Osteoblast-Specific Deletion of Pkd2 Leads to Low-Turnover Osteopenia and Reduced Bone Marrow Adiposity

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Abstract

Polycystin-1 (Pkd1) interacts with polycystin-2 (Pkd2) to form an interdependent signaling complex. Selective deletion of Pkd1 in the osteoblast lineage reciprocally regulates osteoblastogenesis and adipogenesis. The role of Pkd2 in skeletal development has not been defined. To this end, we conditionally inactivated Pkd2 in mature osteoblasts by crossing Ostecalcin (Oc)-Cre;Pkd2+/null mice with floxed Pkd2 (Pkd2flox/flox) mice. Oc-Cre;Pkd2flox/null (Pkd2Oc-cKO) mice exhibited decreased bone mineral density, trabecular bone volume, cortical thickness, mineral apposition rate and impaired biomechanical properties of bone. Pkd2 deficiency resulted in diminished Runt-related transcription factor 2 (Runx2) expressions in bone and impaired osteoblastic differentiation ex vivo. Expression of osteoblast-related genes, including, Osteocalcin, Osteopontin, Bone sialoprotein (Bsp), Phosphate-regulating gene with homologies to endopeptidases on the X chromosome (Phex), Dentin matrix protein 1 (Dmp1), Sclerostin (Sost), and Fibroblast growth factor 23 (FGF23) were reduced proportionate to the reduction of Pkd2 gene dose in bone of Oc-Cre;Pkd2flox/+ and Oc-Cre;Pkd2flox/null mice. Loss of Pkd2 also resulted in diminished peroxisome proliferator-activated receptor γ (PPARγ) expression and reduced bone marrow fat in vivo and reduced adipogenesis in osteoblast culture ex vivo. Transcriptional co-activator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP), reciprocally acting as co-activators and co-repressors of Runx2 and PPARγ, were decreased in bone of Oc-Cre;Pkd2flox/null mice. Thus, Pkd1 and Pkd2 have coordinate effects on osteoblast differentiation and opposite effects on adipogenesis, suggesting that Pkd1 and Pkd2 signaling pathways can have independent effects on mesenchymal lineage commitment in bone.
Introduction

PKD1 encodes polycystin-1 (PC1), a transmembrane receptor-like protein, which links environmental clues to intracellular processes regulating cell growth and development. PKD2 encodes polycystin-2 (PC2), a transient receptor potential channel. The PC1 binds to PC2 in a ratio of 1 to 3 through their respective C-terminal coiled-coiled domains to form the functional signaling polycystin complex in cell surface membranes [1–5]. Typically, the functions of PC1 and PC2 are interdependent and concordant, as evidenced by common Autosomal Dominant Polycystic Kidney Disease (ADPKD) phenotype caused by inactivation of either PKD1 or PKD2 [6, 7].

Although the functions of polycystins have largely been derived from the study of inactivating mutations of either PKD1 or PKD2 in the kidney, Pkd1 and Pkd2 are widely expressed in many tissues and cell types, including the osteoblast lineage in bone. Recent studies indicate that PC1 (Pkd1) and PC2 (Pkd2) also form a complex that co-localize to primary cilia in osteoblasts/osteocytes to create a “sensor” that regulates bone mass [8]. Osteoblast lineage specific deletion of Pkd1 in mice establishes a direct role for PC1 in regulating both osteoblast development and transducing the bone response to mechanical loading [8–15]. Indeed, the selective genetic ablation of Pkd1 in osteoblasts and osteocytes results in osteopenia that is caused by diminished osteoblast-mediated bone formation and increased bone marrow adipogenesis. Loss of Pkd1 reciprocally decreases Runx2 and increases expression of PPARγ transcription factors that direct the commitment of mesenchymal stem cells to the osteoblastic and adipocytic lineages, respectively. Calcium-dependent signal transduction pathways link Pkd1 to Runx2 expression, but the cellular mechanisms mediating the reciprocal regulation of PPARγ have not been defined. The phenotype in the bone specific Pkd1-deficient mice resembles age-related bone loss, suggesting that understanding the function of polycystins in bone may be important in understanding the pathogenesis and treatment of senile osteoporosis.

Whether loss of Pkd2 function results in a bone phenotype in mice similar to Pkd1 deficiency has not be investigated. However, recent siRNA mediated knockdown of Pkd2 in osteoblasts resulted in impaired osteoblasts differentiation in vitro [16]. In addition, data from a GWAS meta-analyses found that the PKD2 SNP rs12511728 was significantly associated with femoral neck bone mineral density (BMD) [16] and mutations in PKD2 are associated with abnormal shape of craniofacial bones in patients with ADPKD [17]. Global homozygous Pkd2\(^{null/null}\) mice die early in utero [18], which precludes assessing the direct effects of Pkd2 on osteoblast function.

To define the osteoblast specific functions of Pkd2, we conditionally inactivated Pkd2 in postnatal mature osteoblasts. We found that loss of Pkd2 suppressed both osteoblast-mediated bone formation and adipogenesis, leading to osteopenia and decreased bone marrow fat. Thus, Pkd1 and Pkd2 have concordant effects on osteoblastogenesis and opposite effects on adipogenesis, consistent with both overlapping and independent signaling functions in osteoblasts.
Materials and Methods

Animal breeding and genotyping

All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The University of Tennessee Health Science Center’s Animal Care and Use Committee approved all animal studies (Protocol number: 12–160.0). The mice were anesthetized with Ketamine (90 mg/kg) and Xylazine (10 mg/kg) for a bone densitometry scan, and the mice not useful for experimental purposes were sacrificed by CO₂ inhalation plus cervical dislocation. We obtained the floxed Pkd2 (in exon 3) mice and heterozygous Pkd2null/+ (in exon 1) mice from Dr. Guanqing Wu at Vanderbilt University Medical Center [19] and Osteocalcin (Oc)-Cre mice from Dr. Thomas Clemens at University of Alabama [20]. These mice were bred and maintained on a C57BL/6J background. At first, we created double heterozygous Oc-Cre;Pkd2null/+ mice and homozygous Pkd2flox/flox mice. Then double heterozygous Oc-Cre;Pkd2null/+ mice were mated with homozygous Pkd2flox/flox mice to generate excised floxed Pkd2 heterozygous (Oc-Cre;Pkd2flox/null or Pkd2Oc-cko), as well as Pkd2 heterozygous mice (Pkd2null/+ and Oc-Cre negative control mice (Pkd2flox/+), equivalent to wild-type). These mice were used to collect samples at 6 weeks of age for phenotypic analysis. For genotyping PCR and Cre-mediated recombination, genomic DNAs were prepared from tail clips, bone, and other tissue specimens using a Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA). Mice were genotyped for Oc-Cre using previously described primers [12], for the Pkd2flox allele using forward primer 5′-TCT GAC TTG CAG ACT GTG GG-3′ and reverse primer 5′-AGG TAG GGG AAG GTC AGG GTT GG-3′ (355 bp product for the Pkd2+ wild-type allele, 575 bp product for the Pkd2flox floxed allele), for the Pkd2flox delta floxed allele using forward primer 5′-AGC TTG GCT GGA CGT AAA-3′ and reverse primer 5′- AGG TAG GGG AAG GTC AGG GTT GG-3′ (427 bp product for the Pkd2flox delta floxed allele), and for the Pkd2null allele using two forward primers 5′-GCG GCC TAG CTG TCC C-3′ and 5′-GTG CTA CTT CCA TTT GTC AGC TCC TGC-3′ and one reverse primer 5′-GTT GTC GCG GCT CCA CG-3′ (150 bp product for the Pkd2+ wild-type allele, 350 bp product for the Pkd2null null allele) as previously described [19]. All Pkd2 alleles were identified in 2% agarose gels (Figure 1).

Bone densitometry, histomorphometric and micro-CT analysis

Bone mineral density (BMD) of femurs was assessed at 6 weeks of age using a small animal bone densitometer (Lunar Corp, Madison, WI). Calcein (Sigma-Aldrich, St. Louis, MO) double labeling of bone and histomorphometric analyses of periosteal mineral apposition rate (MAR) in tibias were performed using the osteomeasure analysis system (Osteometrics, Inc., Decatur, GA). The distal femoral metaphyses were also scanned using a micro-CT 40 scanner (Scanco Medical AG, Brüttsisellen, Switzerland). A 3D images analysis was done to
determine bone volume (BV/TV) and cortical thickness (Ct.Th) as previously described [12, 21].

Three-point bending to failure

Femurs were harvested from freshly euthanized mice. The femurs (1520 slices per femur) were scanned with a micro-CT 40 scanner (Scanco Medical AG, Brüttisellen, Switzerland), and saved in a DICOM file format. To characterize the biomechanical properties of the bones, displacement-controlled three-point bending to failure tests were conducted on excised femurs ex vivo. For each femur, all associated soft tissues were removed; the bone was individually wrapped in saline-soaked gauze, and then stored at −20°C until testing. On the day of testing, samples were removed from −20°C storage, and allowed to thaw and reach room temperature. Prior to, and throughout the tests, bone samples were kept hydrated.
with saline soaked gauze and not allowed to dry out. To allow unbiased comparison between the samples, consistent span lengths of 6mm and crosshead displacement rates of 0.1 mm/s were used during testing. The femurs were placed into the test fixtures so they would be impacted in what would be the anterior to posterior direction \textit{in vivo} (ElectroForce 3200, Bose Corp., Minnetonka, MN). Crosshead displacement and axial load were recorded at a rate of 70 Hz. The stiffness and ultimate force were calculated from the resulting load versus displacement curves for each sample. The Young’s modulus (E) for each bone was calculated using the following equation:

\[ E = \frac{SL^3}{48I} \]

Where \( S \) is the stiffness, \( L \) is the span length, and \( I \) is the area moment of inertia. The area moment of inertia was calculated across the midspan at the fracture location using 10 slices midspan of the realigned microCT scans using the BoneJ plugin for the image-processing program ImageJ (Bone J, NIH, Bethesda, MD, USA) \[8, 22, 23]\.

**Bone microindentation testing (BMT)**

The BMT was performed using a microindentation device (ActiveLife Tech, Inc., Santa Barbara, CA, USA) as previously described \[24–26\]. Briefly, the periosteum of isolated femurs was scratched and a probe assembly placed on the anterior surface of the mid-femur performed measurements. A 10-cycle indentation with a maximum 2N force and 2 Hz frequency at a touchdown force of 0.1N and without any preconditioning was performed and the average value of three measurements was recorded. The indentation distances were analyzed by specific software and four parameters were obtained to use as outcome variables: indentation distance increase (IDI) between the first and last indentation cycle; total distance between the bone surface and the last indentation cycle (Total ID); creep indentation distance (Creep ID), the progressive indentation distance during the stable force phase of the first indentation cycle at the maximum 2N force; and average unloading slope, the average slope of unloading portion during three measurements. IDI values were then normalized by the IDI of polymethylmethacrylate (PMMA) tested with the same probe. Throughout testing samples were kept moist with 1xPBS.

**Detection of bone marrow adipocytes in long bones by oil red O lipid staining and micro-CT analysis**

Whole intact femurs and tibias with encapsulated marrow were dissected from 20 weeks old mice, fixed for 48 hours in phosphate buffered paraformaldehyde, decalcified in 14% EDTA for two weeks. For oil red O lipid staining, the tibias were embedded in tissue freezing medium. Cryosectioning was performed on a frozen sectioning device (Leica, Nussloch, Germany) equipped with a rapid frozen
sectioning kit (Instrumedics, Hackensack, NJ). 10 μm thick sections were then stained with oil red O (ORO) for bone marrow adipocytes as previously described [9, 12]. Briefly, the sections were rinsed in 60% isopropanol, stained for 20 minutes in 0.5% ORO-isopropanol solution, differentiated in 60% isopropanol, rinsed in tap water and mounted in glycerin jelly. Sections were examined with an inverted fluorescent microscope equipped with a digital camera (Leica, Nussloch, Germany). For detection of bone marrow fat cells by Micro-CT, the femurs were stained for 2 hours in 2% aqueous osmium tetroxide (OsO4). The Bones were rinsed in water for 48 hours and then scanned at 6 μm resolution using a micro-CT 40 scanner, 45 KeVp and 177 μA. Quantification of fat volume, density, and distribution throughout the marrow was registered to low contrast decalcified bone as our Lab previously described [9, 12].

Real-time RT-PCR
For quantitative real-time RT–PCR, 1.0 μg total RNA isolated from either the long bone of 6-week-old mice or 10-days cultured primary osteoblasts in differentiation media was reverse transcribed as previously described [9, 27]. PCR reactions contained 100 ng template (cDNA or RNA), 300 nM each forward and reverse primers, and 1XqPCR Supermix (Bio-Rad, Hercules, CA) in 50 μl. The threshold cycle (Ct) of tested-gene product from the indicated genotype was normalized to the Ct for cyclophilin A. Expression of total Pkd2 transcripts was performed using the following Pkd2-allele-specific primers: In exon 3, forward primer of normal Pkd2+ transcript: 5'-GCA TGA TGA GCT CCA ATG TG-3', and reverse primer: 5'-TCG ACA CTG GGG TGT CTA TG -3'. In exon 1, forward primer of normal Pkd2+ transcript: 5'-TTG AGG CAG AGG AGG ATG AC-3', and reverse primer: 5'-CAT CCA TCT CTA CCA CCA TCC-3'. The normal Pkd2+ vs cyclophilin A is normalized to the mean ratio of 5 control mice, which has been set to 1. The percentage of conditional and global deleted transcripts was calculated from the relative levels of the normal Pkd2+ transcripts in different Pkd2 exons.

Serum biochemistry
Serum urea nitrogen (BUN) was determined using a BUN diagnostic kit from Pointe Scientific, Inc. Calcium was measured using a calcium cresolphthalein complexone kit (Stanbio Laboratories, Boerne, TX, USA) and phosphorus was measured using the phosphomolybdylate-ascorbic acid method, as previously described [9, 11, 28]. Serum Osteocalcin levels were measured using a mouse Osteocalcin EIA kit (Biomedical Technologies Inc. Stoughton, MA, USA). Serum parathyroid hormone (PTH) levels were measured using the Mouse Intact PTH ELISA kit (Immutopics, Carlsbad, CA, USA). Serum full-length FGF23 levels were measured using the FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan). Serum OPG and Rank ligand (RankL) were measured using mouse ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA), and serum tartrate resistant
acid phosphatase (TRAP) was assayed with a mouse TRAP ELISA kit (Immunodiagnostic Systems, Fountain Hills, AZ).

Primary osteoblast culture for proliferation, differentiation, and western blot analysis

Calvaria from E17.5 control and Pkd2-deficient embryos were used to isolate primary osteoblasts by sequential collagenase digestion at 37°C. To engineer immortal osteoblast cell lines, isolated primary osteoblasts were infected using a retroviral vector carrying SV40 large and small T antigen as previously described [10, 29]. Briefly, cells were grown in 100-mm plates at 50–60% confluence the day before infection. On the day of infection, the medium was removed and replaced with medium containing SV40 large and small T antigen-helper-free viral supernatant in the presence of 4 mg/ml of polybrene (Sigma, St. Louis, MO, USA) for 48 h. The cells were allowed to recover for 72 h followed by selection with 1 mg/ml puromycin (Sigma-Aldrich) for up to 15 days. The immortalized osteoblasts were cultured in α-MEM containing 10% FBS and 1% penicillin and streptomycin (P/S) and characterized following the protocols below. Cell proliferation was detected by BrdU incorporation assays following the manufacturer’s directions (QIA58, Calbiochem, Gibbstown, NJ, USA). To induce differentiation, the immortalized osteoblasts were plated at a density of 2 × 10^4 cells per well in a 12-well plate and 4 × 10^4 cells per well in a 6-well plate and grown up to 21 days in α-MEM containing 10% FBS supplemented with 5 mM β-glycerophosphate and 25 μg/ml ascorbic acid. ALP activity and Alizarin red-S histochemical staining for mineralization were performed as previously described [10, 29]. Total DNA content was measured with a double-strand DNA quantitation reagent and kit (Molecular Probes, Eugene, OR, USA). Protein concentrations of the supernatant were determined with a total protein assay kit (Bio-Rad, Hercules, CA, USA). For signaling mechanism study, the cells were cultured in the osteogenic differentiation media for 48 hours. Then the cells were lysed with 150 μl of tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) with 1 x Halt protease inhibitor and 1 mM phenylmethanesulfonyl fluoride (PMSF) per well, After three 30-second sonications, total cell lysates were centrifuged at 13,000 × g for 10 minutes and supernatants were stored at -80°C until use. Protein concentrations of the supernatant were determined with a total protein assay kit (Bio-Rad, Hercules, CA). Equal quantities of protein were subjected to 4–12% Bis-Tris gradient Gels (Invitrogen, Carlsbad, CA) and were analyzed with standard western blot protocols (HRP-conjugated secondary antibodies from Santa Cruz Biotechnology and ECL chemiluminescent immunodetection system from GE Healthcare Bio-Sciences). Anti-YAP/TAZ (D24E4, #8418) and Anti-pYAP antibody (Ser127) (D9W2I, #13008) were purchased from Cell Signaling Technologies (Danvers, MA). Purified mouse anti-TAZ (560236) was purchased from BD Biosciences (San Jose, CA). Anti-p-TAZ (Ser 89) (sc-17610) and Anti-β-actin (sc-47778) antibodies were from Santa Cruz
Biotechnology (Paso Robles, CA). The intensity of bands was quantified using Image J software (http://rsb.info.nih.gov/ij/).

**Transient transfection**

The immortalized osteoblasts were cultured in α-MEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). To examine if PC2 regulates Hippo signaling pathway, a number of 1×10^6 of the immortalized osteoblasts were transfected with 3.0 μg of 8xGTIIc luciferase reporter (8xGTIIc-Luc) constructs in combination with 3.0 μg of empty pcDNA3.1 expression vector, and 0.6 μg of Renilla luciferase-null (RL-null) as internal control plasmid by electroporation using a cell line optimal transfection kit according to the manufacturer’s protocol (Amaxa Inc, Gaithersburg, MD). A total of 6.6 μg of plasmid DNAs was used for each electroporation. The transfected cells were plated in 12-well plates and harvested in 32 hours after transfection. Cells were lysed in 100 μl of reporter lysis buffer (Promega, Madison, WI). A luciferase assay (20 μl of cell lysed) was performed using a dual luciferase assay kit (Promega, Madison, WI), and activity was measured with a tube luminometer (MGM Instruments, Inc., Hamden, CT).

**Statistics**

We evaluated differences between groups by one-way analysis of variance. All values are expressed as means ± S.D. All computations were performed using a commercial biostatistics software (GraphPad Software Inc. La Jolla, CA).

**Results**

**Oc-Cre mediated bone-specific deletion of Pkd2**

We studied four genotypes from the breeding strategy, including Oc-Cre;Pkd2^flox/null or Pkd2^Oc-ckO, Oc-Cre;Pkd2^flox/+, Pkd2^flox/null, and Pkd2^flox/+ . These mice were born at the expected Mendelian frequency and all genotypes exhibited survival indistinguishable from wild-type mice over the period of study. Oc-Cre expression is limited to cells of the osteoblast lineage (late osteoblasts, osteocytes) with onset of expression just before birth [20]. To confirm that the Pkd2 floxed allele in Exon 3 was selectively deleted in bone, we performed PCR analysis using a combination of primers that specifically detect floxed Pkd2 alleles (Pkd2^flox) and the excised floxed Pkd2 alleles (Pkd2^Δflox) in Oc-Cre;Pkd2^flox/+ or Oc-Cre;Pkd2^flox/null mice (Figure 1A). We demonstrated that Oc-Cre-mediated floxed recombination occurred exclusively in bone, whereas non-skeletal tissues retained the intact floxed Pkd2 alleles (Pkd2^flox) (Figure 1B). The Pkd2^null null allele in Exon 1 was used in combination with the Cre-recombinase deletion of the floxed Pkd2 allele (Pkd2^Δflox) in Exon 3 to increase the net efficiency of Pkd2 inactivation.

The level of the full-length Pkd2 transcripts in Exon 1 and 3 from the femurs of these four genotypes mice were assessed by real time RT-PCR. As expected, both
Pkd2\textsubscript{flox/null} and Oc-Cre;Pkd2\textsubscript{flox/null} mice displayed 50% reduction of intact Pkd2 transcripts in Exon 1, whereas Oc-Cre;Pkd2\textsubscript{flox/+} and Oc-Cre;Pkd2\textsubscript{flox/null} mice exhibited approximately 25% excision of the floxed Exon 3 from Pkd2, indicating that Oc-Cre mediated bone-specific deletion of the floxed Pkd2 allele is incomplete (Figure 1C). The combined effect Pkd2\textsubscript{null} and (Pkd2\textsubscript{flox}) in Oc-Cre;Pkd2\textsubscript{flox/null}, however, resulted in a net reduction of intact Pkd2 expression by \textasciitilde 75% in bone (Figure 1C). Thus, there was a progressive reduction of functional Pkd2 message in these Pkd2 deficient mice, i.e., Pkd2\textsubscript{flox/+} (100%), Oc-Cre;Pkd2\textsubscript{flox/+} (75%), Pkd2\textsubscript{flox/null} (50%), and Oc-Cre;Pkd2\textsubscript{flox/null} (25%) mice (Figure 1C). In contrast, targeting Pkd2 had no effect on Pkd1 transcript expression (Figure 1D). In addition, Oc-Cre;Pkd2\textsubscript{flox/null}, and Oc-Cre;Pkd2\textsubscript{flox/+} demonstrated no cyst formation in the kidney, consistent with the bone specific inactivation of Pkd2 (data not shown). Pkd2\textsubscript{flox/null} mice also exhibited no renal cysts, as previously reported [18].

Effects of bone specific deletion of Pkd2 on bone structure

At 6 weeks of age, the gross appearance and body weight of all genotypes were indistinguishable. Compared to control Pkd2\textsubscript{flox/+} mice, conditional heterozygous Oc-Cre;Pkd2\textsubscript{flox/+} mice, which had a 25% reduction in Pkd2 expression, exhibited no abnormalities in bone mass. However, both global heterozygous Pkd2\textsubscript{flox/null} and conditional Pkd2\textsubscript{Oc-cKO} null mice, which had respective 50% and 75% reductions in Pkd2 express, were osteopenic, as evidenced by respective 14.4% verse 9.2% and 12.5% verse 9.2% reductions in BMD in both male and female adult mice (Figure 2A). Micro-CT analysis revealed that the reduction in bone mass in both male global heterozygous Pkd2\textsubscript{flox/null} and conditional Pkd2\textsubscript{Oc-cKO} null mice were caused by reductions in trabecular bone volume (19.3% and 30.9%, respectively) and cortical bone thickness (8.4% and 7.3%, respectively) (Figure 2B). In contrast, conditional heterozygous Oc-Cre;Pkd2\textsubscript{flox/+} had no significant difference in both trabecular and cortical bone compared with control mice (Figure 2B). Consistent with a low-bone-mass phenotype by BMD and Micro-CT analysis, we found that loss of Pkd2 in bone from both Pkd2\textsubscript{flox/null} and Pkd2\textsubscript{Oc-cKO} null mice was associated with a decrease in periosteal mineral apposition rate (MAR, 25.1% and 37.3%, respectively) (Figure 2C), indicating a significant reduction of bone formation rate in Pkd2 deficient mice.

Effect of conditional deletion of Pkd2 on femur cortical bone geometry, mechanical and material properties

Conditional deletion of Pkd2 also resulted in site-specific alterations of femur cortical bone geometry. In this regard, single conditional Oc-Cre;Pkd2\textsubscript{flox/+} heterozygous, single global Pkd2\textsubscript{flox/null} heterozygous, and conditional Pkd2\textsubscript{Oc-cKO} null mice showed a gene dose dependent decrease in both total and cortical area at 6 weeks of age when compared with age-matched control Pkd2\textsubscript{flox/+} mice (Table 1), this is in accordance with a significant reduction in moment of inertia, I\textsubscript{x} in these Pkd2 deficient mice (Table 1). Obviously, Pkd2 deficient mice displayed
Figure 2. Pkd2 deficiency results in loss of bone mass. (A) Effects of Pkd2 deficiency on bone mineral density (BMD) at 6 weeks of age. Compared to control Pkd2<sup>fox/+</sup> mice, conditional heterozygous Oc-Cre;Pkd2<sup>fox/+</sup> mice exhibited no abnormalities in bone mass. However, both global heterozygous Pkd2<sup>fox/null</sup> and conditional Pkd2<sup>fox/null</sup> mice were osteopenic, as evidenced by respective 14.4% and 12.5% reduction BMD in male adult mice. (B) Effects of Pkd2 deficiency on structure of femurs. µCT analysis of the distal femoral metaphyses and midshaft diaphyses revealed that the reduction in bone mass in both global
heterozygous Pkd2flox/null and conditional Pkd2Oc-cKO null mice were caused by reductions in trabecular bone volume (19.3% and 30.9%, respectively) and cortical bone thickness (8.4% and 7.3%, respectively). In contrast, conditional heterozygous Oc-Cre;Pkd2flox/+ had no significant difference in both trabecular and cortical bone compared with control mice. (C) Effects of Pkd2 deficiency on bone formation rate. Both Pkd2flox/null and Pkd2Oc-cKO null mice exhibited a significant decrease in periosteal mineral apposition rate (MAR, 25.1% and 37.5%, respectively), indicating a reduction of bone formation rate in Pkd2 deficient mice. Data represent the mean±S.D. from 6–8 individual mice. Values sharing the same superscript in different group are not significantly different at P<0.05. doi:10.1371/journal.pone.0114198.g002

a reduction in the size of the marrow cavity resulting from a smaller midshaft diameter compared with control mice. To examine whether changes of femoral bone geometry in the Pkd2 deficient mice may affect bone mechanical properties, we used these femurs to perform 3-point bending to failure experiments. Compared with the control mice, global Pkd2flox/null heterozygous and conditional Pkd2Oc-cKO null mice exhibited a lower maximum force and stiffness in 3-point bending, but no significant differences in the Young’s modulus (Table 1), indicating that the changes of bone geometry and bone structure at 6 weeks of age may preserve the Young’s modulus in global Pkd2flox/null heterozygous and conditional Pkd2Oc-cKO null mice. There was no difference in these parameters between single conditional Oc-Cre;Pkd2flox/+ heterozygous mice and control mice (Table 1).

To assess whether Pkd2 deficiency may have an impact on bone material properties, we used these femurs to perform reference point indentation (RPI, micro-indentation) measurements. We found significant differences in several RPI variables (Table 1) between Pkd2 deficient and control mice. There was a 226%, 82.5%, and 162% increase in IDI, TID, and CID, respectively, between heterozygous Pkd2flox/null and control mice. A similar 190%, 69%, and 118% increase in IDI, TID, and CID, respectively, was also observed between Pkd2Oc-cKO null and control mice. In contrast, there was a 33.3% decrease in the average US in both global Pkd2flox/null heterozygous and conditional Pkd2Oc-cKO null mice compared with control mice. However, these parameters were not significantly different between heterozygous Oc-Cre;Pkd2flox/+ and control mice (Table 1). These findings that Pkd2 deficiency makes bone more brittle and less stiff.

**Pkd2-regulated gene expression in bone**

Next, we examined by real-time RT-PCR the expression levels of a panel of osteoblast lineage-, osteoclast-, chondrocyte-, and adipocyte-related mRNAs from the femurs of 6-week-old control, heterozygous Pkd2 deficient (Oc-Cre;Pkd2flox/+ and Pkd2flox/null), and Oc-Cre;Pkd2flox/null mice (Table 2). We found that changes in bone transcripts were more sensitive to reductions in Pkd2 and exhibited a gene-dose dependent response on osteoblast-related gene transcripts.

We found significant alterations in osteoblast-related gene expression. Bone derived from heterozygous Oc-Cre;Pkd2flox/+, in spite of having no measurable change in bone structure and only a 25% reduction in Pkd2 message expression,
did have significant reductions in several osteoblast-lineage gene transcripts, including Osteocalcin, Osteopontin, and Cyclooxygenase 2 (Cox2) mRNA levels compared to control mice. Bone from heterozygous Pkd1^flox/null mice with a 50% reduction in Pkd2 expression exhibited significant reductions in Runx2-II, Osteocalcin, Osteopontin, Bsp, Cox2, matrix metalloproteinase 13 (Mmp13), matrix extracellular phosphoglycoprotein (Mepe), and Sost mRNA levels compared to control mice (Table 2). Compared with heterozygous Pkd2^flox/null mice, significantly greater reductions of Osteocalcin, Osteopontin, Cox2, Connexin 43 (Cnx43), Rank ligand (RankL), Phex, Dmp1 and Fgf23 were observed in conditional Pkd2^Oc-cKO null mice that had a 75% reduction in Pkd2 expression.

Changes in gene expression in bone correlated with alterations in serum biomarkers in conditional Pkd2^Oc-cKO null mice. In this regard, further evidence for osteoblast-lineage dysfunction includes reductions in both Fgf23 and RanKL in serum from conditional Pkd2^Oc-cKO null mice at 6 weeks of age (Table 3). However, no changes of serum phosphorus and calcium levels were observed in Pkd2^Oc-cKO null mice compared to control mice. Also, no significant changes of serum BUN, PTH, Osteocalcin, and OPG were observed among these four genotype mice (Table 3).

The Opg/RankL expression ratio was increased in conditional Pkd2^Oc-cKO null mice and was associated with reduced bone expression of Trap and matrix metalloproteinase 9 (Mmp9), markers of bone resorption (Table 2). Serum levels of TRAP were also reduced in conditional Pkd2^Oc-cKO null mice compared to control littermates (Table 3), suggesting that Pkd2-mediated bone loss results from low bone formation rates rather than increased bone resorption. Transcripts of chondrocyte-related genes did not differ between heterozygous Pkd2 deficient and Pkd2^Oc-cKO null mice (Table 2).

Table 1. Femoral bone geometry, biomechanical, and biomaterial properties in 6-week-old mice.

| Parameters                  | Pkd2^flox/+ | Oc-Cre;Pkd2^flox/+ | Pkd2^flox/null | Pkd2^Oc-cKO | p-value |
|-----------------------------|-------------|--------------------|----------------|-------------|---------|
| **Cortical bone Geometry**  |             |                    |                |             |         |
| Total area (mm²)            | 2.5±0.19⁸   | 2.3±0.17⁹         | 2.1±0.27⁹      | 2.1±0.20⁹   | <0.0001 |
| Cortical area (mm²)         | 0.61±0.06⁹  | 0.56±0.06⁸        | 0.52±0.09⁸     | 0.52±0.05⁹  | 0.0017  |
| Moment of inertia, Iₓ (mm⁴)| 0.15±0.04⁹  | 0.11±0.03⁹        | 0.10±0.02⁹     | 0.11±0.03⁹  | 0.0007  |
| **Biomechanical properties**|             |                    |                |             |         |
| Maximum force (N)           | 12.0±0.97⁹  | 10.8±1.2⁸         | 8.9±0.94⁹      | 9.4±1.6⁹    | 0.0077  |
| Stiffness (N/mm)            | 60.2±9.1⁸   | 62.0±3.4⁹         | 46.9±5.6⁹      | 37.1±8.3³   | 0.0001  |
| Young’s modulus (Gpa)       | 2.9±1.0⁹    | 2.9±0.59⁹         | 3.4±0.52⁹      | 2.9±0.72⁹   | 0.5743  |
| **Biomaterial properties**  |             |                    |                |             |         |
| IDI (µm)                    | 7.7±1.6⁹    | 9.7±2.2⁹          | 25.1±3.2⁹      | 22.3±5.3⁹   | 0.0053  |
| Total ID (µm)               | 39.4±4.5⁹   | 47.6±10.6⁹        | 71.9±7.1⁹      | 66.7±7.7⁹   | 0.0015  |
| Creep ID (µm)               | 4.5±0.84⁹   | 5.3±0.82⁹         | 11.8±1.3⁹      | 9.8±3.2⁹    | 0.0003  |
| Average US (N/µm)           | 0.18±0.04⁹  | 0.18±0.02⁹        | 0.12±0.03⁹     | 0.12±0.02⁹  | 0.0077  |

Data are mean ± S.D. from 6-8 serum samples of 6-week-old individual mice. Values sharing the same superscript are not significantly different at P<0.05.
Table 2. Gene-expression profiles in bone in 6-week-old mice.

| Gene          | Accession no. | Oc-Cre; Pkd2^{flox/+} | Pkd2^{flox/null} | Oc-Cre; Pkd2^{flox/null} | p-value |
|---------------|---------------|-----------------------|------------------|--------------------------|---------|
| **Osteoblast lineage** |               |                       |                  |                          |         |
| Runx2-II      | NM_009820.5   | 1.03 ± 0.23           | 0.64 ± 0.14*     | 0.46 ± 0.28*             | 0.0005  |
| Osteopontin   | NM_009263.3   | 0.79 ± 0.19*          | 0.71 ± 0.15*     | 0.45 ± 0.15*             | <0.0001 |
| Osteocalcin   | NM_007541.2   | 0.76 ± 0.16*          | 0.77 ± 0.16*     | 0.51 ± 0.13*             | <0.0001 |
| Bsp           | NM_008318.3   | 0.87 ± 0.12           | 0.68 ± 0.12*     | 0.37 ± 0.24*             | <0.0001 |
| Cox2          | NM_011198.3   | 0.76 ± 0.18*          | 0.77 ± 0.16*     | 0.50 ± 0.15*             | 0.0001  |
| Cnx43         | NM_010286.3   | 0.93 ± 0.23           | 0.95 ± 0.18      | 0.41 ± 0.18*             | <0.0001 |
| Mmp13         | NM_008607.2   | 0.87 ± 0.31           | 0.66 ± 0.21*     | 0.44 ± 0.18*             | 0.0004  |
| Opg           | NM_008764.3   | 0.98 ± 0.24           | 0.87 ± 0.31      | 0.84 ± 0.27              | 0.5987  |
| RankL         | NM_011613.3   | 0.85 ± 0.18           | 0.94 ± 0.19      | 0.56 ± 0.21*             | 0.0014  |
| Phex          | NM_011077.2   | 0.90 ± 0.41           | 0.97 ± 0.39      | 0.43 ± 0.14*             | 0.0040  |
| Dmp1          | NM_016779.2   | 0.91 ± 0.27           | 0.79 ± 0.23      | 0.52 ± 0.21*             | 0.0014  |
| Mepe          | NM_053172.2   | 1.10 ± 0.40           | 0.72 ± 0.12*     | 0.56 ± 0.20*             | 0.0017  |
| Sost          | NM_024449.5   | 0.98 ± 0.35           | 0.68 ± 0.12*     | 0.58 ± 0.18*             | 0.0012  |
| Fgf23         | NM_022657.4   | 0.92 ± 0.33           | 0.88 ± 0.38      | 0.63 ± 0.18*             | 0.0576  |
| Wnt10b        | NM_011718.2   | 0.92 ± 0.42           | 0.71 ± 0.10*     | 0.35 ± 0.24*             | <0.0001 |
| Axin2         | NM_015732.4   | 0.94 ± 0.17           | 0.72 ± 0.26*     | 0.38 ± 0.13*             | <0.0001 |
| Taz           | NM_1168281.1  | 0.75 ± 0.22*          | 0.72 ± 0.16*     | 0.42 ± 0.24*             | 0.0002  |
| Yap           | NM_009534.3   | 1.05 ± 0.11           | 0.74 ± 0.17*     | 0.53 ± 0.34*             | 0.0003  |
| BirC3         | NM_007464.3   | 1.00 ± 0.18           | 0.72 ± 0.18*     | 0.65 ± 0.18*             | 0.0006  |
| Ctgf          | NM_010217.2   | 0.97 ± 0.33           | 0.66 ± 0.18*     | 0.45 ± 0.21*             | <0.0001 |
| Inhba         | NM_008380.1   | 0.98 ± 0.21           | 0.71 ± 0.16*     | 0.69 ± 0.11*             | 0.0002  |
| Hprt          | NM_013556.2   | 1.07 ± 0.15           | 0.95 ± 0.23      | 1.08 ± 0.41              | 0.7324  |
| **Osteoclast** |               |                       |                  |                          |         |
| Trap          | NM_007388.3   | 0.79 ± 0.28           | 0.85 ± 0.29      | 0.46 ± 0.21*             | 0.0005  |
| Mmp9          | NM_013599.3   | 0.91 ± 0.32           | 0.99 ± 0.31      | 0.56 ± 0.14*             | 0.0032  |
| **Chondrocyte** |           |                       |                  |                          |         |
| Collagen II   | NM_031163.3   | 0.91 ± 0.16           | 0.90 ± 0.33      | 1.29 ± 0.07                | 0.2935  |
| VegfA         | NM_009505.4   | 1.15 ± 0.21           | 1.10 ± 0.29      | 0.99 ± 0.37              | 0.7891  |
| **Adipocyte** |               |                       |                  |                          |         |
| PPARγ         | NM_009505.4   | 1.03 ± 0.32           | 0.70 ± 0.15*     | 0.57 ± 0.17*             | 0.0008  |
| aP2           | NM_024406.2   | 1.16 ± 0.31           | 0.98 ± 0.27      | 0.64 ± 0.37*             | 0.0157  |
| Lpl           | NM_008509.2   | 0.77 ± 0.22           | 0.58 ± 0.27      | 0.66 ± 0.29*             | 0.0136  |

Data are mean ± S.D. from 5–6 tibias of 6-week-old individual mice and expressed as the fold changes relative to the housekeeping gene cyclophilin A subsequently normalized to control mice. * indicates significant difference from control Pkd2^{flox/+} mice, † indicates significant difference from Oc-Cre/Pkd2^{flox/+} mice, ‡ indicates significant difference from Pkd2^{flox/+} mice at p < 0.05, respectively.

PPARγ, an adipocyte transcription factor, and adipocyte markers, including lipoprotein lipase (Lpl) and adipocyte fatty acid-binding protein 2 (aP2) were significantly decreased in femurs of in both heterozygous Pkd2^{flox/null} and conditional Pkd2^{Oc-cKO} null mice (Table 2). Consistent with attenuated adipogenic markers, bone marrow exhibited an decreased percentage of fat cells in both Pkd2^{flox/null} and Pkd2^{Oc-cKO} mice at 20 weeks of age, as evidenced by a lower
number of adipocytes and fat droplets in decalcified femurs and tibias stained with Oil Red O (Figure 3A) and Osmium tetroxide (OsO4) (Figure 3B-3C), indicating an impairment of adipogenesis in Pkd2 deficient mice. Thus, the effect of selective deletion of Pkd2 in osteoblasts on adipogenesis is opposite to the effect of Pkd1 deficiency.

Effect of conditional deletion of Pkd2 on osteoblastic function ex vivo

We confirmed that there was a gene dose-dependent reduction of Pkd2 transcripts in osteoblasts derived from control Pkd2\textsuperscript{flox/+}, heterozygous Oc-Cre;Pkd2\textsuperscript{flox/+} and Pkd2\textsuperscript{flox/null}, and Pkd2\textsuperscript{Oc-cKO} mice (Figure 4A). To determine the impact of conditional deleted Pkd2 on osteoblast function ex vivo, we examined cell proliferation and osteoblastic differentiation and gene expression profiles in immortalized primary osteoblast cultures derived from control Pkd2\textsuperscript{flox/+}, heterozygous Oc-Cre;Pkd2\textsuperscript{flox/+} and Pkd2\textsuperscript{flox/null}, and Pkd2\textsuperscript{Oc-cKO} null mice. Consistent with defects in bone formation, we found that Pkd2 deficiency osteoblasts had a higher BrdU incorporation than control osteoblasts, indicating an increased proliferation rate in these Pkd2 deficiency osteoblasts (Figure 4B). In addition, both heterozygous Pkd2\textsuperscript{flox/null} and Pkd1\textsuperscript{Oc-cKO} null osteoblasts displayed impaired osteoblastic differentiation and maturation, as evidenced by lower alkaline phosphatase activity, diminished calcium deposition in extracellular matrix, and reduced osteoblastic differentiation markers such as Runx2-II compared to controls (Figure 4C-4E). In agreement with decreased adipogenic activity in vivo, the cultured immortalized primary osteoblasts under osteogenic condition exhibited a markedly decrease of adipocyte markers such as PPARγ2 in both heterozygous Pkd2\textsuperscript{flox/null} and Pkd1\textsuperscript{Oc-cKO} null osteoblast cultures (Figure 4F), suggesting an impairment of both osteogenesis and adipogenesis in the Pkd2 deficient osteoblasts.

Table 3. Biochemistry analysis in 6-week-old mice.

| Parameters     | Pkd2\textsuperscript{flox/+} | Oc-Cre;Pkd2\textsuperscript{flox/+} | Pkd2\textsuperscript{flox/null} | Pkd2\textsuperscript{Oc-cKO} | p-value |
|----------------|-------------------------------|--------------------------------------|----------------------------------|-------------------------------|---------|
| BUN (mg/dl)    | 18 ± 3.2\textsuperscript{a}  | 19 ± 3.8\textsuperscript{a}          | 18 ± 2.7\textsuperscript{a}     | 17 ± 2.3\textsuperscript{a}   | 0.5972  |
| FGF23 (pg/ml)  | 127 ± 27\textsuperscript{a}  | 125 ± 23\textsuperscript{a}          | 108 ± 29\textsuperscript{a}     | 73 ± 36\textsuperscript{b}    | 0.0066  |
| PTH (pg/ml)    | 44 ± 19\textsuperscript{a}   | 54 ± 26\textsuperscript{a}          | 64 ± 23\textsuperscript{a}      | 32 ± 15\textsuperscript{a}    | 0.0714  |
| P (mg/dl)      | 10.3 ± 0.90\textsuperscript{a} | 11.0 ± 1.01\textsuperscript{a}     | 11.4 ± 0.98\textsuperscript{a}  | 11.1 ± 0.67\textsuperscript{a} | 0.1229  |
| Ca (mg/dl)     | 8.7 ± 0.08\textsuperscript{a} | 8.7 ± 0.11\textsuperscript{a}       | 8.7 ± 0.17\textsuperscript{a}   | 8.7 ± 0.15\textsuperscript{a}  | 0.4528  |
| Osteocalin (ng/dl) | 124 ± 104\textsuperscript{a} | 92 ± 67\textsuperscript{a}          | 122 ± 41\textsuperscript{a}     | 104 ± 59\textsuperscript{a}   | 0.7657  |
| OPG (ng/ml)    | 3.9 ± 0.71\textsuperscript{a} | 3.9 ± 0.48\textsuperscript{a}       | 3.5 ± 0.95\textsuperscript{a}   | 3.9 ± 0.51\textsuperscript{a}  | 0.5027  |
| RanKL (pg/dl)  | 83 ± 14\textsuperscript{a}   | 81 ± 21\textsuperscript{a}          | 77 ± 20\textsuperscript{a}      | 54 ± 17\textsuperscript{a}    | 0.0346  |
| TRAP (U/L)     | 19 ± 1.8\textsuperscript{a}  | 18 ± 1.9\textsuperscript{a}         | 19 ± 3.7\textsuperscript{a}     | 15 ± 1.8\textsuperscript{b}   | 0.0338  |
| 1,25(OH)\textsubscript{2}D (pg/ml) | 59 ± 8.1\textsuperscript{a} | 57 ± 10.3\textsuperscript{a}        | 56 ± 5.4\textsuperscript{a}     | 55 ± 11.3\textsuperscript{a}  | 0.8022  |

Data are mean ± S.D. from 6-8 serum samples of 6-week-old individual mice. Values sharing the same superscript are not significantly different at $P<0.05$. 

doi:10.1371/journal.pone.0114198.t003
Effects of Pkd2 deficiency on signaling pathways in osteoblasts

We found that Pkd2 deficiency had a gene dose effect on basal intracellular calcium ([Ca\(^{2+}\)]\(_i\)) concentration and flow-induced intracellular calcium response in immortalized Pkd2-deficient osteoblasts. In this regard, heterozygous Pkd2\(^{null/+}\) osteoblasts showed a significantly lower basal intracellular calcium ([Ca\(^{2+}\)]\(_i\)) concentration compared with wild type Pkd2\(^{+/+}\) cells, and homozygous Pkd2\(^{null/null}\) osteoblasts had greater reductions of basal [Ca\(^{2+}\)]\(_i\) compared with the heterozygous Pkd2\(^{null/+}\) cells (Figure 5A). To study whether PC2-mediated
Figure 4. Pkd2 deficiency osteoblasts have a developmental defect in vitro. (A) A real-time RT-PCR analysis of total Pkd2 transcripts in osteoblast cultures. A gene dose-dependent reduction of Pkd2 transcripts was observed in immortalized control and Pkd2-deficient osteoblasts. (B) BrdU incorporation. Primary cultured Pkd2 deficient osteoblasts exhibited a higher BrdU incorporation than control Pkd2flox/+ osteoblasts for 6 hours, indicating increased proliferation rate in the Pkd2-deficient osteoblasts. (C) ALP activity. Primary cultured Pkd2flox/null and Pkd2Oc-cKO null osteoblasts displayed time-dependent increments in alkaline phosphatase (ALP) activities during 15 days of culture, but the ALP activity was significantly lower at different time points compared with control Pkd2flox/+ and Oc-Cre;Pkd2flox/+ osteoblasts. (D) Quantification of mineralization. Alizarin Red-S was extracted with 10% cetylpyridinium chloride and quantified as described in Experimental Procedures. Primary cultured Pkd2flox/null and Pkd2Oc-cKO null osteoblasts displayed time-dependent increments in Alizarin Red-S accumulation during 22 days of culture, but the accumulation was significantly lower at different time points compared with control Pkd2flox/+ and Oc-Cre;Pkd2flox/+ osteoblasts. (E and F) Gene expression profiles by real-time RT-PCR. 10-days cultured Pkd2flox/null and Pkd2Oc-cKO null osteoblasts in osteogenic differentiation media showed a significant attenuation in both osteogenesis and adipogenesis compared to control Pkd2flox/+ and Oc-Cre;Pkd2flox/+ osteoblasts, evidenced by a significant reduction in osteoblastic and adipogenic markers, such as Runx2-II and PPARγ. Data are mean ± S.D. from triple three independent experiments. Values sharing the same superscript in different group are not significantly different at P<0.05.

doi:10.1371/journal.pone.0114198.g004
mechanical flow-induced intracellular calcium level changed, these immortalized cells were exposed to 6.24 dynes/cm² pulsatile laminar fluid flow. On fluid stimulation, we detected an immediate rise in intracellular calcium throughout the wild type Pkd2<sup>+/+</sup> cell population, peaking roughly 10–20 s after stimulation (Figure 5B). The [Ca<sup>2+</sup>]<sub>i</sub> levels then rapidly decreased but were maintained at moderate levels for 50–60 s before returning to baseline. In contrast, when we exposed these Pkd2-deficient osteoblasts to an identical flow stimulus, we detected intermediate calcium response curve in the heterozygous cells and greater reduction of calcium influx in either the peak or late phase in the homozygous osteoblasts (Figure 5B). In addition, 100 nM triptolide stimulated normal calcium influx in wild type Pkd2<sup>+/+</sup> cells, moderate calcium signals in heterozygous Pkd2<sup>null/+</sup> cells, and no response in Pkd2 null cells (data not shown) in the loading chamber, consistent with loss of PC2 abolishes PC2 agonist-induced calcium response in osteoblasts.

To explore mechanisms of decreased Runx2 and PPARγ expression in Pkd2-deficient mice, we looked for alterations in the Hippo signaling pathway effectors YAP/TAZ, which play a critical roles in regulating mesenchymal stem cell fate determination into osteoblasts and adipocytes in response to alterations in extracellular matrix rigidity and cell shape [30]. We found that loss of Pkd2 in osteoblasts resulted in significant reduction in TAZ, YAP, and their transcriptional targets, baculoviral IAP repeat containing 3 (Birc-3), connective tissue growth factor (Ctgf), and inhibin beta A (Inhba) (Table 2). Western blot analysis revealed that phosphorylation of YAP and TAZ were significantly increased (indicating retention of p-YAP and p-TAZ in the cytoplasm) (Figure 5C). We also found that the total amount of YAP and TAZ were significantly decreased in Pkd2-deficient osteoblasts (Figure 5C). To examine the effect of Pkd2 inactivation on TAZ/YAP transcriptional activity, we examined a reporter gene construct with multiple copies of the TEAD-binding GTIIC (GGAATG) site (8xGTIIC-luciferase). YAP and TAZ are coactivators of the transcription factor TEAD, which can be used as a read out of YAP/TAZ activity. GTIIC-luciferase activity was reduced in a Pkd2 gene dose-dependent manner in immortalized primary osteoblasts derived from the Pkd2-deficient osteoblasts compared to the wild-type controls (Figure 5D). These findings indicated that loss of PC2 significantly attenuates both YAP and TAZ components of Hippo signaling pathway in immortalized primary osteoblasts.

In addition, Sost is expressed in osteocytes and regulates bone mass through Wnt-dependent signaling. We looked for evidence of activation of Wnt signaling pathway, since Sost message expression was decreased in Pkd2 deficient bone. However, we observed a decrease in Wnt10b and Axin2, suggesting that loss of Pkd2 resulted in decreased Wnt signaling, in spite of reductions in Sost.

**Discussion**

Using an Osteocalcin (Oc) promoter-driven Cre and Pkd2<sup>flox/null</sup> mouse model, we demonstrate that osteoblast-specific deletion of Pkd2 results in decreased...
osteoblast-mediated bone formation and reduction in bone mass in vivo and impaired osteoblast differentiation of primary osteoblasts ex vivo. In addition, there is decreased bone marrow fat in vivo and a reduction of adipogenesis in primary osteoblast cultures derived from Pkd2-deficient mice. The reduction in bone mass was due to both loss of trabecular bone volume and diminished cortical thickness and lead to loss of structural integrity as assessed by reduced ability to resist fracture in response to loading ex vivo. Reductions in osteoblast-mediated mineral apposition rates and decrements in osteoblast gene expression, as well as the lack of evidence for increased osteoclastic markers in bone or serum, are most consistent with bone loss due to decreased bone formation rather than increased bone resorption. Thus, selective Pkd2 deficiency causes a low turnover osteopenia.
Consistent with PC1 coupling to PC2, both Oc-Cre-mediated reductions of Pkd1 and Pkd2 transcripts in osteoblasts cause a low bone mass due to decreased osteoblast-mediated bone formation. However, there are a few notable differences in the bone phenotype of these conditional Pkd1 and Pkd2 deficient mice, suggesting that PC1 and PC2 functions are not identical. In this regard, Pkd1 deficiency in osteoblasts resulted in a more robust gene-dose dependent effects than observed in targeted deletion of Pkd2 in osteoblasts [12]. A greater effect of PC1 compared to PC2 is also seen in other settings. For example, mutations in Pkd1 cause a more severe cystic kidney phenotype than mutations in Pkd2 [31]. In addition, Pkd1 deficient mice have an inverse effect on osteoblastic and adipocytic differentiation, such that decreased osteoblastic function and osteopenia were associated with a reciprocal enhancement of adipogenesis and increased bone marrow fat. In contrast, there was a concordant reduction in osteoblastogenesis and adipogenesis in Pkd2 deficient mice. These differences might reflect the differential and broader signaling pathways coupled to Pkd1 compared to Pkd2. Indeed, Pkd1 deficiency resulted in a decrease in Runx2 and an increase in PPARγ, whereas reductions in Pkd2 resulted in parallel reductions of Runx2 and PPARγ.

We have previously shown that Pkd1 regulation of bone mass is mediated by Runx2 [21]. Compound Runx2 and Pkd1 heterozygous mice have additive effects on reduction of bone mass. Also, Pkd1 effects on Runx2 promoter activity are mediated through coupling to Pkd2 calcium channel activity and regulation of calcium signaling pathways. In this regard, Pkd1 deficient osteoblasts had lower intracellular calcium and Pkd1 responsive enhancer regions of the Runx2 promoter were identified in an area containing AP-1 and NFI binding sites. shRNA-mediated reductions in PKD1 in MG-63 osteoblasts also reduced intracellular calcium, attenuated calcium signaling response to shear fluid stress, and increased cAMP responses [27]. It is likely that reduction in Runx2 message expression in Pkd2 deficient osteoblasts is related to alterations in similar intracellular calcium signaling. Indeed, osteoblasts derived from Pkd2-deficient osteoblasts exhibited lower basal intracellular calcium ([Ca^{2+}]_i) and impaired response to flow-induced intracellular calcium influx, indicating that calcium channel PC2 is coupled to fluid flow sensing PC1 to response to mechanical loading osteoblasts.

The differential effects of selective Pkd1 and Pkd2 deficiency in osteoblasts on bone marrow adipogenesis suggests that PC1 and PC2 signaling can be uncoupled in bone. This possibility led us to investigate the potential interactions between polycystins and YAP and TAZ, components of the Hippo signaling pathway. TAZ/YAP, like polycystins, are regulated by mechanical and cytoskeletal cues [30, 32–36]. In addition, YAP/TAZ differentially regulates mesenchymal precursors toward osteoblastic and adipocytic cell fates [32]. Specifically, TAZ acts as a co-activator of Runx2 and a direct inhibitor of the transcriptional activity of PPARγ [37, 38]. Indeed, TAZ overexpression in osteoblasts stimulates Runx2 expression, osteoblast differentiation and increases bone mass [39]. YAP, on the other hand, acts as a co-repressor of Runx2 and co-activator of PPARγ transcriptional activity [40–42]. PKA-induced adipogenesis also involves cAMP dependent phosphor-
ylation of YAP [43]. Finally, TAZ binds to PC2 leading to its degradation and to PC1 to both modify its interactions with PC2 and possibly enhance nuclear translocation and transcriptional functions of the PC1 C-terminal tail [44]. We observed reductions in both TAZ and YAP in Pkd2-deficient mouse bone. PC-2-dependent stimulation of TAZ could work in concert with calcium- and PC-1-C-terminal tail dependent stimulation of Runx2 to stimulate osteoblastogenesis; whereas PC-2 stimulation of YAP and promotion of PPARγ activity could stimulate adipogenesis (Figure 6). In this schema, activation of PC-1 results in stimulation of osteoblastogenesis and inhibition of adipogenesis through coordinate effects on Runx2 and TAZ signaling. Further studies are needed to investigate this schema and establish the regulation and functional roles of TAZ and YAP in polycystin control of osteoblast and adipocyte differentiation.

Interestingly, the reduction in YAP in Pkd2 deficient mice is opposite to the up-regulation of YAP in ADPKD [45], suggesting differences in tissue specific regulation of Hippo signaling.

In addition, we observed that both Fgf23 transcripts in bone and serum Fgf23 levels were decreased in Pkd2 deficient mice, consistent with the effects of loss of Pkd2 to suppress osteoblast/osteocyte functions. Recent studies, however, show that that serum FGF23 levels are increased in ADPKD patients [46–48] and in a PKD orthologous mouse model [9]. These results imply that the observed FGF23 elevation is due to effects of chronic kidney disease (CKD) to increase FGF23, possibly due to systemic and/or local factors that stimulate FGF23 production or end-organ resistance to FGF23 action in CKD [49]. Alternatively, FGF23 mRNA and protein expression was detected in cell lining renal cysts, but not in bone of

![Figure 6. Schema showing potential interactions between polycystins and Hippo signaling pathways in osteoblasts.](https://doi.org/10.1371/journal.pone.0114198.g006)
the cy/+ Han:SPRD rat model of PKD; similar finding was also observed in an inducible Pkd1 knockout mouse model, suggesting ectopic expression of FGF23 in CKD [49]. The Col4a3 null mouse model of CKD also exhibited increased circulating FGF23 levels prior to increased expression of FGF23 in bone [50, 51]. Further studies are needed to understand the mechanism leading to increased FGF23 in CKD, but our studies indicate that specific loss of Pkd2 in bone results in decreased FGF23 expression, along with other gene products produced by osteocyties.

In conclusion, osteoblast-targeted deletion of Pkd2 result in significant bone loss associated with impaired osteoblastic and adipocytic differentiation. Reductions in intracellular calcium and alterations in the YAP/TAZ transcriptional modulators of mesenchymal stem cell differentiation are downstream mediators linking Pkd2 and regulation of Runx2 and PPARγ expression. These findings contrast with prior studies showing that Pkd1 deficiency results in reciprocal downregulation of Runx2-mediated osteoblastogenesis and upregulation of PPARγ-mediated adipogenesis. Thus, Pkd1 and Pkd2 separately regulate osteogenic and adipogenic pathways. Polycystins in partnership with the Hippo signaling pathway in osteoblasts may have the fundamental function of maintaining the osteoblast differentiation state and by regulating adipocytes differentiation/transdifferentiation in response to environmental factors that include mechanical loading.

Acknowledgments

We are particularly grateful to Dr. Guanqing Wu at Vanderbilt University Medical Center for providing the floxed Pkd2 mice. We also thank Active Life Scientific for their technical support during this study.

Author Contributions

Conceived and designed the experiments: ZSX ARS MJ LDQ. Performed the experiments: ZSX LC YJL JSH MD. Analyzed the data: ZSX JSH ARS. Contributed reagents/materials/analysis tools: ZSX ARS MJ LDQ. Wrote the paper: ZSX ARS LDQ.

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