Conditional Mesenchymal Disruption of Pkd1 Results in Osteopenia and Polycystic Kidney Disease

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Abstract

Conditional deletion of Pkd1 in osteoblasts using either Osteocalcin(Oc)-Cre or Dmp1-Cre results in defective osteoblast-mediated postnatal bone formation and osteopenia. Pkd1 is also expressed in undifferentiated mesenchyme that gives rise to the osteoblast lineage. To examine the effects of Pkd1 on pre- and postnatal osteoblast development, we crossed Pkd1\textsuperscript{floxed/floxed} and Col1a1\textsuperscript{(3.6)}-Cre mice, which has been used to achieve selective inactivation of Pkd1 earlier in the osteoblast lineage. Control Pkd1\textsuperscript{floxed/floxed} and Pkd1\textsuperscript{floxed/+}, heterozygous Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/+} and Pkd1\textsuperscript{floxed/null}, and homozygous Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/null} mice were analyzed at ages ranging from E14.5 to 8-weeks-old. Newborn Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/null} mice exhibited defective skeletonogenesis in association with a greater reduction in Pkd1 expression in bone. Conditional Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/+} and Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/null} mice displayed a gene dose-dependent decrease in bone formation and increase in marrow fat at 6 weeks of age. Bone marrow stromal cell and primary osteoblast cultures from heterozygous Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/+} mice showed increased proliferation, impaired osteoblast development and enhanced adipogenesis ex vivo. Unexpectedly, we found evidence for Col1a1\textsuperscript{(3.6)}-Cre mediated deletion of Pkd1 in extraskeletal tissues in Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/+} mice. Deletion of Pkd1 in mesenchymal precursors resulted in pancreatic and renal, but not hepatic, cyst formation. The non-lethality of Col1a1\textsuperscript{(3.6)}-Cre/Pkd1\textsuperscript{floxed/+} mice establishes a new model to study abnormalities in bone development and cyst formation in pancreas and kidney caused by Pkd1 gene inactivation.

Introduction

Polycystin-1 (PC1), the PKD1 gene product, is a highly conserved, multi-domain membrane protein widely expressed in various cell types and tissues [1,2]. The specific biological functions of PC1 (PKD1) in different tissues are currently being elucidated; best understood are the kidney functions. In renal epithelium, PKD1 forms a complex with the calcium channel, PKD2 that co-localizes to primary cilia and functions as a flow sensor regulating cell proliferation and polarity. Loss of PKD1 or PKD2 function in renal tubular epithelial cells causes Autosomal Dominant Polycystic Kidney Disease (ADPKD) [3,4] that is the result of abnormal cell proliferation and cell polarity and leads to cystic kidney disease. PKD1 mutations also lead to cystic disease of liver and pancreas in some patients with ADPKD, also because of abnormal proliferation of ductal epithelial cells in these tissues [5,6,7]. PKD1 may also have a role in the development and function in endothelial- and mesenchymal-derived cells. PKD1 mutations lead to vascular (intraocular and aortic aneurysms) [8,9,10,11] and lung (bronchiectasis) abnormalities [12]. PKD1 and PKD2 are required for placental development [13]. Pkd1 mutations in mouse models also cause abnormalities of the skeleton [14,15,16,17,18] and human subjects with polycystic kidney disease appear to have earlier elevation of the bone-derived hormone FGF23 [19].

It has been difficult to determine the specific extra-renal functions of Pkd1/PKD1 using mutations of Pkd1/PKD1 in mice and humans. Global ablation of Pkd1 in mice leads to a complex, embryonically lethal, phenotype [7]. Multiple abnormalities, including renal and pancreatic cysts and pulmonary hyperplasia, are observed in Pkd1\textsuperscript{−/−} mice when they survive to embryonic day 15.5 post coitum (E15.5). In global knockout mice it is difficult to differentiate between indirect extra-renal abnormalities due to the effects of the complex metabolic alterations caused by renal cystic disease from direct effects caused by loss of Pkd1 functions in affected tissues. In addition, in humans, ADPKD is a heterozygous state, whereby mutations leading to loss of one PKD1 or PKD2 allele is combined with somatic mutations in the kidney (i.e., a second hit) to cause renal cystic disease [20,21,22,23,24]. The resulting residual function of the non-mutated PKD1 or PKD2 allele in extra-renal tissues may also mask discovery of PKD1 or PKD2 functions in non-renal tissues.

The Pkd1\textsuperscript{floxed/+} mouse model has been used to define the tissue selective function of Pkd1 in vivo [25]. A low frequency of renal Pkd1 gene inactivation and only a few renal cysts and more frequent hepatic cysts is reported from the conditional deletion of

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Phd1 in MMTV-Cre mice [25], whereas the broadly expressed tamoxifen-Cre inducible inactivation of the Phd1 gene in mice resulted in massive cystic transformation of renal tissue [26]. The selective deletion of Phd1 in kidney by using Ksp-Cre, or more broadly Nestin-Cre, also leads to the formation of polycystic kidneys resembling human ADPKD [27,28].

In addition, use of Phd1^{flox/flox} mice and bone-specificCre mice has defined previously unrecognized functions of polycystin-1 in bone. In this regard, the selective deletion of Phd1 in osteoblasts by using Osteocalcin(Oc)-Cre and in osteocytes by using Dmp1-Cre results in osteopenia in adult mice because of defects in osteoblast-mediated bone formation [17,18]. At present, however, it is unclear whether the functions of Phd1 are limited to mature osteoblasts and osteocytes or involve earlier stages in osteoblast development.

To explore the effects of Phd1 on early pre-osteoblast stage and prenatal bone development, we used the Col1a1(3.6)-Cre promoter to drive Cre-recombinase expression (Col1a1(3.6)-Cre) in mesenchymal precursors. Col1a1(3.6)-promoter driven Cre expression begins at E10 and peaks between E12.5 and E14.5 in developing skeletal elements [29,30]. Col1a1(3.6)-Cre is thought to be specific for the osteoblast lineage and it has been extensively used to conditionally delete genes early in osteoblastic development and to study bone-specific function of many genes [29,30,31,32].

We found that conditional deletion of Phd1 from osteoblasts precursors within the mesenchymal lineage resulted in defective bone formation that was associated with abnormal osteoblastic development and enhanced adipogenesis. Unexpectedly, we found that Col1a1(3.6)-Cre was not bone specific and resulted in deletion of Phd1 in multiple tissues, leading to cyst formation in the kidney and pancreas, but not the liver of adult mice.

**Results**

Col1a1 (3.6)-Cre-mediated conditional deletion of Phd1 in different tissues

The four genotypes from the chosen breeding strategy (Col1a1(3.6)-Cre; Phd1^{flox/flox}, Col1a1(3.6)-Cre; Phd1^{flox/+}, Phd1^{flox/+}, and Phd1^{flox/+}) were born at the expected Mendelian frequency. We investigated Col1a1(3.6)-Cre; Phd1^{flox/flox} (Col1a1(3.6)-KO), heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+}, and Phd1^{flox/+} mice. Phd1^{flox/flox} was used as the control group. Col1a1(3.6)-Cre; Phd1^{flox/+} mice exhibited normal survival indistinguishable from control mice (Phd1^{flox/flox}), whereas Pkd1Col1a1(3.6)-KO mice displayed a 50% mortality rate in association with development of multiple cysts in the kidney and pancreas from newborn to 6-week-old mice (Fig. 1A). We observed no differences in body weight between heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} and control Phd1^{flox/flox} littersmates; however, Phd1^{Col1a1(3.6)-KO} mice were smaller and had a significantly lower body weight compared with Col1a1(3.6)-Cre; Phd1^{flox/+} and Phd1^{flox/+} littersmates at 6 weeks-of-age (Fig. 1B). Using an alternative breeding strategy with Phd1^{flox/null} mice, we also generated Col1a1(3.6)-Cre; Phd1^{flox/null} mice, which had greater perinatal mortality that prevented collection of adult animals for analysis (data not shown). Therefore, the analysis of the skeletal phenotype of Col1a1(3.6)-Cre; Phd1^{flox/null} mice is limited to newborn mice.

Col1a1(3.6)-Cre-mediated excision occurs in the developing mesenchymal tissues between E12.5 and E14.5 [29,30] and would be expected to carry forward into tissues developed from mesenchymal precursors. To determine the tissue distribution of Col1a1(3.6)-Cre-mediated deletion of Phd1 in adult mice, we performed PCR analysis in different tissues by using a combination of primers that specifically detect floxed Phd1 alleles (Phd1^{flox}) and the excised floxed Phd1 alleles (Phd1^{flox/null}), as well as wild type alleles (Phd1^{+/+}) in Phd1^{Col1a1(3.6)-KO} (Fig. 1C). We found that Col1a1(3.6)-Cre-mediated floxed recombination occurred in both skeletal and non-skeletal tissues including pancreas, liver, and kidney (Fig. 1C), consistent with prior reports [31] that Col1a1(3.6)-Cre mRNA was highly expressed in calvaria, long bone, and tendon, but was also detected in brain, kidney, liver and lung [31].

To quantify the excised efficiency of floxed Phd1 by Col1a1(3.6)-Cre-recombinase, we examined the percentage of Phd1 transcripts in exons 2–4 in calvarias, kidney, and liver from 6-week-old mice by real-time RT-PCR. We found that Col1a1(3.6)-Cre; Phd1^{flox/+} mice exhibited approximately 18–41% excision of the floxed exons 2–4 from total Phd1 transcripts, whereas Phd1^{flox/null} mice resulted in a net reduction of Phd1 expression by 45–60% in calvarias, kidney, and liver (Fig. 1D, 1E, and 1F). In addition, there is a gene-dose-dependent reduction in Phd1 transcripts in calvaria (Fig. 1D) that correlated with a more severe bone phenotype in Col1a1(3.6)-Cre; Phd1^{flox/null} newborn mice compared to Col1a1(3.6)-Cre; Phd1^{flox/+} mice.

A gene dose-dependent effect of Col1a1 (3.6)-Cre-mediated conditional deletion of Phd1 in newborn and postnatal bone formation

Indeed, we failed to observe abnormalities of skeletal development in homozygous Phd1^{Col1a1(3.6)-KO} newborn mice (Fig. 2A, 2C, 2E and 2G). In contrast, bone structural abnormalities were observed in homozygous Col1a1(3.6)-Cre; Phd1^{flox/+} newborn mice, including delayed bone mineralization in calvarial and vertebral bone tissues (Fig. 2B), a short and less mineralized femur (Fig. 2D), and a significant reduction in both trabecular bone volume (Fig. 2F) and cortical bone thickness (Fig. 2H). Because the Cre; Phd1^{flox/+}; null strategy leads to greater Cre-mediated reduction in Phd1 conditional deletion compared to Cre; Phd1^{flox/+} approach [33], the more severe phenotype that we observed is likely the result of greater reductions in Phd1 gene dose during embryogenesis.

We also observed a Phd1 gene dose dependent reduction in bone mineral density (BMD) in heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} and homozygous Phd1^{Col1a1(3.6)-KO} mice. A significant reduction in BMD of 21–22% was observed in both male and female heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice at 6 weeks of age compared with age-matched control mice (Phd1^{flox/+}) (Fig. 3A). Homozygous Phd1^{Col1a1(3.6)-KO} mice had greater loss in BMD, with respective reductions in BMD of 35% and 36% reduction in male and female adult mice (Fig. 3A). μCT analysis revealed that the lower bone mass in male heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice was caused by reduced trabecular bone volume (BV/TV, 40%) and cortical bone thickness (Ct.Th, 15%) (Fig. 3B), and homozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice had greater loss in both trabecular (73%) and cortical bone (41%) than did heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice (Fig. 3B). These reductions in bone volume and cortical thickness were associated with a significant Phd1 gene dose-dependent decrease in periosteal mineral apposition rate (MAR)(Fig. 3C). In this regard, periosteal MAR was reduced by 19% in heterozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice and 41% in homozygous Col1a1(3.6)-Cre; Phd1^{flox/+} mice compared with age-matched Phd1^{flox/+} controls (Fig. 3C). In addition, the femurs of homozygous Phd1^{Col1a1(3.6)-KO} mice were 17% shorter in length, indicating a role of Phd1 in growth plate of metaphyseal bone (Fig. 3D). Interestingly, the severity of BMD reductions in more limited number of Col1a1(3.6)-Cre; Phd1^{flox/null} mice that were available for examination was similar to that of Phd1^{Col1a1(3.6)-KO} mice (data not shown).
Figure 1. Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1 from the floxed Pkd1 allele (Pkd1\textsuperscript{flox}) in different tissues. (A) Kaplan-Meier survival curve. Heterozygous Col1a1(3.6)-Cre; Pkd1\textsuperscript{flox/+} had normal survival identical to control (Pkd1\textsuperscript{flox/flox}) mice, homozygous Col1a1(3.6)-Cre; Pkd1\textsuperscript{flox/flox} mice began to die 1 week after birth and only half of these mice survived beyond 6 weeks. (B) Body weight of Col1a1(3.6)-Cre; Pkd1\textsuperscript{flox/+} and control (Pkd1\textsuperscript{flox/flox}) mice were not different, but the body weight of both male and female Col1a1(3.6)-Cre;Pkd1\textsuperscript{flox/flox} mice was reduced by ~30% and ~36% compared with the other two genotypes. (C) Genotyping PCR analysis of different tissues harvested from 6-week-old homozygous Col1a1(3.6)-Cre;Pkd1\textsuperscript{flox/flox} mice showed that both Pkd1\textsuperscript{flox} and Pkd1\textsuperscript{Dflox} alleles existed in all tested tissues including bone and nonskeletal tissues, indicating that Col1a1(3.6)-Cre promoter is not specific for bone. (D–E) Real-time RT-PCR analysis of total Pkd1 transcripts in calvaria from both Col1a1(3.6)-Cre;Pkd1\textsuperscript{flox/flox} and Col1a1(3.6)-Cre;Pkd1\textsuperscript{flox/null} models, and in kidney and liver from Col1a1(3.6)-Cre;Pkd1\textsuperscript{flox/flox} model at 6 weeks of age.
To investigate the effects of Pkd1 deficiency on gene expression profiles in bone, we examined by real-time RT-PCR the expression levels of a panel of osteoblast lineage- and adipocyte-related mRNAs from the tibias of 6-week-old control Pkd1<sup>flox/fox</sup>, heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup>, and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice (Table 1). Consistent with a low bone mass phenotype by BMD and μCT analysis, we found a significant Pkd1 gene dose-dependent decrease in osteoblast-lineage gene transcripts in these Pkd1-deficient mice, including Runt2, Osterix, FGF23, Osteoprotegerin (Opg), Rank ligand, and alkaline phosphatase 2 (Akp2) mRNA levels, but no obvious change was observed in osteocalcin expression compared to control mice (Table 1). Consistent with a ratio of Opg/RankL, which would predict reduced osteoclastogenesis, bone expression of tartrate-resistant acid phosphatase (Tralp) and matrix metalloproteinase 9 (Mmp9), markers of bone resorption, were also reduced in heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice (Table 1). The changes in bone mRNA expression did not correlate with serum levels of these biomarkers, except serum RankL, which was reduced in Pkd1<sup>Col1a1(3.6)-cKO</sup> mice. Serum osteocalcin and OPG were elevated and TRAP was in the normal range in homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice compared to age-matched control Pkd1<sup>flox/fox</sup> mice (Table 2).

In accordance with decreased osteogenesis in bone, we also observed an increased adipogenesis in bone marrow and in bone marrow stromal cultures from homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice. In this regard, homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice showed a higher number of adipocytes and volume of fat droplets in decalcified tibias stained with Oil Red O and OsO4 (Fig. 4A). BMSC cultures derived from Pkd1<sup>flox/fox</sup> mice exhibited a marked increase of Oil Red O stained adipocytes (Fig. 4B). In addition, PPAR<sub>γ</sub>, an adipocyte transcription factor, and adipocyte markers such as PPAR<sub>γ</sub> (adipocyte fatty acid-binding protein 2) were also significantly increased in BMSC cultures of Pkd1<sup>Col1a1(3.6)-cKO</sup> mice compared to Pkd1<sup>flox/fox</sup> control mice (Fig. 4C). In agreement with decreased osteogenesis in bone, we also observed a significant increase of adipogenic markers including adiponectin, aP2, and Lpl (lipoprotein lipase) in long bone samples of Pkd1<sup>Col1a1(3.6)-cKO</sup> mice (Table 1).

**Effect of Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1 on osteoblastic function ex vivo**

To determine the impact of Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1 on osteoblast function ex vivo, we isolated primary calvarial osteoblasts from E17.5 control Pkd1<sup>flox/fox</sup>, heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup>, and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> fetuses. Primary calvarial osteoblasts under osteogenic culture condition undergo progressive alterations in cell proliferation and osteoblastic differentiation that recapitulates the osteoblastic developmental program [34]. Consistent with our previous report [17], we found that heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> osteoblasts increased BrdU incorporation that were proportionate to the reduction of Pkd1 gene dose (Fig. 5A and 5B). In addition, heterozygous Col1a1(3.6)-Cre;Pkd1<sup>flox/+</sup> and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> osteoblasts had a gene dose-dependent impairment of osteoblastic differentiation and maturation, as evidenced by culture duration-dependent reductions in ALP activity (Fig. 5C), diminished calcium deposition in extracellular matrix (Fig. 5D), and reduced osteoblastic differentiation markers, including Runx2 and Aplp2, compared to control Pkd1<sup>flox/fox</sup> osteoblasts (Fig. 5E and 5F). A similar reduction of FGF23 transcripts was also observed in vitro cultures osteoblasts at day 18 (Fig. 5 G). In agreement with increased adipogenic markers observed in bone in vivo, heterozygous Col1a1(3.6)-Cre;Pkd1<sup>flox/+</sup> and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> osteoblasts exhibited a gene dose-dependent increase of adipocyte markers such as PPAR<sub>γ</sub>2 and aP2 (Fig. 5H and 5I), suggesting that impairment of osteogenesis was associated with enhancement of adipogenesis in Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1 osteoblast cultures.

**Development of polycystic kidney and pancreatic disease in homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> embryos and mice**

Besides skeletal abnormalities, we also observed that homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice developed severe renal and pancreatic cysts at 6 weeks-of-age. In contrast, no cyst formation was observed in the kidney or pancreas of age-matched control Pkd1<sup>flox/fox</sup> or heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> mice (Fig. 6A). Hematoxylin-eosin (H&E)-stained sections showed no cyst formation in liver tissues from all of three groups (Fig. 6B). In spite of 35% and 54% reduction in Pkd1 mRNA expression in heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> and homozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/fox</sup> mice, respectively (Fig. 1F), homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice developed massive cysts in both the pancreas and kidney; however, glomeruli formation in the kidney and endocrine islet formation in pancreas were unaffected (Fig. 6C and 6D). Expansion of pancreatic ducts formed large pancreatic cysts that led to massive acinar cell loss, formation of abnormal tubular structures, and appearance of endocrine cells in ducts (Fig. 6C and 6D).

Timed pregnancies were generated to analyze heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> and homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> fetuses at various developmental stages. We did not observe renal or pancreatic cysts in heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> mice during embryogenesis or at 6-weeks-of-age (data not shown). Pancreatic cysts first became evident in homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> embryos was at E15.5 (Fig. 7A and 7B). In addition, the dilatation of pancreatic cysts progressed as a function of age in homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> embryos (Fig. 7A and 7B). The size of renal cystic lesions also increased as a function of age. Masson-trichrome stained sections of the kidney showed that the cystic fibrosis of polycystic kidney started at P7 and became progressively more severe by 6-weeks of age (Fig. 8A and 8B). Real-time RT-PCR showed that mesenchymal-to-epithelial transition and fibrosis formation genes were significantly up-regulated in cystic kidney from homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice compare with control Pkd1<sup>flox/fox</sup> mice (Fig. 9A–9F), but no such alterations were observed in heterozygous Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup> mice (Fig. 9A–9F). In addition, homozygous Pkd1<sup>Col1a1(3.6)-cKO</sup> mice had significantly higher levels of serum BUN, PTH, and FGF23, but lower levels of phosphorus, and no changes in calcium levels at 6 weeks of age (Table 2), consistent with the development of renal impairment caused by polycystic kidney disease and secondary induction of compensatory hormonal changes (Table 2).
Figure 2. Col1a1(3.6)-Cre-mediated deletion of Pkd1 results in osteopenia in Col1a1(3.6)-Cre;Pkd1^{floxed/null} newborn mice. The whole skeleton mineralization (A and B), full-length mineralized femurs (C and D), bone volume of metaphyseal region (E and F), and cortical thickness of cortical bone (G and H) of femurs from Col1a1(3.6)-Cre;Pkd1^{floxed/null} and Col1a1(3.6)-Cre;Pkd1^{floxed/null} newborn mice by μCT analysis. A Pkd1 gene dose
Discussion

Pkd1, Pkd2, and primary cilia are present in mature osteoblasts and osteocytes [16], where the primary cilia-polycystin complex plays an important role in postnatal osteoblast and osteocyte regulation of bone formation and mechanosensing [17,18]. Primary cilia-polycystin complexes are known to have a role in regulating developmental pathways in other tissues, such as left-right patterning in embryos and kidney development [35], but the role of Pkd1 in osteoblast development and bone embryogenesis, while suggested by skeletal alterations in global Pkd1 knockout mice [16,17,18,36], has not been confirmed by selective ablation of Pkd1 early in the osteoblast lineage. In the current study, Pkd1 was conditionally deleted in mesenchymal precursors that are destined to become multiple cell types including osteoblast lineage [37], renal tubule cells [38], pancreas duct epithelial cells [39], and bile ductal plate cells [40] by creating Pkd1Col1a1(3.6)-cKO or Col1a1(3.6)-Cre;Pkd1fl/fl mice, which differ in the degree of Pkd1 deletion [33].

We found that reduction of Pkd1 in mesenchymal precursors in Pkd1Col1a1(3.6)-cKO mice resulted in impaired osteoblast-mediated bone formation and low bone mass in the adult mouse, consistent with the observations in Oc-Cre- and Dmp1-Cre-mediated conditional knockout of Pkd1 [17,18]. Primary osteoblast cultures showed higher cell replication and lower osteoblastic differentiation markers in E17.5 homozygous Pkd1Col1a1(3.6)-cKO mice, similar to the defects of osteoblastic maturation in Oc-Cre-mediated conditional knockout of Pkd1 [17]. The increased proliferation observed in Pkd1-deficient osteoblasts is similar to increased renal effect was observed during skeletogenesis between Col1a1(3.6)-Cre;Pkd1fl/fl and Col1a1(3.6)-Cre; Pkd1fl/null newborn mice. Data represent the mean ± SD from three to four individual samples. *Significant difference from control mice (Pkd1fl/fl or Pkd1fl/fl) at P<0.05.

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Figure 3. Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1 leads to severe osteopenia in Col1a1(3.6)-Cre;Pkd1fl/fl adult mice. (A) Bone mineral density (BMD), (B) Bone structure of femurs, (C) Bone mineral apposition rate (MAR), and (D) Femur length at 6 weeks of age. There was a Pkd1 gene dose-dependent reduction in BMD in both male and female heterozygous Col1a1(3.6)-Cre; Pkd1fl/+ and homozygous Col1a1(3.6)-Cre; Pkd1fl/fl mice compared with age-matched control mice (Pkd1fl/fl). μCT analysis revealed that the lower bone mass in male Col1a1(3.6)-Cre-mediated mice with conditional deletion of Pkd1 resulted from reductions in both trabecular BV/TV and cortical CtTh that were proportionate to the reduction of Pkd1 gene dose. These reductions in bone mass and structure were associated with a 19% and 41% reduction in mineral apposition rate (MAR) in male heterozygous Col1a1(3.6)-Cre; Pkd1fl/+ and homozygous Col1a1(3.6)-Cre; Pkd1fl/fl mice compared with age-matched control mice, respectively. In addition, the femurs of homozygous Col1a1(3.6)-Cre; Pkd1fl/fl mice were 17% shorter in length, indicating a postnatal bone growth retardation. Data represent the mean ± S.D. from five to six individual mice. *Significant difference from control (Pkd1fl/fl) and # significant difference from Col1a1(3.6)-Cre; Pkd1fl/+ mice at P<0.05, respectively.

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cell proliferation caused by loss of Pkd1 in renal epithelial cells, suggesting that an important function of Pkd1 is to regulate cell proliferation, which is typically inversely correlated with differentiation [41,42]. In addition, the marrow fat content of bone was increased and enhanced adipogenesis was found in bone marrow stromal cell cultures, which expressed decreased levels of Runx2 and increased levels of PPARγ [peroxisome-proliferator-activated receptor-γ], which regulates adipocyte development. These findings are consistent with previous studies showing that Pkd1 stimulates osteogenesis and inhibits adipogenesis through a Pkd2-calcium dependent Runx2 expression and that compound heterozygous Pkd1 and Runx2 deficient mice have additive effects to induce osteopenia [17,18,36]. Further studies are needed to determine the signaling pathways linking Pkd1 inactivation in pre-osteoblasts to increments in PPARγ expression.

We were surprised by the difference in skeletal abnormalities between newborn Col1a1(3.6)-Cre;Pkd1flox/null and Pkd1flox/+Cre;Pkd1flox/null mice. Col1a1(3.6)-Cre;Pkd1flox/null had evidence of abnormal skeletogenesis, albeit less severe than the previously described global Pkd1−/− mouse models [14,15,16]. Col1a1(3.6)-Cre, which is highly active between E12.5 and E14.5, and the resulting bone abnormalities are consistent with known effects of Pkd1 to regulate Runx2, an essential transcriptional factor controlling osteoblast development [43,44]. In contrast, newborn Pkd1Col1a1(3.6)-Cre;Pkd1flox/null mice lacked a demonstrable bone phenotype, consistent with studies in Osx-Cre;Pkd1flox/null mice, which also failed to find evidence for a role of Pkd1 in skeletogenesis [45]. A gene-dose dependent effect on skeletogenesis and differences in

### Table 1. Gene-expression profiles in 6-week-old mice.

| Gene          | Accession no. | Pkd1flox/flox | Col1a1(3.6)-Cre;Pkd1flox/+ | p-value |
|---------------|---------------|---------------|----------------------------|---------|
| Osteoblast lineage |
| Pkd1         | NM_013630     | 1.00±0.29     | 0.75±0.21*                  | 0.53±0.19*   | 0.0005            |
| Runx2        | NM_009820     | 1.00±0.25     | 0.76±0.17*                  | 0.54±0.11*   | 0.0003            |
| Osx          | NM_130458     | 1.00±0.24     | 0.78±0.21*                  | 0.49±0.11*   | 0.0005            |
| Osteocalcin  | NM_007541     | 1.00±0.43     | 0.90±0.33                   | 1.10±0.76    | 0.8078            |
| Opg          | NMU94331      | 1.00±0.25     | 0.94±0.37                   | 0.45±0.24*   | 0.0041            |
| Rank ligand  | NM_011613     | 1.00±0.34     | 0.58±0.19*                  | 0.26±0.13*   | 0.0002            |
| Akp2         | NM_007431     | 1.00±0.27     | 0.74±0.21*                  | 0.48±0.11*   | 0.0006            |
| FGF23        | NM_022657     | 1.00±0.33     | 0.84±0.11                   | 0.56±0.21*   | 0.0076            |
| Osteoclast    |
| Trap         | NM_007388     | 1.00±0.31     | 0.73±0.15*                  | 0.40±0.13*   | 0.0007            |
| Mmp9         | NM_013599     | 1.00±0.41     | 0.68±0.12*                  | 0.44±0.11*   | 0.0006            |
| Adipocyte     |
| Adiponectin  | NM_009505     | 1.00±0.21     | 1.04±0.43                   | 1.82±0.56*   | 0.0077            |
| aP2          | NM_024406     | 1.00±0.36     | 1.22±0.33                   | 2.45±1.51*   | 0.0268            |
| Lpl          | NM_008509     | 1.00±0.15     | 1.17±0.21                   | 1.63±0.36*   | 0.0010            |

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 β-actin subsequently normalized to control mice. * indicates significant difference from control Pkd1flox/flox mice, and † indicates significant difference from heterozygous Col1a1(3.6)-Cre;Pkd1flox/+ mice at p<0.05, respectively.

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### Table 2. Biochemistry analysis of serum in 6-week-old mice.

| Genotype   | Pkd1flox/flox | Col1a1(3.6)-Cre;Pkd1flox/+ | p-value |
|------------|---------------|----------------------------|---------|
| BUN (mg/dl)| 21±1.8        | 20±2.8                     | 36±10.4*   | 0.0002 |
| Ca (mg/dl) | 9.6±0.24      | 9.9±0.31                   | 9.9±0.59          |         |
| P (mg/dl)  | 7.3±0.42      | 7.1±0.15                   | 6.6±0.24*, †   |         |
| Osteocalcin(ng/ml)| 40±17 | 88±26                     | 169±91*, †      |         |
| OPG (pg/ml)| 3.2±0.38      | 3.6±0.51                   | 5.1±1.15*, †    |         |
| RankL (pg/ml)| 89±24 | 94±19                     | 53±23*, †       |         |
| TRAP (U/L)| 4.0±0.43      | 3.5±0.82                   | 3.6±0.83       |         |
| PTH (pg/ml)| 42±16         | 40±5.6                     | 92±54*, †      |         |
| FGF23 (pg/ml)| 94±25 | 88±24                     | 173±99*, †     |         |

Data are mean ± S.D. from 6–8 individual mice. * and † indicates significant difference from control Pkd1flox/flox and Col1a1(3.6)-Cre;Pkd1flox/+ mice at p<0.05, respectively. Osteocalcin is produced by osteoblasts, and TRAP is produced by osteoclasts.

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methods of conditional gene targeting might explain these discrepancies. In this regard, the less severe skeletal phenotype in newborn Pkd1+/Col1a1(3.6)-cKO and Osx-Cre;Pkd1flox/null mice compared to Col1a1(3.6)-Cre;Pkd1flox/null mice may be due to insufficient reductions in Pkd1 expression to cause abnormal osteoblast development [33]. Additional studies are needed using the heterozygous floxed allele paired with a "null" mutant allele along with earlier osteoblast lineage specific and less leaky promoters to define the function of Pkd1 in osteoblast development during different stages of embryogenesis [17,33,46].

The role of Pkd1 in post-natal bone remodeling is further documented by the current studies. Bone remodeling that occurs postnatally is characterized by the recruitment of bone marrow mesenchymal stem cells to differentiate into osteoblasts that refill resorptive cavities. Our studies suggest that a reduction of Pkd1 expression of more than 50% results in abnormalities in osteoblast-mediated bone formation in adult mice through Pkd1 regulation of critical transcription factors involved in osteoblastogenesis and adipogenesis [47,48,49]. We have previously shown that the bone-specific deletion of Pkd1 using Osx-Cre or Dmp1-Cre had a direct role in adult bone formation [17,18]. However, we observed a more severe osteopenia in adult Pkd1+/Col1a1(3.6)-cKO compared to Osx-Cre or Dmp1-Cre-mediated Pkd1 deletion, which could result from the effects of Col1a1(3.6)-Cre to delete Pkd1 during embryogenesis or to the leakiness of this promoter leading to alterations in systemic factors that lead to secondary effects on bone. Although we observed significant increases of serum PTH and FGF23 levels in association with elevated BUN in adult Pkd1+/Col1a1(3.6)-cKO mice, the expected PTH-induced increases in bone transcripts were not observed, rather we found that Akt2, FGF23, Trap, Osterix and Runx2 transcripts were decreased in Pkd1+/Col1a1(3.6)-cKO, suggesting a direct effect of Pkd1 on bone. In addition, our finding of abnormal skeletogenesis in newborn Col1a1(3.6)-Cre;Pkd1flox/null mice and impairment in osteoblast differentiation maturation in E17.5 immortalized osteoblast cell cultures (A) Histology of adipocytes in decalcified tibia. Oil Red O staining (upper panel) showed that the numbers of adipocytes and fat droplets in tibia bone marrow were greater in 6-week-old Col1a1(3.6)-Cre; Pkd1flox/null mice compared with age-matched control Pkd1flox/null mice. Osmium tetroxide (OsO4) staining by μCT analyses (lower panel) showed that adipocyte volume/marrow volume (Ad.V/Ma.V, %) and adipocyte number (Ad.N, mm−2) were much higher in the proximal tibia from 6-week-old Col1a1(3.6)-Cre; Pkd1flox/null mice compared with age-matched control Pkd1flox/null mice. (B) Adipocytic differentiation in BMSC cultures. An increase of adipogenesis potential was observed in 6-week-old Col1a1(3.6)-Cre; Pkd1flox/null BMSC cultures, evidenced by a significant increase of Oil Red O staining in adipogenic cultures. (C and D) Expression of adipogenic markers by real-time RT-PCR. Significantly increased levels of PPARγ and aP2 mRNAs were observed in 6-week-old Col1a1(3.6)-Cre; Pkd1flox/null BMSC cultures compared with control (Pkd1flox/null) cultures. Data are expressed as the mean ± SD from three independent experiments. * Significant difference from control (Pkd1flox/null) at P<0.05.

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cultures from Pkd1Col1a1(3.6)-cKO mice, are consistent with direct effects of Pkd1 in osteoblasts, as previously reported [16].

Interestingly, in this model, serum phosphate was low and FGF23 was high, consistent with other reports that FGF23 regulation may be abnormal in ADPKD [19]. However, we failed to find evidence for increased FGF23 mRNA expression in bone or in osteoblasts cultures derived from Pkd1Col1a1(3.6)-cKO mice; rather FGF23 mRNA levels were decreased in bone and osteoblasts.
Figure 6. *Col1a1(3.6)-Cre*-mediated conditional deletion of *Pkd1* causes polycystic pancreas and kidney. (A) Gross appearance of liver, kidney, and pancreas. There was no cyst formation in the liver, kidney, and pancreas in heterozygous *Col1a1(3.6)-Cre; Pkd1<sup>flox/+</sup>* mice, whereas age-matched homozygous *Col1a1(3.6)-Cre; Pkd1<sup>flox/flox</sup>* mice developed severe renal and pancreatic cysts at 6 weeks of age. (B–D) Hematoxylin-eosin-stained sections (5X) of liver, pancreas, and kidney from 6-week-old mice. Cysts were not observed in the livers from heterozygous and homozygous mice, and renal and pancreatic cysts were also not found in kidney and pancreas tissues from heterozygous *Col1a1(3.6)-Cre; Pkd1<sup>flox/</sup>mouse. However, homozygous *Col1a1(3.6)-Cre; Pkd1<sup>flox/flox</sup>* mice exhibited massive cyst formation in both the pancreas and kidney. Interestingly, glomeruli formation in
cultures from these mice, suggesting that reduction of Pkd1 in osteoblast lineage diminishes FGF23 production. The disparity between serum FGF23 and bone expression of FGF23 is consistent with recent findings that FGF23 is regulated by both transcriptional and post-transcriptional mechanisms [50].

In addition to skeletal abnormalities, we found that Col1a1(3.6)-Cre-mediated deletion of Pkd1 resulted in cyst formation in the kidney and pancreas. Although Pkd1 is expressed in undifferentiated mesenchyme during embryogenesis [1,2,51] and Pkd1 mutations lead to cyst formation in the kidney, pancreas and liver in hereditary polycystic diseases [52,53,54,55,56], extraskeletal abnormalities due to deletion of Pkd1 in mesenchymal precursors using Col1a1(3.6)-Cre was unexpected. Indeed, the Col1a1(3.6) promoter has been purported to specifically target cells of the osteoblastic lineage, and in previous reports was found to have minimal expression of Cre-recombinase in liver, pancreas, and kidney [30,32]. However, unlike our studies, the prior reports did not examine Cre-mediated recombination in extraskeletal tissues. Indeed, we found evidence for Col1a1(3.6)-Cre deletion of Pkd1 in all tissues tested, including kidney, pancreas and liver. Pancreatic and renal cysts developed by E15.5, just after highly expression of Col1a1(3.6)-Cre activity in skeletal tissues at E12.5 [29,30,31]. The size of polycystic pancreas and kidney varied between individual mice, and only 30% of individual homozygous Pkd1Col1a1(3.6)-cKO mice had both polycystic pancreas and kidney, indicating the

Figure 7. Development of cysts in pancreas and kidney caused by Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1. Hematoxylin-eosin (H&E) staining (5X) for pancreas (A) and kidney (B) between E14.5 and P14. Both pancreatic duct and renal tubule cysts started at E15.5 in homozygous Col1a1(3.6)-Cre/Pkd1floxflox embryos, and the size of renal cystic lesions developed rapidly between E15.5 and P14.

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Figure 8. Development of fibrosis in kidney caused by Col1a1(3.6)-Cre-mediated conditional deletion of Pkd1. Masson-trichrome staining for fibrosis in polycystic kidney sections (A)1X, (B) 20X magnification between E16.5 and 8 weeks (8w). Masson-trichrome staining was observed in polycystic kidney tissue at P7 and became more severe at 8 weeks of age, indicating a renal fibrosis formation occurring in Col1a1(3.6)-Cre/Pkd1floxflox mice.

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possible effects of a mixed genetic background or other factors affecting cyst formation in these mice. This observation, along with the fact that mesenchymal cells give rise to both osteoblasts during bone development and renal tubular epithelia through mesenchymal-to-epithelial transition during kidney development [37,57,58,59], and pancreatic mesenchyme gives rise to pancreatic ducts by epithelial-mesenchymal interaction during pancreas development [60,61,62], suggests a broad role of Pkd1 in mesenchymal development pathways. Also, consistent with the known gene-dose dependent effect of Pkd1 in cystogenesis that requires a second hit in humans to cause ADPKD, there was no evidence of cyst formation in the pancreas or kidney of heterozygous Col1a1(3.6)-Cre;Pkd1^flox/+ mice. Approximately 30% of homozygous Pkd1^Col1a1(3.6)-cKO adult mice exhibited a polycystic pancreas containing solitary unilocular cysts, whereas pancreatic cysts have been reported in only 5% of patients with ADPKD [63,64].

Interestingly, we did not observe cyst formation in the liver of 6-week-old homozygous Pkd1^Col1a1(3.6)-cKO mice, in spite of the known propensity for liver cyst formation in hereditary cystic disorders. The reason for the absence of liver cysts in our study was a consequence of limitations of the Col1a1(3.6)-Cre or the need for additional time for cyst development in the liver. Regardless, the broad expression of Col1a1(3.6)-Cre in multiple
tissues limits the conclusion that can be drawn from this targeting strategy [32]. Regardless, unlike the embryonic lethality of global Pkd1 null mice [7,15], the conditional Pkd1 null mice are born alive and exhibit a 50% 6-week survival rate, thereby creating a new model to study polykystic kidney and pancreatic cystic disease postnatally. We found that the expression of growth factor such as TGF-β was significantly increased in homozygous Pkd1Col1a1(3.6)-KO kidney at 6 weeks of age, which would stimulate the epithelial cell phenotype’s transformation, producing myofibroblasts and secreting extracellular matrix (ECM). In fact, snail1 and vimentin, the EMT markers, and z-SMA and Col1a1, the fibrosis markers, were markedly upregulated in homozygous Pkd1Col1a1(3.6)-Cre;Pkd1flox/flox kidneys compared with controls at 6 weeks of age. These findings agree with previous reports that polykystic kidney disease triggers the onset of epithelial-mesenchymal transition (EMT) and renal fibrosis [65,66,67,68,69,70].

In conclusion, the conditional deletion of Pkd1 from mesenchymal lineage results in both defective bone formation and polykystic kidney and pancreatic but not liver disease, indicating that role function in mesenchymal precursors to regulate skeletal, renal, and pancreatic development. The long-term survival of Pkd1Col1a1(3.6)-Cre;Pkd1flox/null mice establishes a potential model to study postnatal interventions to retard cyst formation.

Materials and Methods

Mice

We obtained the floxed Pkd1 mouse (Pkd1flox/flox) which harbors two loxP sites flanking exon 2–4 of the Pkd1 gene from Dr. Germano Germino at Johns Hopkins University [25] and Col1a1(3.6)-Cre transgenic mouse, which has activity in mesenchymal precursors, from the University of Missouri-Kansas City [31]. We crossed the floxed heterozygous Pkd1flox/+ mice with heterozygous Col1a1(3.6)-Cre mice to generate double heterozygous Col1a1(3.6)-Cre;Pkd1flox/+ mice. Then the resulting Col1a1(3.6)-Cre; Pkd1flox/+ mice were bred with homozygous Pkd1flox/flox mice to generate conditional Pkd1 heterozygous mouse (Col1a1(3.6) -Cre;Pkd1flox/+), conditional Pkd1 heterozygous mouse (Col1a1(3.6)-Cre;Pkd1flox/+), homozygous Pkd1flox/flox mice, and heterozygous Pkd1flox/+ mice. To achieve greater Cre-mediated reduction in Pkd1 conditional deletion, we bred double heterozygous Col1a1(3.6) -Cre;Pkd1flox/+ mice with homozygous Pkd1flox/flox mice to generate excised floxed Pkd1 heterozygous (Col1a1(3.6) -Cre;Pkd1flox/+), null mice (Col1a1(3.6) -Cre;Pkd1flox/null), as well as Pkd1 heterozygous mice (Pkd1flox/+) and Col1a1(3.6) negative control mice (Pkd1flox/+), equivalent to wild type. These mice were used for phenotypic analysis. Mouse embryos between embryonic day 14.5 (E14.5) and E18.5 were collected from timed pregnant mice. 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: 18053R2).

Genotyping polymerase chain reaction (PCR) to detect deletions

Genomic DNA was prepared from different tissues using standard procedures, and genotyping PCR was performed to detect Col1a1(3.6)-Cre-mediated deletions of the Pkd1 gene as previously described [17,18]. In this regard, Pkd1 wild-type (Pkd1WT) and floxed (Pkd1flox) alleles were identified in 2% agarose gels as 130- and 670-bp bands, respectively. The conditional deleted Pkd1 (Pkd1flox) allele was detected as a 0.85-kb band in 1% agarose gels as previously described [17,18].

Bone densitometry, histomorphometric, marrow adipocyte staining, and microcomputed tomography analyses

BMD of femurs was assessed at 6 weeks of age with a LUNARPXIMUS bone densitometer (Lunar Corp., Madison, WI, USA). Calcine (Sigma-Aldrich, St. Louis, MO, USA) double labeling of bone and histomorphometric analyses of periosteal MAR in tibias were performed using the osteomeasure analysis system (OsteoMetrics, Decatur, GA, USA) [71,72]. The distal femoral metaphyses were also scanned with a Scanco µCT 40 (Scanco Medical AG, Brüttisellen, Switzerland). 3D-images were analyzed to determine bone volume/trabecular volume and cortical thickness as previously described [71]. For detection of bone marrow adipocytes, whole intact femurs or tibiae with encapsulated marrow were dissected from 6-week-old mice, fixed for 48 h in phosphate-buffered paraformaldehyde, decalcified in 14% EDTA, and then stained with aqueous osmium tetroxide (OsO4) for quantification of fat volume, density, and distribution by µCT analysis [17]. In addition, the crossectioning was performed for Oil Red O lipid staining as previously reported in our laboratory [17].

Tissue histology and kidney fibrosis staining

The kidneys, livers, and pancreases from E14.5-, E15.5-, E16.5-, E18.5-, newborn (P0), postnatal day 3 (P3), 7 (P7), 14 (P14), 4-, 6-, and 8-week-old mice were collected and fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin. Eight-micrometer tissue sections were used for hematoxylin-cosin staining as described previously [17]. Masson-Trichrome staining for collagen fibers on kidney sections was also performed according to the manufacturer’s instructions (Polysciences Inc., Warrington, PA, USA).

Serum biochemistry

Serum osteocalcin levels were measured using a mouse Osteocalcin EIA kit (Biomedical Technologies, Inc., Stoughton, MA, USA). Serum BUN was determined using a BUN diagnostic kit from Pointe Scientific Inc (Canton, MI, USA). Serum calcium (Ca) was measured by the colorimetric cresolphthalein binding method, and phosphorus (P) was measured by the phosphomolybdate–ascorbic acid method (Stanbio Laboratory, Boerne, TX, USA). Serum osteoprotegerin (OPG) and Rank ligand (RANKL) were measured using mouse ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA), and serum osteoblast-targeted acid phosphatase (TRAP) assay (Immunodiagnostic Systems, Tarrytown, AZ, USA). Serum parathyroid hormone (PTH) levels were measured using the Mouse intact PTH ELISA kit (Immutopics, Carlsbad, CA, USA). Serum FGF23 levels were measured using the FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan).

Bone RNA isolation and real-time reverse transcriptase (RT)-PCR

For quantitative real-time RT-PCR, 1.0 μg total RNA isolated from calvaria, kidney, liver, and whole tibia of 6-week-old control and Col1a1(3.6)-Cre-mediated Pkd1-deficient mice was reverse transcribed as previously described [34]. PCR reactions contained 20 ng template (cDNA or RNA), 375 nM each forward and reverse primers, and 1X SsoFast™ EvaGreen® supermix (Bio-
was calculated from the relative levels of the normal GTG AAC CT-3' normalized to the mean ratio of five control mice, which was set to 1. The percentage of conditional deleted Pkd1(+)/Pkd1(-) transcripts was calculated from the relative levels of the normal Pkd1(-) transcripts in different Pkd1-deficient mice [73]. All primer information of other genes used in real-time RT-PCR can be found in our previous report [18].

Cell proliferation, osteoblast, and Pkd1-deficient phenotypes in immortalized osteoblast cultures

Calvaria from E17.5 control and Pkd1-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-helper-free viral supernatant in USA) for 48 h. The cells were allowed to recover for 72 h followed by selection with 1 μg/ml puromycin (Sigma) for up to 15 days. The immortalized osteoblasts were cultured in 10% 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 manufacturers' directions (QIA58, Calbiochem, Gibbstown, NJ, USA). To induce differentiation, the immortalized osteoblasts were plated at a density of 2 × 10^5 cells per well in a 12-well plate and 4 × 10^5 cells per well in a 6-well plate and grown up to 21 days in 9% 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 [16,34]. Total DNA content was measured with a PicoGreen® dsDNA quantitation reagent and kit (Molecular Probes, Eugene, OR, USA). Protein concentrations of the supernatant were determined with a Bradford protein assay kit (Bio-Rad). PCR reactions contained 20 ng template (cRNA or cDNA), 375 μmol each forward and reverse primers, 1X SoFast™ EvaGreen® supermix (Bio-Rad) in a total of 10 μl reaction volume. The Ct of tested gene product from the indicated genotype was normalized to the Ct for cyclophilin A as previously described [16,34,36].

Statistical analysis

We evaluated differences between two groups by unpaired t-test and multiple groups by one-way analysis of variance. All values are expressed as means ± SD. All computations were performed using GraphPad Prism5 (GraphPad Software Inc. La Jolla, CA, USA).

Author Contributions

Conceived and designed the experiments: NQ ZSX LDQ. Performed the experiments: NQ LC YD ZH. Analyzed the data: NQ ZSX LDQ. Contributed reagents/materials/analysis tools: NQ LC YD ZH. Wrote the paper: NQ ZSX LDQ.

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