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Mini-Review

Novel roles of protein kinases D1 and D2 in bone formation

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ABSTRACT

Protein kinase D (PKD), a member of the Ca^{2+}/calmodulin-dependent kinase (CaMK) superfamily, is a serine/threonine kinase expressed as three isoforms (PKD1, 2, and 3). PKD has been suggested at the crossroads for the Bone Morphogenetic Protein-Insulin-like Growth Factor1 (BMP-IGF1) signaling axis, which plays a major role in bone formation. This article focuses on the roles of PKD1 and 2 in the mouse skeleton. Dual-energy X-ray absorptiometry (DEXA) scan analysis of male and female pubescent mice revealed a significantly decreased bone mineral density (BMD) in the whole body and femoral bone of mice with one inactive PKD allele PKD1 (+/-), compared to their wild-type littermates. The body weight, nasal-anal length, and percent body fat of the mice were not significantly different from their wild-type littermates. In PKD2 (+/-) male, but not female mice, significantly decreased BMD in the whole body, spine, and femoral bone was detected, compared to their wild-type littermates. Lower expression levels of osteoblast and osteoclast differentiation markers were detected in cultured bone marrow stromal cells (BMSC) from PKD1 (+/-) male mice compared to wild-type mice. In female mice, only markers of osteoblast differentiation were reduced. Cultured mouse calvarial cells from PKD1 (+/-) mice demonstrated substantially reduced BMP responsiveness and BMP type II receptor mRNA expression levels. Cultured BMSC from male PKD2 (+/-) mice exhibited lower mRNA expression levels of osteoblast differentiation markers and higher levels of osteoclast differentiation markers than wild-type. However, osteoblast and osteoclast differentiation markers were reduced in female PKD2 (+/-) mice. BMC cultures from male and female PKD2 (+/-) mice displayed a reduced BMP responsiveness without any significant changes in BMP receptor mRNA expression levels. These data suggest that PKD1 and 2 share common but also distinct mechanisms in the regulation of osteoblastogenesis and osteoclastogenesis that are manifested during mouse puberty in a gender-specific manner.

KEYWORDS: protein kinase D, BMP, IGF1, osteoblastogenesis, osteoclastogenesis

1. General introduction to protein kinase D

Protein kinase D (PKD) is a serine/threonine kinase that belongs to the Ca^{2+} calmodulin-dependent kinase superfamily [1], but exhibits distinct structure and substrate specificity from the other known PKC family members [2]. Three mammalian PKD isoforms have been identified: PKD1 (human PKCμ), PKD2, and PKD3 (PKCν) [2-6]. Several conserved structural motifs are found in these isoforms. The highest sequence homology is located in the catalytic kinase domain at the C-terminus and the regulatory region at the N-terminus. The regulatory region consists of two cysteine-rich diacylgylcerol (DAG)-binding domains and an auto-inhibitory PH (pleckstrin homology) domain [1]. The regulatory region of PKD1 is also involved in interaction with cellular membranes. Whereas PKD1 and 2 share high homology in their structure, PKD3 lacks some of the regulatory elements, such as a PDZ binding motif [7] and a Src kinase phosphorylation

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motif [8]. The lesser-conserved regions have been proposed to account for isoform-specific functions. All PKD isoforms can be phosphorylated and activated by novel PKCs [2] that are themselves activated by phosphoinositide-dependent kinase-1 (PDK-1) [9]. PKD activity can be regulated by protein phosphorylation, intracellular localization, level of expression and interaction with tissue-specific interacting proteins [9-11]. In addition, PKDs can be activated by a wide variety of biological molecules, including bioactive lipids, chemokines, growth factors, and hormones [1]. Moreover, the different PKD isoforms have been shown to regulate surface localization of distinct subsets of substrates, leading to different physiological responses [7, 11].

The biological functions of PKD are diverse, including regulation of cell growth and survival, cell motility, protein trafficking, NF-κB-dependent gene regulation, and JNK activity [1, 3, 12, 13]. Different PKD isoforms have been shown to play distinct functions [2, 13]. An excellent assessment of the functions and regulation of PKD in numerous cell types and organ systems in health and disease can be found in a recent review article [1]. The role of PKD in the nervous system has been recently reviewed [14]. The roles of PKD associated with various cancers, such as breast, prostate, pancreatic and skin cancers, have also been recently reviewed [15-17]. In the current article, we will focus on the roles of PKD1 and 2 in the mouse skeleton associated with osteoblast cell differentiation and bone biology *in vivo* and *in vitro*.

2. Protein kinase D and bone metabolism

Recent *in vitro* and *in vivo* studies have revealed that PKD plays an important role in bone metabolism and differentiation [18-24]. A published report showed that PKD activity can be stimulated by bone morphogenetic protein (BMP)-2 and IGF-1 in mouse osteoblastic MC3T3-E1 cells [18]. Treatment with antisense PKD1 blocked basal and BMP-2-induced osteoblast differentiation marker alkaline phosphatase (ALP) activity and osteocalcin (OCN) mRNA expression [19]. Prolonged dimethyl sulfoxide (DMSO) treatment of MC3T3-E1 induced mRNA expression of transcription factors Runx2 and Osx and ALP, and bone nodule formation [24]. In the primary human malignant bone cell osteosarcoma, PKD1 levels were lower than normal. Molecular genetic manipulation to overexpress PKD1 in these cells resulted in cells with lower cell invasion, migration and proliferation [25], suggesting that the expresion level of PKD could affect cell fate.

Celil & Campbell [19] reported that PKD activation by BMP-2 and IGF-1 resulted in an increased Osx expression in human bone marrow progenitor cells. PKD inhibitor Gö6976 blocked BMP-2, IGF-1, and the combination of BMP-2 and IGF-1 in the stimulation of mRNA expression of ALP and Osx, and bone mineralization. These studies, however, did not establish the identity of the PKD isoforms nor the mechanism(s) by which BMP-2 and IGF-1 activated PKD.

Jensen [20] reported that BMP-2 stimulated HDAC 7 nuclear export in MC3T3-E1 and C2C12 cells. Gö6976 blocked basal and BMP-2-induced HDAC 7 nuclear export. PKD1 inhibited HDAC7 repression of Runx2 expression, suggesting that “BMP-2 signaling regulates Runx2 activity *via* PKD-dependent inhibition of HDAC7 transcription repression” [20].

Using pharmacological inhibitors and molecular genetic approaches, Yeh *et al*. [21] discovered the importance of PKD in the synergistic induction of osteoblast differentiation and mineralized nodule formation by BMP-7 and IGF-1 in fetal rat calvarial (FRC) cells. The study showed that BMP-7 alone and the BMP-7 and IGF-1 combination synergistically stimulated protein kinase D (PKD) phosphorylation at Ser744/748 and Ser916. These residues are phosphorylated by PKCs, whereas phosphorylation at Ser916 is auto-phosphorylated and is considered to be an indication of increased catalytic activity [2, 26, 27]. Moreover, transfection of FRC cells with a constitutively active PKD stimulated osteoblast differentiation marker expression, while transfection with a catalytically inactive PKD did not. Furthermore, treatment of FRC cultures with the PKD inhibitor Gö6976, which inhibited protein kinase C (PKC) α and β1 [27, 28], blocked PKD phosphorylation and the synergistic action of the BMP-7 and IGF-1 combination on osteoblast differentiation as measured by ALP enzymatic activity and mRNA expression of biochemical markers of osteoblast differentiation osteocalcin (OCN) and bone sialoprotein (BSP). Formation of mineralized bone nodules was also

inhibited. In contrast, treatment of FRC cultures with Gö6983, which inhibited PKCα, β, γ, δ, and ζ, did not block osteoblast differentiation. Published studies also show that Gö6983 inhibits conventional PKCα, β and γ as well as novel PKCδ and atypical PKCξ [27]. Since both Gö6976 and Gö6983 block PKCα and β, it is likely that Gö6983-sensitive PKCs other than PKCα and β are responsible for promoting osteoblast mineralization in FRC cells. Taken together, these results suggest that induction of osteoblast differentiation in FRC cells by BMP-7 alone and the combination of BMP-7 and IGF-1 requires PKD activity. The data are consistent with a novel mechanism in which the combination of BMP-7 and IGF-1 activates upstream signaling by PKC(s) which subsequently phosphorylates and activates PKD leading to osteoblastic differentiation and bone nodule formation *in vitro*.

3. Protein kinase D1 and bone metabolism

Studies described above revealed that PKD plays an important role in osteoblastic differentiation, but have not identified and elucidated the role of the different PKD isoforms. Recently published *in vivo* studies using mice deficient in PKD1 catalytic activity [29] have shown that PKD signaling affects the acquisition of bone mass in young mice [22]. Specifically, longitudinal dual-energy X-ray absorption (DEXA) assessment of pubescent male and female mice revealed decreased acquisition of bone mass in the heterozygous PKD1 (PKD1 +/-) mice. Haplodeficiency of PKD1 male mice, at 5 and 6 weeks of age, revealed no measurable change in bone mineral density (BMD) of the whole body and femoral bone compartment between wild-type and PKD1 (+/-) mice (Table 1). However, at the age of 7 weeks, the PKD1 (+/-) male mice exhibited significant decreases in whole body BMD with no statistically significant changes in femoral bone density. At the age of 8 weeks, the PKD1 (+/-) male mice showed significant decreases in femur BMD, but only a small decrease in whole body BMD, compared to their wild-type littermates. PKD1 (+/-) female mice at 5 weeks of age revealed no detectable change in whole body and femoral bone density compared to the wild-type littermates. However, beginning 6 to 7 weeks of age, they showed detectable time-dependent small decreases in BMD. At the age of 8 weeks, the female PKD1 (+/-) mice displayed significant decreases in whole body and femoral bone density (Table 1).

Subsequent *in vivo* and *in vitro* studies further showed that PKD1 action contributes to the gender-specific regulation of bone density by altering osteoblastic gene expression, affecting skeletal matrix acquisition during puberty [22]. Specifically, *in vitro* studies of bone marrow mesenchymal cells cultured under osteogenic conditions revealed that early-stage osteoblastic differentiation cultures (CFU-F) from PKD1 (+/-) male animals displayed a marked decrease in mRNA expression of bone sialoprotein (BSP), osterix (OSX), and type I collagen (T1C) with no detectable change in osteocalcin (OCN) mRNA expression [22] (Table 1). By comparison, CFU-F cultures from PKD1 (+/-) female animals revealed a marked decrease in the expression of OCN, OSX, and T1C with no significant change in BSP mRNA expression. Under osteogenic conditions, late-stage, functionally mature cultures (CFU-OB) from PKD1 (+/-) male and female animals presented a marked decrease in BSP expression (Table 1). Thus, this data supports the notion that PKD1 signaling is necessary throughout cell differentiation and maturation of the osteoblast. In this regard, Celil and Campbell [19] and Jensen *et al.* [20] also demonstrated the importance of PKD1 signaling in the regulation of Osterix and Runx2, respectively. Furthermore, using cells from the PKD1 (+/-) mice, Ford *et al.* [22] observed attenuated mineralized nodule formation by cells deficient in PKD1 signaling in both male and female mice. Taken together, these findings strongly suggest that PKD1 signaling, by presently unknown mechanisms, functions to promote osteoblast differentiation leading to bone matrix mineralization in a gender-dependent manner.

As described above, cells from tibia of PKD1 (+/-) mice showed significantly reduced mRNA expression of osteoblast differentiation markers. Additionally, mouse calvarial cells from PKD1 (+/-) mice also showed significantly decreased mRNA expression of osteoblast markers BSP, OCN, OSX, and TIC [22]. Taken together, these findings suggest that PKD1 signaling is essential in both modes of bone formation, endochondral and intramembranous.

Bone morphogenetic proteins (BMPs) are important growth factors essential for normal growth and development and for bone differentiation and repair [30-32]. Multiple studies have demonstrated that PKD signaling is involved in the synergy between BMP and IGF1 in promoting osteoblast differentiation [18-22]. Taking advantage of the availability of the PKD1 (+/-) mouse, our laboratories [22] further showed that osteoblasts deficient in wild-type PKD1 signaling were less responsive to BMP-7, presumably by virtue of the reduced receptor number and/or disruption of the downstream BMP-IGF1-PKD1 signaling cascade. Specifically, calvarial cell cultures from PKD1-deficient mice were significantly reduced in BMP-7-stimulated mRNA expression of osteoblastic differentiation markers, compared to the wild-type mice (Table 1). IGF-1 alone minimally stimulated the expression of these markers in these cells, compared to the solvent-treated control. The reduced responsiveness to BMP in the calvarial cell from PKD1 (+/-) might be a consequence of a significant decrease in the mRNA expression levels of the type II BMP receptor (BMPR-II) in BMSC from PKD1-deficient male and female mice, compared to the wild-type BMPR-II levels (Table 1).

Little is known about the role of PKD1 in osteoclastogenesis until now. Ford *et al*. [22] first reported that mRNA expression of osteoclast differentiation markers tartrate-resistant acid phosphatse (TRAP), receptor activator of nuclear factor kappa-B (RANKL), and osteoprotegerin (OPG) in PKD1-deficient male was lower, compared to the male wild-type (Table 1). However, only RANKL in female PKD1-deficient mice was lower with no effects on TRAP expression, compared to wild-type (Table 1). The findings imply that PKD1 may play a role in the formation and function of osteoclasts and that the role may be gender specific, with female mice able to compensate, by currently unknown mechanism, for the interrupted signaling pathway. These data support the supposition that PKD1 haplodeficiency uniformly depressed osteoblastic signaling, differentiation, and function in both female and male mice but exerted differential effects on osteoclast differentiation resulting in an overall decreased BMD phenotype. It is possible that female osteoclasts are less sensitized to the effects of PKD1 reduction by a mechanism involving levels of sex hormones, which differ in peak and average concentrations between the two genders. That the precise role of PKD1 signaling in the osteoclast and its gender specific role could in part be due to hormonal differences between male and female mice in the pubertal stage is supported by the alterations of BMP and IGF-1 receptor levels which are known targets of hormone pathways [33-35].

4. Protein kinase D2 and bone metabolism

The effects of PKD2 on bone *in vivo* during pubertal period were assessed using PKD2 heterozygous, i.e., knock-in of one catalytically inactive allele, [PKD2 (+/-)], mice [29], at 5 weeks of age until 8 weeks of age (Yeh *et al*, unpublished). Dual-energy X-ray absorptiometry scan analysis revealed that PKD2 (+/-) male mice showed significant decreases in BMD of whole body, spine and particularly femur, compared to the wild-type at the age of 7 weeks but with no detectable differences in mice at 5, 6 and 8 weeks of age. In contrast, PKD2 (+/-) female mice did not exhibit statistically significant differences in the BMD of whole body, spine and femur, compared to the wild-type, between 5 to 8 weeks of age (Table 1). In this respect, the gender difference in BMD was also observed in the PKD1-deficient mice [22]. Moreover, deficiency in the two PKD isoforms (PKD1 vs PKD2) appeared to affect BMD of different bone components in a gender-dependent manner (Table 1).

Recent *in vivo* and *in vitro* studies from our laboratory further showed that PKD2 action contributes to the gender-specific regulation of bone density by altering osteoblastic gene expression (Yeh *et al*. unpublished). Specifically, CFU-F cultures from PKD2 (+/-) male mice displayed a significant decrease in mRNA expression levels of osteoblast differentiation markers ALP, BSP, and OCN, compared to the wild-type litter-mates (Table 1). CFU-F cultures from PKD2 (+/-) female mice displayed a significant decrease in mRNA expression levels of BSP and OCN without alteration in the ALP mRNA levels, compared to the wild-type litter-mates (Table 1). By comparison, CFU-F

cultures from PKD1 (+/-) male animals displayed a marked decrease in mRNA expression of BSP, OSX, and T1C but not OCN [22]. CFU-F cultures from PKD1 (+/-) female animals revealed a marked decrease in the expression of OCN, OSX, and T1C with no significant change in BSP mRNA expression. Under osteogenic conditions, late-stage, functionally mature cultures (CFU-OB) from PKD2 (+/-) male and female mice exhibited attenuated mRNA expression of osteoblast differentiation markers ALP, BSP, and OCN (Table 1). By comparison, CFU-OB from PKD1 (+/-) mice also showed reduced mRNA expression of ALP and OCN. Accordingly, our *in vivo* data strongly suggest that PKD1 and PKD2 signaling is necessary throughout cell differentiation and maturation of the osteoblast [21, 22]. Our findings thus are congruent with but have further extended those of Celil and Campbell [19] and Jensen *et al.* [20] who demonstrated the importance of PKD1 signaling in the regulation of osteoblastic gene transcription in cultured cells. It is noteworthy to point out that Avriyanti *et al.* [36] reported that the morphology of different regions of the brain from PKD1 and PKD2 knock-out mice was similar to the wild-type control, suggesting a redundancy between PKD1 and PKD2 in certain neuronal function. By comparison, Matthews *et al.* reported that homozygous deficiency of PKD1 activity in mice is lethal [29].

Published studies indicated that PKD2 expression or enzymatic activity is involved in osteoclast formation and maturation [37, 38]. Mansky *et al.* [37] reported detection of PKD2 expression in monocytes, a key precursor of osteoclasts. The same study also reported that, in bone marrow macrophages (BMMs) treated with M-CSF and RANKL, PKD2 was most highly expressed, with a lower PKD3 level and a very low PKD1 level expression in osteoclasts. Furthermore, inhibition of PKD2 using RNAi and the PKD inhibitor CID755673 showed that PKD activity is unnecessary for TRAP-positive pre-osteoclast formation in culture, but is required for the transition from mono-nucleated pre-osteoclasts to multinucleated osteoclasts. Tan *et al.* [38] further showed that PKD2 is involved in the migration of monocytes. In the study by Yeh *et al.* (unpublished), a significant increase in the mRNA expression of RANKL and OPG in BMC cells from PKD2 (+/-) male mice was detected (Table 1). On the contrary, in PKD2 (+/-) female mice, RANKL and OPG mRNA levels decreased, compared to wild-type (Table 1). These findings imply that PKD2 plays a unique role in the formation and function of osteoclasts and that the role is likely gender specific. The observation is consistent with the finding that BMD, though a static measure of the balance of bone formation and resorption, is decreased in male but not in female PKD2 (+/-) mice. Taken together, these results further suggest possible differences in the roles for PKD1 and PKD2 in osteoclast formation and maturation. It is not surprising that the different PKD isoforms may play different roles in a tissue-specific and gender-specific manner. For example, Matthew *et al.* [29] reported unique functions of PKD1 and PKD2 in T cells. Furthermore, a recent extensive study by the same laboratory on PKD2 substrates identified 15,871 phosphorylations on 3505 proteins in cytotoxic T cells. In PKD2 null cytotoxic T cells, 281 phosphoproteins were down-regulated and 196 phosphoproteins were up-regulated. Additional analyses of these PKD2 substrates revealed that these proteins could be classified into two distinct biological functions: regulation of protein sorting and intracellular vesicle trafficking, and control of chromatin structure, transcription, and translation [39]. By contrast, in other cell types, proteins such as HDAC7 and actin regulatory proteins such as slingshot were identified as PKD substrates but they were not PKD substrates in primary T cells. Taken together, currently available data suggest that PKD isoforms are likely to play diverse functional roles in different cell types [39]. Recent findings from our laboratory on PKD1 and PKD2 in bone from pubertal mice are consistent with this supposition.

A published report showed that fetal rat calvarial (FRC) cell differentiation induced by BMP *in vitro* requires PKD stimulation [20]. Studies on the effects of PKD2 deficiency *in vivo* on the BMP-induced osteoblast differentiation further revealed that the basal levels of markers ALP,

BSP, and OCN in the solvent-treated control cells from PKD2 (+/-) male mice were significantly lower than in the solvent-treated cells from wild-type mice (Yeh *et al*. unpublished data). Moreover, BMP-2 did not stimulate mRNA expression of these markers in cells from PKD2 (+/-) male and female mice (Table 1).

Published studies have implicated altered expression of BMP receptors in PKD1 signaling cascades in bone [21, 22]. Moreover, the altered response to BMP of the BMC cultures from PKD2-deficient mice might also result from the influence of PKD2 on BMPR expression. However, BMPR-IA, -IB, and –II mRNA expression levels in BMC cultures from male and female pubertal PKD2 (+/-) mice were not significantly altered, compared to the wild-type (Table 1). It is possible that the reduced response of BMC cultures from the PKD2-deficient mice to BMP-2 might be the result of a disruption of the downstream BMP-PKD signaling cascade or other receptors, such as the Activin receptors, which have been shown to bind BMP as well, though with different affinities.

5. Do PKD isoforms have differential effects on bone?

The following table compares and contrasts the bone characteristics of male and female pubertal mice with PKD1 and 2 haplodeficiency.

6. Concluding remarks

Earlier studies indicated that PKD plays an important role in osteoblast differentiation. Recent findings have advanced our understanding by showing that both PKD1 and PKD2 isoforms are essential for pubertal mouse bone development. Nevertheless, the differential roles of each PKD isoform suggest that they utilize distinct as well as similar mechanisms in regulating bone development. These observations have provided a molecular basis for us to determine more fully the mechanisms by which PKD1 and PKD2 are activated in bone and what signaling mechanisms are used by the two PKD isoforms to regulate osteoblastogenesis and osteoclastogenesis. It is increasingly apparent that members of the PKD subfamily are key players in events involved in bone remodeling. Identifying the two PKD isoforms as requirements for acquisition of peak bone density will likely provide insights into general mechanisms of bone formation and the pathophysiology and pathogenesis of osteoporosis. Future studies on the role of the third PKD isoform PKD3 during pubertal development should further advance our understanding of the individual role of all three PKD isoforms in osteoblastogenesis and osteoclastogenesis. The current data on the role of individual PKD in bone remodeling is expected to pave the way for further study on their role in skeletal development, maturation and aging.

Table 1. A summary of the bone characteristics of male and female mice with haplodeficiency of PKD1 and PKD2*.

Characteristics	**PKD1**		**PKD2**	
	Male	**Female**	**Male**	**Female**
Femur BMD	↓	↓	↓	↔
Spine BMD	↔	↔	↓	↔
Whole body BMD	↓	↓	↓	↔
mRNA expression of osteoblast differentiation markers	↓	↓	↓	↓
mRNA expression of osteoclast differentiation markers	↓	↔	↑	↓
BMPRI mRNA expression	↔	↔	↔	↔
BMPRII mRNA expression	↓	↓	↔	↔
BMP Responsiveness	↓	↓	↓	↓

*Footnote: ⇩Reduced, ⇧Increased, ⇔No significant change

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

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