GRK2 in EO during skeletal development.

METHODS: To determine the role of GRK2 in the regulation of EO we induced GRK2 deletion in chondrocytes (GRK2-icKO) of mice at P3 and studied changes in skeletal development at P10 and P28 (*Fig. 1a*). To evaluate whether the primary effect of GRK2 occurs via PTH1R desensitization, we deleted PTH1R in combination with GRK2 at P3 and euthanized mice at P10 (*Fig. 2a,b*). Furthermore, we challenged GRK2-icKO and CTRL mice with exogenous PTH treatment following the same timeline (P3-P10) (*Fig. 2c*). Finally, we investigated the interplay between GRK2 and phosphate mediated ERK phosphorylation during chondrocyte terminal differentiation in the GP. GRK2 deletion was induced at P29, and mice were maintained on either a normal chow or a low-phosphate diet (LPD) to induce hypophosphatemia from P28 till sacrifice at P40. For all animal experiments, tissues were fixed and embedded in paraffin and histological sections were used for further analysis. We used Hematoxylin and Eosin (H&E) and Safranin Orange/Fast Green stains. TUNEL staining was performed to assess apoptosis. RNAscope/ISH was performed to evaluate gene expression and immunofluorescent staining (IF) for protein expression analysis. We performed in vitro studies using primary chondrocytes that were harvested from the ribs of P4 mice, cultured and differentiated for 3, 7 and 14 days for cAMP analysis (to measure GPCR desensitization) and Alizarin-Red staining (to measure mineralization). Mouse GRK2 siRNA was used to knock down GRK2, while control cells was treated with negative control siRNA. All statistical analyses (unpaired or paired t-test, One or Two -way ANOVA) were performed using GraphPad Prism.

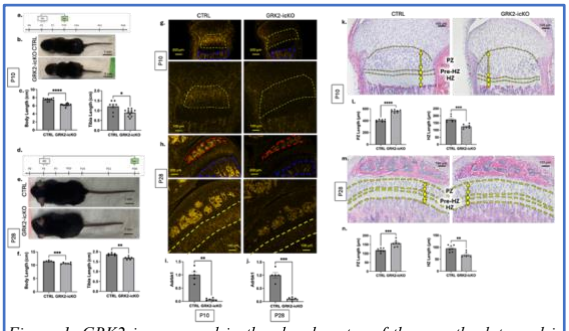

*Figure 1. GRK2 is expressed in the chondrocytes of the growth plate and is essential for skeletal elongation. (a) Timeline for the experiment: Tamoxifen (Tmx) injection at P3 and P4 and sacrifice (Sac) at P10. (b) Gross morphology of P10 control (CTRL, GRK2f/f) and GRK2-icKO (GRK2$^{f/f}$/Agc1$^{tm(IRES\text{-}CreERT2)}$) mice, with (c) body and tibia length quantified. (d) Timeline for studies of late postnatal skeletal development, with first Tamoxifen (Tmx) injection at P3 and sacrifice (Sac) at P28. (e) Gross morphology of P28 mice, with (f) body and tibia length quantified. GRK2 (adrbk1) RNA expression in (g) P10 and (h) P28 control (CTRL) and GRK2-icKO proximal tibiae using RNAScope. yellow dashed lines indicate the proliferative and prehypertrophic zones, blue dashed lines indicate the primary ossification center, and red dashed lines indicate the secondary ossification center; with quantification in (i) P10 and (j) P28. H&E staining of proximal tibiae of (k) P10 and (m) P28 control and GRK2-icKO mice. Yellow lines and arrows delineate proliferative (PZ), prehypertrophic (pre-HZ) and hypertrophic zones (HZ). Quantification of (l) P10 and (n) P28 PZ and HZ length (μm). *P < 0.05, **P < 0.01, ***P<0.001, ****P < 0.0001 using unpaired student's t test with Welch's correction, values are expressed as mean ± SEM.*

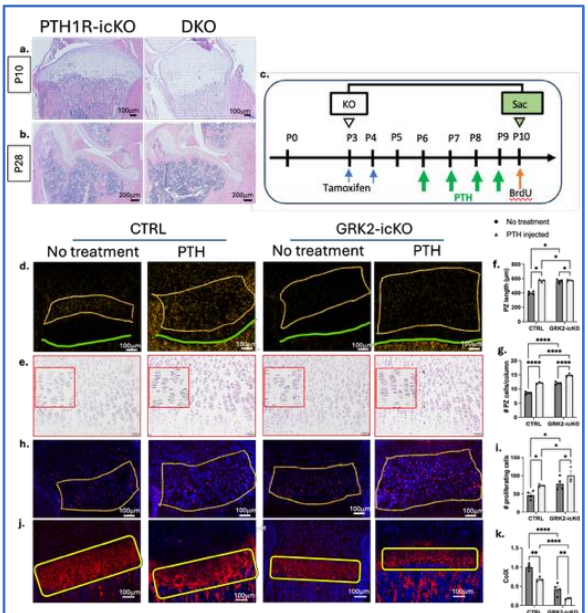

*Figure 2. Effects of GRK2-icKO in combination with PTH1R deletion and stimulation on the postnatal growth plate. H&E staining images of proximal tibiae of (a) P10 and (b) P28 PTH1R-icKO (PTH1R$^{f/f}$/Agc1$^{tm(IRES\text{-}CreERT2)}$) and DKO (double knockout: PTH1R$^{f/f}$; GRK2$^{f/f}$/Agc1$^{tm(IRES\text{-}CreERT2)}$) mice, following the experimental timeline in Fig. 1a. (c) Timeline for the PTH challenge experiment: Tamoxifen (Tmx) injection at P3 & P4, PTH injection P6-P9, and BrdU injection 2 hours before sacrifice (Sac) at P10. (d) GRK2 RNA expression in proximal tibiae using RNAScope; yellow line indicates the proliferative zone; green line delineates the end of the hypertrophic zone. (e) H&E staining images; quantification of the (f) proliferative zone (PZ) length (μm) and (g) number of PZ cells/column. (h) BrdU/DAPI IF staining of the proliferative zone (area marked with a yellow line), with (i) quantification of number of proliferative cells. (j) Col X/DAPI IF staining of the proximal tibiae, with (K) quantification. *P < 0.05, **P < 0.01, ****P < 0.0001 using 2-way ANOVA test, values are expressed as mean ± SEM.*

RESULTS: GRK2-icKO mice GP had longer proliferative zone with increased proliferation, short and disarranged hypertrophic zone with increased chondrocyte proliferation (*Fig. 1*), defective differentiation, and decreased apoptosis and mineralization, causing shorter bones at both P10 and P28 (n=6-7/group). In vitro experiments in primary chondrocyte corroborated these results with GRK2 knockdown leading to increased cAMP production and decreased mineralization (n= triplicates with 3 repetitions/group). PTH1R expression was increased in the GP of GRK2-icKO mice at P10 and P28, suggesting that GRK2 desensitizes PTH1R in the GP to promote chondrocyte differentiation. The deletion of GRK2 in combination with PTH1R did not rescue the GP from fusion at P28, despite slower differentiation at P10 (*Fig. 2a,b*), supporting PTH1R as the major GPCR regulated by GRK2 in GP chondrocytes. In contrast, the administration of exogenous PTH promoted the proliferation and decreased the hypertrophic differentiation of GP chondrocytes, which was potentiated in GRK2-icKO mice (*Fig. 2c-k*); (n=3/group). Furthermore, we found a direct regulatory role for GRK2 in GP chondrocyte differentiation through ERK phosphorylation, where GRK2-icKO mice demonstrated diminished ERK phosphorylation and apoptosis at the GP chondro-osseous junction, independent of dietary phosphate levels (n=3/group).

DISCUSSION: These findings present GRK2 as a key regulator of endochondral ossification that ensures proper chondrocyte differentiation through PTH1R dependent and independent mechanisms. SIGNIFICANCE/CLINICAL RELEVANCE: Any disturbance or dysregulation in EO leads to a variety of chondrodysplasias that can be fatal. This study uncovers GRK2 as a novel regulator and therapeutic target for conditions in which pathological or dysregulated EO is a disease feature.