PYK2: POTENTIAL REGULATOR OF POST MENOPAUSAL BONE LOSS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Bone mass is controlled by the actions of osteoclasts which degrade bone and osteoblasts with form new bone. Osteoporosis is a pathologic condition of bone commonly associated with aging in males and females, and is accelerated in women following menopause. After menopause, the bone resorbing activity of osteoclasts exceeds bone formation by osteoblasts, resulting in decreased trabecular and cortical bone mass. Reduced bone mass increases the risk of pathologic fracture of bones.

One of the major hormones regulating bone mass is estrogen. Estrogen plays a role in regulating bone remodeling by controlling remodeling activation, osteoblast and osteoclast numbers, and their respective effectiveness in bone formation and resorption, respectively. As a result of declining estrogen levels after menopause, the elegant balance between the actions of osteoclasts and osteoblasts is altered, and bone resorption exceeds bone formation, resulting in bone loss and increased bone fragility. However, the intracellular proteins that control the effects of estrogen on bone cell function are not completely understood.

Pyk2 is a protein tyrosine kinase that plays an important role in regulating bone resorption by osteoclasts [1, 2] as well as osteoblast proliferation and differentiation [3, 4]. Deletion of the *Pyk2* gene in mice leads to an increase in bone mass, in part due to dysfunctional osteoclast and osteoblast activity [1, 3]. However, the mechanism of action of Pyk2 and its role in estrogen signaling is unknown. Moreover, the role of Pyk2 in the protection of bone loss associated with menopause is unclear. The aim of this study was to examine the role of Pyk2 in a mouse model of post-menopausal bone loss using ovariectomized (OVX) wild type (WT) and Pyk2 KO mice.

PURPOSE OF THE STUDY

The purpose of the study was to examine and compare the effects of ovariectomy with or without estrogen supplementation on trabecular and cortical bone mass in Pyk2 KO and WT mice.

HYPOTHESIS

- Pyk2 KO mice will have higher bone mass after ovariectomy than WT mice.
- Ovariectomized Pyk2 KO mice supplemented with estrogen will have increased bone density compared to WT ovariectomized mice supplemented with estrogen.

REVIEW OF THE LITERATURE

Osteoporosis is a pathologic condition of bone that leads to decreased bone mass, increased fragility of bone, and increased risk of pathologic fracture of bone. Nearly half of all women and one fifth of all men will have an osteoporotic-related fracture in their lifetime, contributing to 1.5 million osteoporotic fractures annually [5, 6]. Current standards of care utilize drugs known as bisphosphonates to inhibit the activity of osteoclasts which resorb bone, anabolic pharmaceutical agents such as parathyroid hormone that stimulate bone formation by osteoblasts, and selective estrogen receptor modulators which act selectively at estrogen receptors as agonists or antagonists in various tissues to prevent bone loss [7]. Historically, estrogen replacement therapy was prescribed to prevent post-menopausal bone loss, but due to elevated risk of stroke, blood clots, breast and uterine cancer, this therapeutic approach is on the decline [7]. Despite these medical interventions, fracture rates are only reduced by approximately 50-70% and treatments are not without untoward side effects [8, 9]. Bisphosphonates have been shown to be associated with rare but significant side effects such as pathologic fractures, osteonecrosis of the jaw, and other concomitant adverse effects [10]. Recombinant parathyroid hormone such as teriparatide has been linked with osteosarcoma in preclinical animal studies, so two year usage limits have been applied to this drug [11]. Selective estrogen receptor modulators (SERMs) such as raloxifene, though promising in reducing vertebral fractures, were associated with side effects such as hot flashes, leg cramps as well as deep vein thrombosis and pulmonary embolisms [7] Therefore, research scientists and medical professionals continue to pursue alternative forms of treatment for bone loss associated with osteoporosis.

Bone mass is controlled by genetic factors, diet, hormones, cytokines, mechanical loading, as well as other environment conditions [12, 13]. The steroid hormones, estrogen and testosterone, are critical for bone mass homeostasis, and depletion of either hormone leads to decreased bone mass and elevated risk of osteoporosis [12]. Estrogen controls bone remodeling through hormonal signaling pathways (Figure1) [14]. Estrogen produced by the ovaries is stimulated by follicle stimulating hormone (FSH) which is secreted from the pituitary gland. A negative feedback loop produced by elevated estrogen levels controls FSH secretion and is modulated by the pituitary gland. When estrogen levels are reduced, FSH secretion is increased and production of cytokines by the bone marrow and immune cells is increased. These cytokines promote osteoclast differentiation from hematopoietic precursors found in bone marrow. Estrogens and growth hormone from the pituitary gland also prompt hepatic production of insulin-like growth factor 1 (IGF1) that stimulates osteoblast differentiation [14]. Both males and females are affected by hormone-related bone loss, but females are more dramatically affected due to estrogen depletion after menopause [12]. Bone loss in females occurs in two stages [12]. During the first stage, trabecular bone is predominantly lost due to increased osteoclast-mediated bone resorption; whereas, during the second phase of menopause which occurs four to eight years later, loss of both trabecular and cortical bone occurs, mostly attributed to decreases in bone formation [12].

In adults, bone density is maintained by balancing the activity of osteoblasts which form new bone, and osteoclasts, which degrade bone. These two cell types compose the bone multi-cellular unit, a tightly coupled functional unit where both cells types communicate. The mechanism of bone remodeling within the bone multi-cellular

unit is initiated by osteoclasts whose catabolic activity degrades the mineralized bone. The control of osteoclast differentiation and number is controlled by osteoblasts, which release macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). RANKL binds to the RANK receptor on osteoclast progenitor cells, and with the combined actions of M-CSF, will lead to an increase in osteoclast numbers. The osteoclastogenic effects of RANKL are blocked by osteoprotegerin (OPG) which is also secreted by osteoblasts and acts as a decoy receptor for RANKL. Osteoblasts embedded in the mineralized bone matrix further differentiate to become osteocytes which play a role in mechano-signaling and mechano-transduction [15]. Recently, it was also shown that osteoclast differentiation is regulated by osteocyte-derived M-CSF, RANKL and OPG [16-18]. Therefore, the local ratio of RANKL/OPG, whether secreted by osteoblasts or osteocytes, helps keep osteoclast numbers in check by decreasing the ability of RANKL to bind to its receptor on early osteoclasts [12, 19]. Following bone resorption, osteoclast die by apoptosis and osteoblast precursor cells are recruited to the resorbed site, possibly by growth factors released from the degraded bone. The osteoblastic precursors undergo differentiation into mature osteoblasts that secrete un-mineralized osteoid which is later mineralized to form new bone [12, 16, 19]. Under normal homeostatic conditions, the amount of bone formed at a given site closely approximates the amount resorbed.

Estrogen depletion affects the bone cells in different ways. Estrogen depletion increases the proliferation of the osteoblast cell lineage, which regulates osteoblastogenesis and bone formation [20-24]. However, osteoblasts have reduced ability to form new bone, offsetting the balance in favor of bone resorption at each

remodeling site. This results in a decrease in bone mass [12]. Estrogen negatively affects osteoclasts at the receptor level by decreasing bone resorbing activity [25, 26] and inducing osteoclast apoptosis [12, 27]. With declining estrogen levels after menopause, the regulatory control of osteoclast numbers and activity is removed. This promotes osteoclast differentiation, leading to increased numbers of osteoclasts, increased bone resorption and subsequently to a decrease in bone mass and bone strength. Estrogen-deficiency also promotes osteoclast survival which is the predominant means of increasing osteoclast numbers after menopause [24, 28].

The actions of estrogen are mediated by binding to its receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα and ERβ are differentially expressed in various skeletal and non-skeletal tissues. In the skeleton, ERα is found predominantly in cortical bone and ERβ is expressed in trabecular bone [29, 30]. ERα expression is increased during osteoblast differentiation of pre-osteoblasts, whereas ERβ levels remain relatively constant throughout differentiation [29]. ERα and ERβ have compensatory actions on skeletal bone mass in female mice, and both influence bone remodeling. However, in male mice, ERα appears to have a more predominant effect on bone remodeling [31]. The activation of the ERs in bone cells also activates a number of other signaling cascades which are important for cellular function [32-35]. ERs can activate signaling through the nucleus or cytoplasm. Nuclear signaling is the classical pathway of estrogen signaling and it can take 30 to 60 minutes to affect changes in gene expression [36]. In the classical nuclear activation pathway of osteoblasts, circulating estrogen binds to ERα, which is subsequently translocated to the nucleus where it acts as a nuclear transcription factor [37, 38] and promotes expression of proteins such, c-fos, c-jun, jun-

D, which have important effects in osteoblasts [24, 28, 39]. In addition, estrogen leads to increased secretion of Fas ligand (FasL) by osteoblasts, which subsequently binds to the pro-apoptotic Fas receptor on osteoclasts. This promotes osteoclast apoptosis and decreases osteoclast cell number [24, 27, 28, 39-42]. Alternatively, estrogen can act in the cytoplasm to activate proteins such as receptor tyrosine kinases that result in rapid intracellular responses. Examples of these non-canonical estrogen signaling pathways are activation of mitogen-activated protein kinases, adenylyl cyclase and G-protein coupled receptors [36, 43].

Estrogen binding to ERα also initiates a signaling cascade involving the focal adhesion kinase (FAK) and other proteins [44]. It was previously established that estrogen depletion reduces breast cancer cell motility via FAK [44] and that ERα promotes endothelial cell motility through FAK [45, 46]. The tyrosine kinase Pyk2 is highly-related to FAK at the nucleotide and amino acid level. However, Pyk2 is expressed predominantly in neural and hematopoietic tissues, whereas FAK is expressed more ubiquitously [47]. The role of Pyk2 in estrogen signaling is unclear. In one study, it was shown that 17β-estradiol stimulation of platelet cells induced the translocation and activation of Pyk2 [48], which is mediated by activation of ERβ and the integrin $\alpha_{iib}\beta_3$ [49].

Deletion of Pyk2 in mice leads to osteopetrosis (increased bone mass) as a result of defective osteoclast activity and increased osteoblastic bone formation [1, 3]. Pyk2 influences osteoclast differentiation and bone resorption by interacting with various protein signaling cascades involved in osteoclast cell movement and bone resorbing activity [2, 50, 51]. Several groups have reported that Pyk2 is localized to actin-rich

adhesion structures called podosomes in osteoclasts, which are organized as a ring-like structure around the cell periphery [27, 52]. Upon cell attachment and integrin activation [53], Pyk2 associates with several proteins associated with actin ring formation, including $p130^{CAS}$ [54], Src [52], Cbl [55], integrins [52], gelsolin [56], and paxillin [57].

Pyk2 also plays a role in osteoblast function [3, 53, 58]. By quantification of a fluorochrome label, which integrates into actively mineralizing bone, it was demonstrated that Pyk2 KO mice have a 139% increase in bone formation rate in tibia from compared to wild-type mice [3]. This increase was the result of both increased mineralizing surface per bone surface and mineral apposition rate. In addition, it has been reported that bone marrow cells from Pyk2 KO mice cultured in osteogenic media containing ascorbic acid and β-glycerolphosphate exhibit elevated alkaline phosphatase levels, a marker of osteoblast activity [3, 59]. Importantly, unpublished studies from Dr. Bruzzaniti's laboratory revealed that deletion of Pyk2 results in a much greater increase in bone mass in female mice compared to males. Moreover, Pyk2 was found to form a molecular complex with the ERs in calvarial-derived osteoblasts *in vitro*. Together, these studies suggest that Pyk2 plays a role in regulating osteoclast and osteoblast activity. Furthermore, these studies support a potential role for Pyk2 in regulating estrogen receptor signaling in bone cells.

In the current study, we examined the role of Pyk2 in the maintenance of bone mass in estrogen-depleted and estrogen-replete female mice. To determine the role of Pyk2 in the regulation of bone mass, we compared femoral bone morphometrics in WT and global Pyk2 KO mice following ovariectomy or sham surgeries. We hypothesized that deletion of the *Pyk2* gene in mice which increases bone mass, would confer a

protective effect against bone loss in ovariectomized mice compared to WT mice. We also proposed that estrogen supplementation would rescue bone loss in ovariectomized mice, and increase the magnitude of the bone mass response in Pyk2 KO mice.

MATERIALS AND METHODS

Pyk2 KO Mice: The IACUC approval number is DS000885R and the biohazard approval number is 1085. Pyk2 KO mice have been backcrossed >15 generations onto a C57Bl6 background. Mice were bred as heterozygotes and crossed to generate knockout mice and wild-type littermates.

Ovariectomy: OVX was performed on 12 week old WT and Pyk2 KO female mice. Sixty mice were divided into six groups of 10 that are wild type or Pyk2 KO, and groups were treated with sham surgery (ovaries are gently lifted and replaced into the abdominal cavity), OVX plus placebo implant, or OVX plus estrogen pellet implantation. For OVX, mice were given a subcutaneous pre-operative dose of the analgesic buprenorphine (1-.5 mg/kg), and anesthetized using inhaled isoflurane. After shaving, a small dorsal incision was made 1-2 cm lateral to the midline and below the last rib. The cutting point of the ovaries was tied with Ethicon coated vicryl braided polygalactan 910 absorbable sutures, and the organs were removed using scissors or a scalpel. Estrogen (E2) releasing pellets were placed at the time of surgery subcutaneously in a dorsal location for 4 weeks into two of the groups, the estrogen replacement dose of 167 ng 17β-estradiol/mouse/day (Innovate Research of America, Sarasota, Florida, USA). The estrogen pellet supplementation was used to replicate hormone replacement therapy following menopause, and to determine the resultant effects of estrogen on bone mass after OVX in WT and Pyk2 KO mice. The peritoneum was closed with a few sutures and skin closed with wound clips. Mice were housed for 28 days post-surgery, food and water ad libetum, and sacrificed at 16 weeks of age. To reduce postoperative pain, analgsia

(Buprenorphine 0.05-0.1 mg/kg SQ every 12 hours) was provided for two days or for as long as deemed necessary by veterinary staff.

Micro-computed Tomography: After euthanization by inhaled isoflurane and cervical dislocation, posterior legs were amputated and stored in formalin. All soft tissue was removed from bone, and the femur was separated from the tibia and fibula and stored in 70% ethanol. Uterine wet weight of mice was also recorded at necropsy to the nearest milligram. Micro-computed tomography (micro-CT) studies were performed on the distal femoral metaphysis and cortical diaphysis using the Skyscan 1172 high-resolution desk-top micro-CT system (Skyscan, Aartselaar, Belgium). Samples were wrapped in parafilm (Brand, Germany), and affixed to the scanning stage. Scans were acquired using an x-ray source set at 60kV over an angular range of 180 degrees (rotational steps of 0.7 degrees) with a 6 μm pixel size. Projection images were reconstructed using standard Skyscan software (NRecon, Skyscan). The trabecular bone of the distal femora secondary spongiosa was segmented from the cortical shell for 1mm of tissue (165 slices) beginning at the point where the femoral condyle disappears and the cortex is relatively intact and moving proximally (Figure 2A). A single slice of cortex was analyzed at a site 3mm proximal from the trabecular region. Trabecular site outcomes included bone volume/total volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th). Cortical parameters included mean total cross-sectional bone area (Ct.Ar), cortical thickness (Ct.Th), and mean polar moment of inertia (CSMI polar).

Serology: In addition to bone analyses, at necropsy serum was collected and uterine wet weight measured, as a measure of effectiveness of estrogen-depletion and estrogen-replacement. For serum collection, animals fasted for 12 hours prior to blood draw. Blood was collected and stored in a glass tube for 30 to 60 minutes at room temperature, and then the clot was separated from the walls of the tube to allow for clot contraction. After overnight storage at 4°C, the clot was removed and the sample centrifuged at 2000 to 3000 rpm for 10 minutes and stored at -80°C. Serum levels of circulating estradiol in WT and Pyk2 KO mice was measured using commercially available enzyme-linked immunosorbant assays (ELISA) (Calbiotech #ES180S-100).

Statistical analysis: The effects of Pyk2-KO and estrogen status (sham, OVX+Placebo, OVX+estrogen) and their interaction the bone parameters were analyzed using two-way ANOVA, with Tukey's multiple comparisons procedure used to control the overall significance level of the pair-wise comparisons at 5%. Distributions of the outcomes were examined, and transformations of the data were necessary for the analyses: natural logarithm of the trabecular bone parameters and cortical mean polar moment of inertia and the ranks of cortical bone parameters were used for the analyses. Our group sizes had sufficient power (>0.80) to detect a 13.6% difference between any two groups.

RESULTS

ROLE OF PYK2 ON TRABECULAR BONE

To assess the role of Pyk2 on the regulation of bone mass in response to estrogen status, we performed OVX or sham surgeries on 12 week old female WT and Pyk2 KO mice. One group of mice for each genotype also received OVX surgery plus estrogen supplementation (Table 1). After 4 weeks, we performed micro-CT analysis of the distal femoral metaphysis of mice to examine the effects of estrogen and/or Pyk2 status on trabecular bone mass and bone geometry. Specifically, we quantified changes in bone volume to total volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N).

Micro-CT analysis revealed a higher overall bone mass in Pyk2 KO mice than WT mice as expected. Micro-CT analysis also revealed a higher bone mass in Pyk2 KO OVX mice compared to WT OVX mice. Representative micro-CT images of our study groups are shown in Figure 2. The mean ± standard deviation (SD) of the trabecular data is shown in Table 2 and graphically in Figure 3. Analysis of our findings revealed a drastic difference between sham WT and Pyk2 KO mice for BV/TV, Tb.Th, and Tb.N (Figure 3A-D). Similarly, Pyk2 KO OVX and OVX+E2 mice had higher BV/TV, Tb.Th and Tb.N than WT OVX and WT OVX+E2 groups, respectively. Overall, there was a significant effect of OVX on trabecular bone parameters, with lower BV/TV values in OVX animals compared to sham when all genotypes and groups were combined (Table 3). However, in both WT and Pyk2 KO mice, we did not find a statistically significant decrease in BV/TV between sham and OVX surgery groups (Table 4). This can possibly be explained by an unexpectedly high standard deviation, insufficient animal numbers or

not allowing enough time to pass after OVX surgery to produce statistically significant differences in bone geometry. In contrast, there was significantly higher trabecular BV/TV, Tb.Th, and Tb.N after estrogen supplementation, compared to sham or OVX mice for both Pyk2 KO and WT mice (Figure 3A-C and Table 4).

We next examined if the difference in BV/TV between OVX+E2 and OVX was statistical significant between Pyk2 and WT mice. For these studies, we calculated the difference (delta) for Pyk2 KO mice versus WT mice for all trabecular parameters using the analysis (OVX+E2 minus OVX). The results are shown in Table 5. These analyses revealed that the magnitude of the BV/TV, Tb.Th and Tb.N response in Pyk2 KO mice after estrogen supplementation was significantly greater than the effects of estrogen on BV/TV Tb.Th and Tb.N in WT mice. That is, there was a greater increase in trabecular bone parameters for Pyk2 KO mice than WT mice following estrogen supplementation in OVX mice. Together, these findings reveal that Pyk2 KO sham, OVX and OVX+E2 mice have higher trabecular bone mass than WT sham, OVX or OVX+E2 mice, respectively. In addition, Pyk2-KO mice appear to respond better to the effects of estrogen supplementation than WT mice, resulting in a greater increase in bone mass.

ROLE OF PYK2 ON CORTICAL BONE

We next examined the effect of Pyk2 on cortical area (Ct.Ar), cortical thickness (Ct.Th), and polar cross-sectional moment of inertia (CSMI). In both Pyk2 KO and WT mice, we found that estrogen depletion (OVX mice) did not affect Ct.Th, Ct.Ar and CSMI compared to sham mice for each genotype (Table 4 and Figure 4). We also found

that estrogen supplementation in WT mice led to a significant increase in Ct.Th, Ct.Ar compared to either WT sham mice or OVX mice, but sham and OVX were not different from each other. In contrast, in Pyk2 KO mice had significantly higher Ct.Th, Ct.Ar, and CSMI than WT for OVX and sham but Pyk2 and WT did not have significantly different, Ct.Th, Ct.Ar, or CSMI for OVX+E2 (Table 3).

We also examined if there was any change in the difference (delta) between the OVX+E2 and OVX groups for Pyk2 KO and WT mice. The results are shown in Table 5. This data reveals that for cortical bone, the difference between Pyk2 KO and WT groups was significantly different ($p<0.05$) for Ct.Ar, Ct.Th and CSMI, but not for total bone perimeter and tissue perimeter. However, WT mice showed the greater increase in cortical parameters following estrogen supplementation than Pyk2 KO mice. This suggests that in Pyk2 KO mice, cortical bone was more resistant to change in response to estrogen status than WT mice.

In summary, when looking at overall cortical data, it appears that sham Pyk2 KO mice have greater cortical bone parameters compared to sham WT mice, and that cortical bone mass in Pyk2 KO mice is preserved in the presence of low estrogen (OVX) or high estrogen (OVX+E2), suggesting that Pyk2 may play a role in the maintenance of cortical bone mass.

UTERINE WEIGHT AND CIRCULATING ESTRADIOL LEVELS

The uterus is an estrogen-responsive organ and net uterine wet weight increases or decreases in parallel with changes in estrogen levels [60]. Therefore, to monitor the effect

of OVX and OVX+E2 in our mice, we measured uterine weight in Pyk2 KO and WT mice at the time of necropsy.

Overall, Pyk2 animals had higher uterine weights compared to WT. When OVX was performed both mouse genotypes decreased in uterine weight and when supplemented with estrogen, the uterine weights of both mouse genotypes increased.

Changes in circulating serum levels of 17β-estradiol are an alternative measure of the efficacy of OVX and estrogen supplementation. Therefore, we collected whole blood at necropsy and measured 17β-estradiol levels in serum using a commercial enzyme-linked immunosorbant assay (ELISA). No differences were detected in estrogen levels between WT and Pyk2 KO mice receiving sham surgeries or OVX surgeries. Although, estradiol levels were significantly higher in mice supplemented with estrogen, we found no difference in serum estradiol levels between Pyk2 OVX+E2 and WT OVX+E2 groups (Figure 6).

For both uterine weight and serum 17β-estradiol levels, the difference in the effects of estrogen supplementation (OVX+E2) compared to OVX mice was not statistically significant between Pyk2 KO and WT mice (see Table 5).

TABLES

Study group	WT	Pyk2 KO
Sham	10	10
OVX	10	9
OVX + E2	11	10

TABLE 1. Surgical groups for WT and Pyk2 KO mice

A total of 31 WT mice and 29 Pyk2 KO mice at twelve weeks of age were used. Mice received sham surgery, ovariectomy (OVX) plus placebo pellet, or OVX plus 17-β-estradiol pellets (OVX+E2).

Bone	Outcome	Mouse	Estrogen	Mean (SD)
Trabecular	BVTV	Pyk2	OVX	11.7 (6.1)
			OVX+E2	78.3 (15.5)
			Sham	18.3 (5.5)
		WT	OVX	3.4 (2.2)
			OVX+E2	44.9 (22.5)
			Sham	4.5 (1.7)
Trabecular	Tb#	Pyk2	OVX	1.72 (0.86)
			OVX+E2	6.30 (1.35)
			sham	2.50 (0.72)
		WT	OVX	0.71 (0.45)
			OVX+E2	6.19 (2.61)
			sham	0.92 (0.31)
Cortical	Ct. Th	Pyk2	OVX	0.191 (0.016)
			OVX+E2	0.214 (0.036)
			sham	0.200 (0.034)
		WT	OVX	0.165 (0.007)
			OVX+E2	0.200 (0.016)
			sham	0.171 (0.007)
Cortical	T. Ar	Pyk2	OVX	1.00 (0.08)
			OVX+E2	1.10 (0.34)
			sham	1.03 (0.07)
		WT	OVX	0.76 (0.04)
			OVX+E2	0.94 (0.08)
			sham	0.78 (0.07)
Cortical	B. Ar	Pyk2	OVX	0.99 (0.07)
			OVX+E2	1.03 (0.25)
			sham	1.02 (0.07)
		WT	OVX	0.76 (0.04)
			OVX+E2	0.93 (0.07)
			sham	0.78 (0.07)
Cortical	CSMI polar	Pyk2	OVX	0.45 (0.04)
			OVX+E2	0.43 (0.08)
			sham	0.49 (0.14)
		WT	OVX	0.34 (0.05)
			OVX+E2	0.40 (0.03)
			sham	0.34 (0.07)

TABLE 2. Mean and SD data from micro-CT analyses mice.

Calculated bone data for trabecular and cortical bone parameters.

	Outcome	Mouse Comparison			All	OVX	OVX+E2	sham
Trabecular	ln(BVTV)	Pyk2	vs.	WT	<.0001	<.0001	0.0216	<.0001
	ln(Tbth)	Pyk2	vs.	WT	<.0001	<.0001	<.0001	<.0001
	ln(Tb#)	Pyk2	vs.	WT	<.0001	0.0002	0.9976	0.0001
Cortical	Ct. Th	Pyk2	vs.	WT		0.0405	0.7876	0.0474
	rank(T. Ar)	Pyk2	vs.	WT	<.0001	0.0001	0.9939	<.0001
	rank(B. Ar)	Pyk2	vs.	WT	<.0001	<.0001	0.9982	<.0001
	ln(CSMI polar)	Pyk2	vs.	WT	<.0001	0.0049	0.9764	0.0004
	Endocortical Perimeter	Pyk2	vs.	WT	<.0001			
Non-Bone	Uterine Weight	Pyk2	vs.	WT	0.0371			
	Estrogen	Pyk2	vs.	WT	0.7515			

TABLE 3: Statistical Analysis of micro-CT data for WT and Pyk2 KO Mice

Chart comparing mouse to mouse interactions between WT and Pyk2 KO mice.
This chart shows comparisons between WT and Pyk2 KO mice to demonstrate difference in bone density parameters which can be attributed to the mouse genotype. Darkened boxes indicate statistical significance (p-value < 0.05).

					p-values		
Bone	Outcome	Surgery Comparison			All	Pyk2	WT
Trabecular	ln(BVTV)	OVX	vs.	OVX+E2	<.0001	<.0001	<.0001
		OVX	vs.	sham	0.0074	0.1472	0.3780
		OVX+E2	vs.	sham	<.0001	<.0001	<.0001
Trabecular	ln(Tbth)	OVX	vs.	OVX+E2	<.0001	<.0001	<.0001
		OVX	vs.	sham	0.1385	0.4592	0.9445
		OVX+E2	vs.	sham	<.0001	<.0001	<.0001
Trabecular	ln(Tb#)	OVX	vs.	OVX+E2	<.0001	<.0001	<.0001
		OVX	vs.	sham	0.0135	0.2347	0.4154
		OVX+E2	vs.	sham	<.0001	0.0002	<.0001
Cortical	Ct. Th	OVX	vs.	OVX+E2		0.7166	0.0095
		OVX	vs.	sham		0.2608	0.5395
		OVX+E2	vs.	sham		0.7876	0.0115
Cortical	rank(T. Ar)	OVX	vs.	OVX+E2	0.0518	0.9860	0.0015
		OVX	vs.	sham	0.7423	0.9906	0.9978
		OVX+E2	vs.	sham	0.2380	0.8000	0.0062
Cortical	rank(B. Ar)	OVX	vs.	OVX+E2	0.0518	0.9747	0.0011
		OVX	vs.	sham	0.8003	0.9977	0.9973
		OVX+E2	vs.	sham	0.1991	0.8328	0.0049
Cortical	ln(MMI polar)	OVX	vs.	OVX+E2	0.7374	0.9056	0.2709
		OVX	vs.	sham	0.8936	0.9813	1.0000
		OVX+E2	vs.	sham	0.9570	0.5081	0.2345
Non-Bone	Uterine Weight	OVX	vs.	OVX+E2	<.0001		
		OVX	vs.	sham	<.0001		
		OVX+E2	vs.	sham	<.0001		
Non-Bone	Estrogen	OVX	vs.	OVX+E2	0.0045		
		OVX	vs.	sham	0.9923		
		OVX+E2	vs.	sham	0.0046		

TABLE 4. Statistical Analysis of effects of estrogen on WT and Pyk2 KO OVX mice.

This chart shows comparisons between WT and Pyk2 KO mice surgical groups to assess the effects of estrogen bone density**.** Darkened boxes denote statistical significance (p-values < 0.05).

Statistical significance of difference (delta) between WT and Pyk2 KO mice
Comparison of (OVX+E2 - OVX) for Pyk2 versus (OVX+E2 - OVX) for WT

		Difference (SE)	p-value
Trabecular	ln(BVTV)	-0.63 (0.29)	0.0329
Trabecular	ln(Tb.Th)	0.21 (0.06)	0.0010
Trabecular	ln(Tb.N)	-0.85 (0.27)	0.0027
Cortical	rank(T. Ar)	-25.86 (7.73)	0.0015
Cortical	rank(B. Ar)	-26.95 (7.74)	0.0010
Cortical	ln(CSMI polar)	-0.24 (0.10)	0.0294
Cortical	Cortical Bone Perimeter	-0.28 (0.41)	0.5027
Cortical	Tissue Perimeter	-0.24 (0.13)	0.0766
Non-Bone	Uterine Weight	9.40 (19.03)	0.6234
Non-Bone	Estrogen	-5.31 (15.78)	0.7396

TABLE 5. Differences in the effects of estrogen supplementation on trabecular and cortical bone parameters for WT versus Pyk2 KO mice.

Comparison of the differences calculated between surgical groups and mouse genotypes. Table shows the difference between data groups for Pyk2 KO versus WT mice for the OVX+E2 minus OVX groups for all parameters. The differences (delta) were found to be statistically significant between genotypes for BV/TV, Tb.Th, TbN (bigger difference for Pyk2 KO mice) and for cortical analysis of T.Ar, B.Ar and CSMI (bigger difference for WT mice) **(**$p<0.05$).

FIGURES

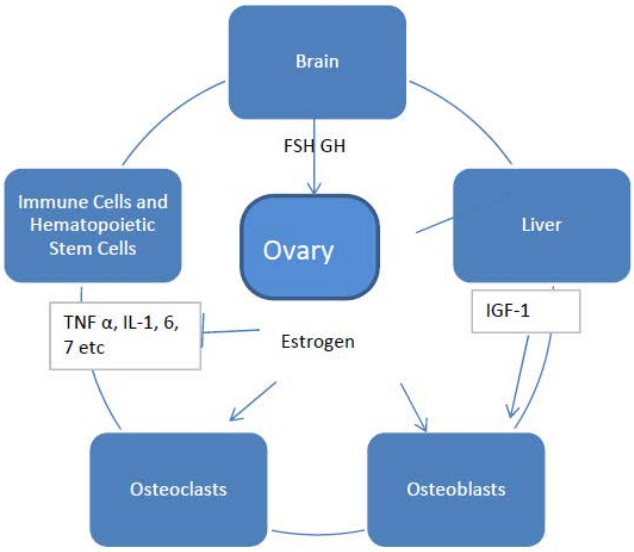
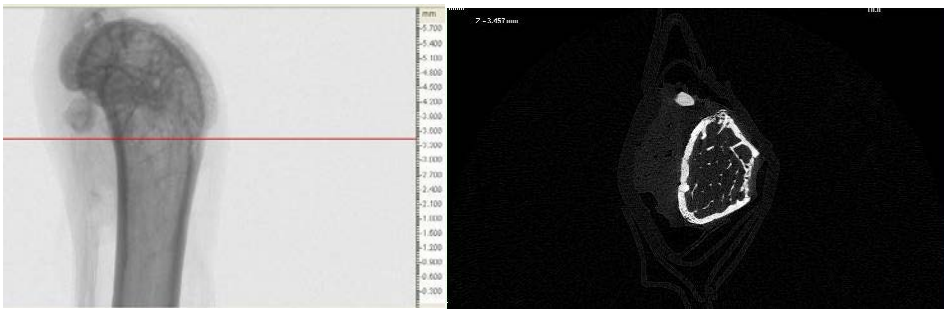


FIGURE 1. Schematic illustration of the role of estrogen on bone remodeling.

The pituitary gland secretes follicle-stimulating hormone (FSH) which stimulates estrogen secretion by the ovaries. Estrogens inhibit FSH secretion through negative feedback regulation mediated by the pituitary ER. After menopause, estrogen deficiency increases FSH secretion which causes the bone marrow and immune cells to release cytokines, interleukins (IL) and tumor necrosis factor (TNF). These cause the osteoclasts to differentiate from hematopoietic stem cells. In contrast, estrogen and growth hormone (GH) from the pituitary gland cause the liver to release insulin-like growth factor 1 (IGF-1) which causes the osteoblasts to differentiate. [14]

A.



B.

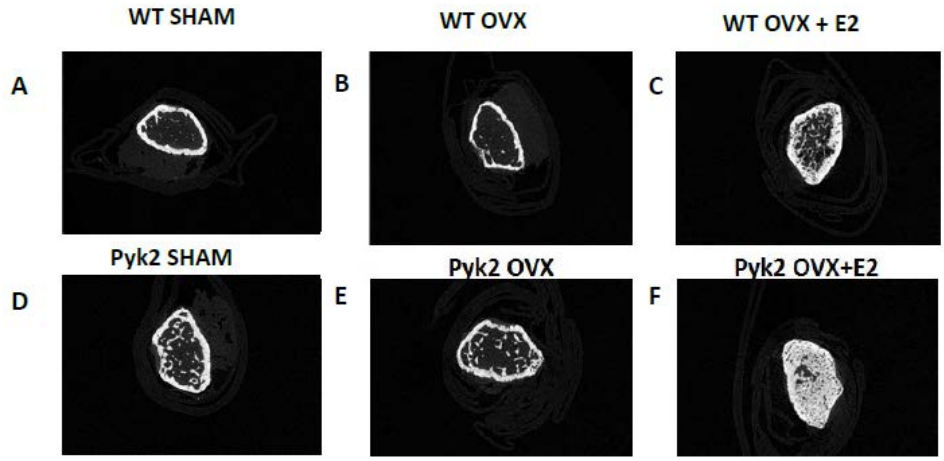


FIGURE 2. Representative micro-CT images of trabecular bone in WT and Pyk2 KO mice

Micro-CT studies were performed on the distal femoral metaphysis. Scans were acquired using an x-ray source set at 60kV over an angular range of 180 degrees (rotational steps of 0.7 degrees) with a 6 µm pixel size. **A.** Representation of region of interest used for analysis of trabecular bone. **B.** Projection images of bone slices from each study group are shown and were generated by image reconstructed using standard Skyscan software.

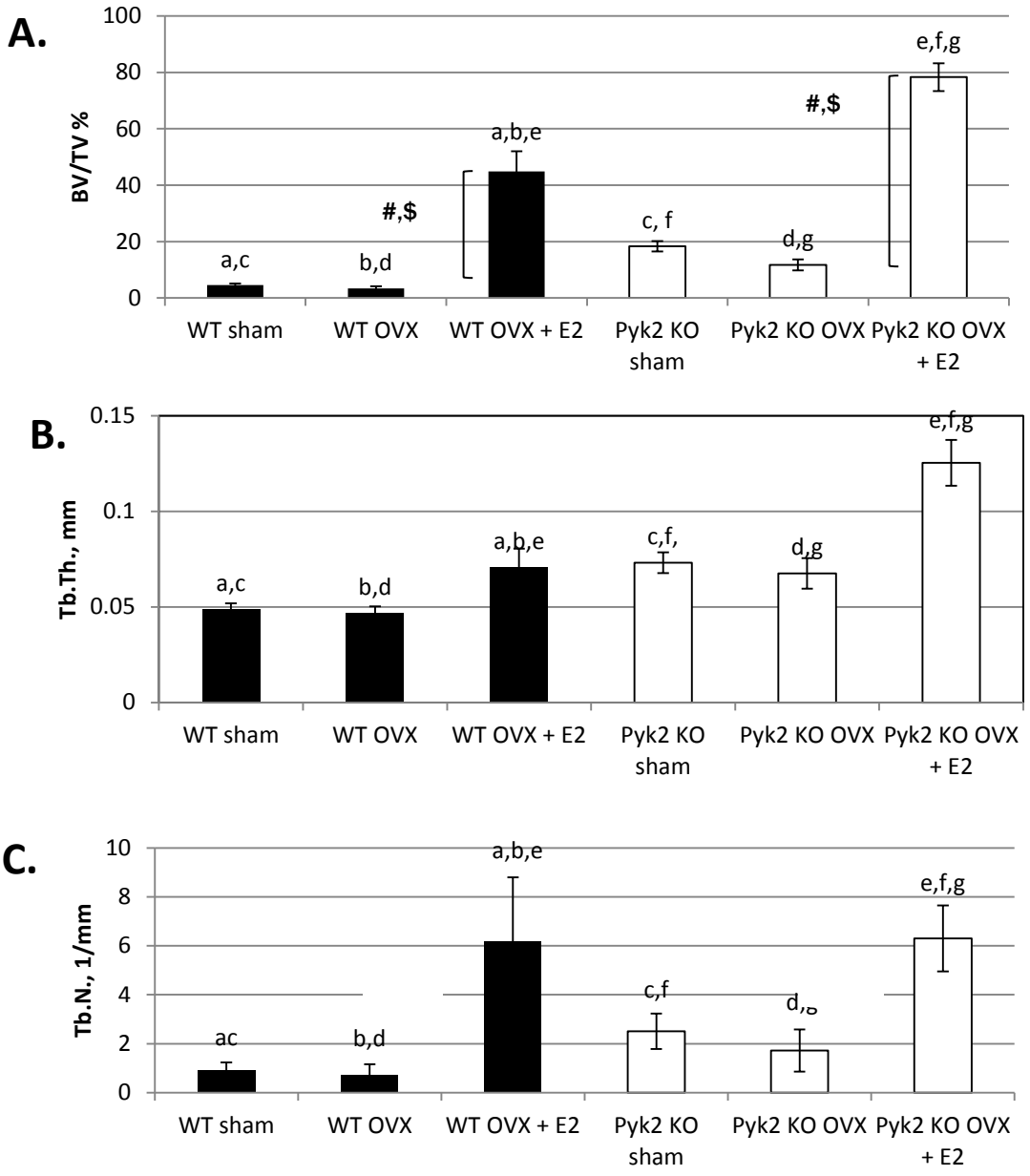


FIGURE 3. Micro-CT analysis of trabecular bone in WT and Pyk2 KO mice.

Trabecular bone parameters were measured by micro-CT on WT and Pyk2 KO 16 week old mice. **A.** Bone volume/total volume (BV/TV). **B.** Trabecular thickness (Tb.Th.). **C.** Trabecular number (Tb.N.). Results are mean ± SD from female mice (n=9 WT sham, 10 WT OVX, 10 WT OVX+E2) (n=10 Pyk2 KO sham, 10 Pyk2 KO OVX, 10 Pyk2 KO OVX+E2). $p<0.05$ using 2-way ANOVA for genotypes/groups (a-g). The relative change in BV/TV for Pyk2 KO OVX+E2 (#) relative to OVX ($) or sham was significantly greater than the change in BV/TV calculated for WT mice (see D on next page).

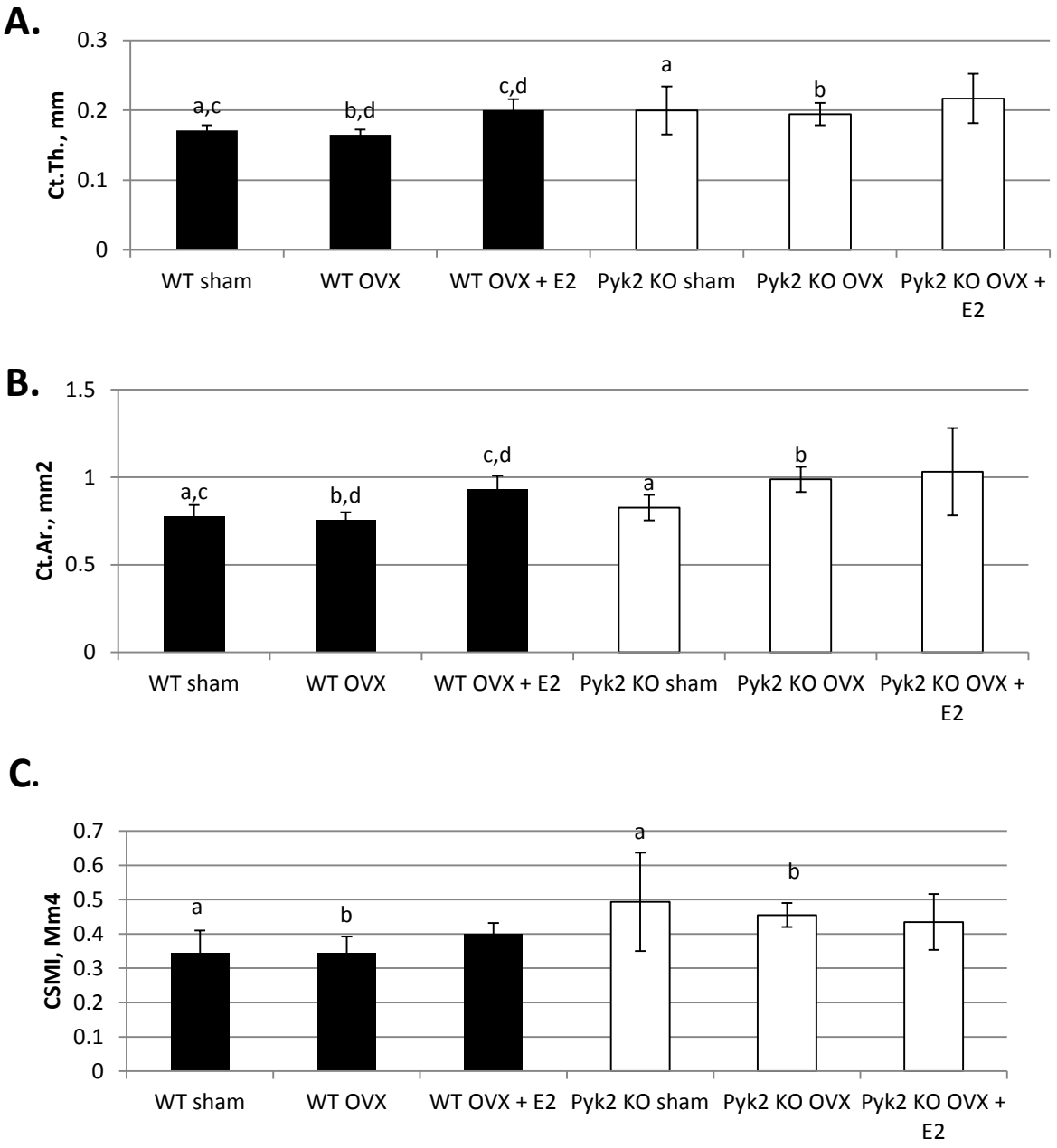
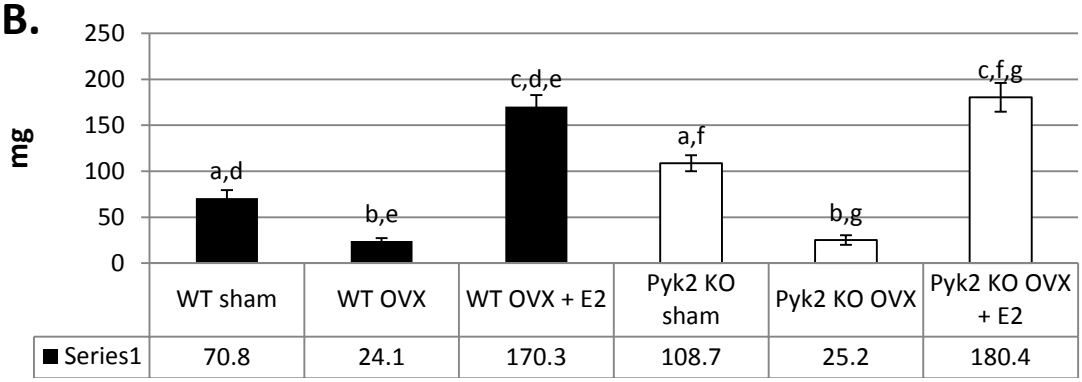


FIGURE 4. Effect of OVX and estrogen on cortical bone mass.

Micro-CT analysis of cortical bone in WT and Pyk2 KO mice 4 weeks after surgery. **A.** Cortical thickness (Ct.Th.). **B**. Mean total cross sectional area(Ct.Ar). **C.** Mean polar moment of inertia (CSMI)**.** Graph C is shown on following page. All graphs are mean ± SD. N=9 WT sham, 10 WT OVX, 10 WT OVX+E2. N=10 Pyk2 KO sham, 10 Pyk2 KO OVX, 10 Pyk2 KO OVX+E2). $p<0.05$ using 2-way ANOVA for genotypes/groups. Significance indicated by symbols "a-e". **C.** Mean polar moment of inertia (CSMI**).**

A.

B.



C.

	Mean ± SEM	% change within genotype relative to sham ± SEM
WT sham	70.8 ± 8.6	100 ± 12.1
WT OVX	24.1 ± 3.1	34.0 ± 4.3
WT OVX + E2	170.3 ± 12.5	240.5 ± 17.6
Pyk2 KO sham	108.7 ± 8.7	100 ± 8.0
Pyk2 KO OVX	25.2 ± 5.3	23.2 ± 4.9
Pyk2 KO OVX + E2	180.4 ± 15.8	166 ± 14.5

FIGURE 5. Effect of OVX and estrogen on uterine weight

The uteri of Pyk2 KO and WT mice were harvested at the time of necropsy and weighed. **A.** Representative photographs of uteri from Pyk2 KO mice. **B.** The average net wet weight of uteri (mg) ± standard error of the mean (SEM) for each experimental group. N =9 WT sham, 10 WT OVX, 10 WT OVX + E2. N=10 Pyk2 KO sham, 10 Pyk2 KO OVX, 10 Pyk2 KO OVX+E2. $p<0.05$ using 2-way ANOVA for genotypes/groups as indicated (a-e). Statistically significant differences in uterine weight were seen between WT and Pyk2 KO mice, and between sham and OVX +E2 groups. **C.** Percent (%) increase in uterine weight for WT and Pyk2 KO mice. Statistical significance denoted by asterisk.

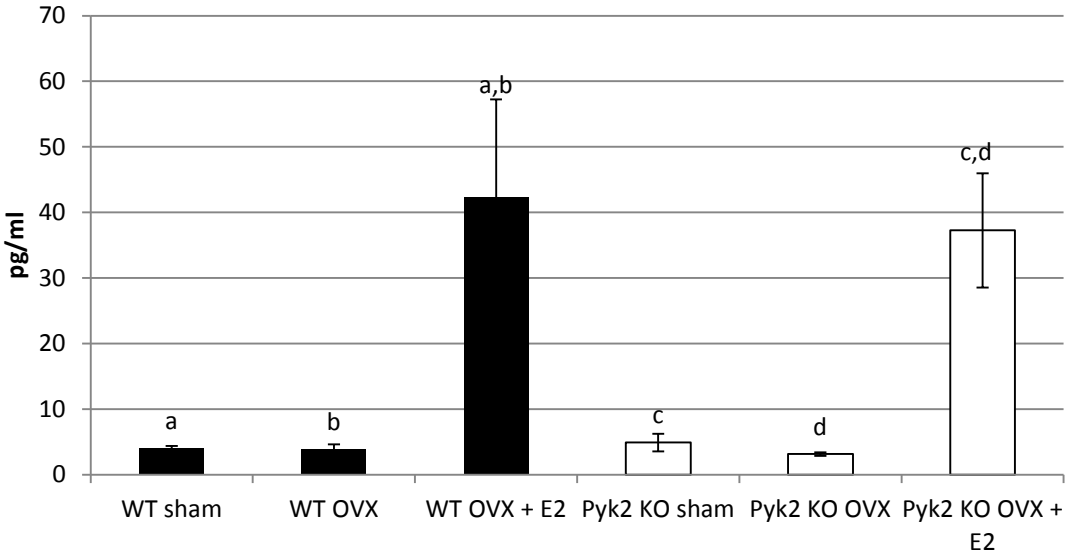


FIGURE 6: Serum estrogen levels in WT and Pyk2 KO mice.

Blood sera from WT and Pyk2 KO mice were assayed for total estrogen levels by ELISA. The graph is mean ± SD of results. For WT mice, n=9 sham, 10 OVX and 10 OVX+E2. For Pyk2 KO mice, n=10 sham, 10 OVX and 10 OVX+E2. $p<0.05$ using 2-way ANOVA of genotypes/groups. Statistical significance for multiple comparisons is indicated by "a-d".

DISCUSSION

The fine balance between bone formation and resorption occurs by the carefully modulated interactions of osteoclasts and osteoblasts, and these interactions are influenced by many agents, including endocrine and paracrine factors. Alterations in bone cells or the factors that regulate them can yield pathologic conditions of bone such as post-menopausal bone loss. Declining estrogen levels during menopause yield both decreases in the osteoblast cell lineage and bone formation, as well as increases in osteoclast differentiation and bone-resorbing activity due to increased life-span. Overall, this leads to a reduction in bone mass and consequently, osteoporosis which makes bones more susceptible to fracture. Therefore, understanding the mechanisms by which bone loss occurs may yield more finely targeted therapeutic approaches to prevent bone loss and replace bone.

Previous studies from our laboratory have revealed that Pyk2, a non-receptor tyrosine kinase, plays an important role in the regulation of osteoclasts and osteoblasts. In the current study, we found that female Pyk2 KO mice, which started with greater bone mass, had an overall higher bone mass after OVX than WT OVX mice. Hence, the increase in high bone mass of Pyk2 KO mice would be predicted to provide some protective effect against some of the negative sequelae of bone loss, such as increased bone fragility. In addition, we found that the Pyk2 KO mice were more responsive to estrogen supplementation than WT mice, resulting in a greater increase in bone mass following estrogen supplementation than WT mice. These findings suggest that Pyk2 regulates bone mass in part by modulating estrogen signaling cascades. Although the molecular mechanism for the role of Pyk2 in estrogen signaling is still unknown, correlations can be drawn between our study and the study of Sims et al., (2002) using

single or double ER knockout mice (ERα, ERβ or ERαβ KO mice) [31]. It was reported that ERα and ERβ have compensatory actions in the skeleton of female mice and both receptors influence bone remodeling. In female mice, deletion of ERα leads to an increase in trabecular bone volume. However, female mice lacking ERβ exhibited a higher BV/TV levels than both WT and ERα KO mice. Moreover, in female mice lacking both ERα and ERβ (double KO) BV/TV was lower than WT mice. In contrast, the bone density of male mice is primarily affected by deletion of ERα [31]. In the Sims study, male mice lacking ERα had higher BV/TV levels than WT mice, while deletion of ERβ left bone unchanged and mice lacking both ERα and ERβ (double KO) had an intermediate bone phenotype that was higher than WT mice [31]. ERβ is expressed in trabecular bone and ERα is primarily expressed in cortical bone [29, 30]. Given that we found marked changes in trabecular bone volume in Pyk2 KO mice in response to estrogen, but little effect on cortical bone parameters, these findings suggest that Pyk2 potentially acts downstream of ERβ signaling pathways in trabecular bone. This is also supported by our unpublished findings which reveal that osteopetrosis (high bone mass) in Pyk2 KO mice was detected in female mice, but not male mice.

As was shown in our study and previously reported [1, 3], Pyk2 KO mice have higher BV/TV, trabecular thickness, and trabecular number than WT mice. Buckbinder *et al.* investigated the possible role of Pyk2 inhibitors in the protection of post-menopausal bone loss. OVX rats were injected for 21 days with either vehicle, a small molecule inhibitor of Pyk2 (PF-431396), or an anti-resorptive agent. Micro-CT analysis of the distal femur of these rats revealed that the Pyk2 inhibitor preserved bone density in

OVX rats, compared to controls. Consistent with this finding, our current studies reveal that Pyk2 KO mice have a higher BV/TV, trabecular thickness and trabecular number than WT mice. However, we were unable to detect a statistical significant change in trabecular bone mass after OVX for either Pyk2 KO or WT mice, when compared to sham mice, most likely due to insufficient passage of time following OVX. When supplemented with estrogen after OVX, we found that both Pyk2 KO mice and WT mice demonstrated a higher trabecular bone mass than sham or OVX mice of the same genotype. Importantly, we also determined whether the difference (delta) between OVX+E2 and sham was different for Pyk2 KO and WT mice. These findings revealed that the magnitude of the bone mass increase in estrogen-supplemented mice was greater for Pyk2 KO mice than WT mice, indicating they are more responsive to estrogen

Pyk2 KO mice also demonstrated higher cortical bone volume parameters than WT mice when comparing OVX groups or sham groups. However, cortical bone for either Pyk2 KO or WT mice did not show statistically significant changes in bone morphometric parameters when exposed to estrogen supplementation, suggesting cortical bone may be more resistant to hormonal changes. Another possible explanation for the lack of changes in cortical bone after estrogen supplementation could be due to insufficient passage of time to allow for detectable changes in bone parameters. Indeed other research groups have used 6 weeks post-OVX mice to study bone morphometrics, again suggesting additional time may have yielded more significant results [41] Of interest, cortical bone changes in humans are detected several years after trabecular bone changes are noted [12].

As a measure of the in vivo effects of estrogen, we examined uterine weights after necropsy. Uterine weights were greater in Pyk2 KO mice than WT mice, implying basal gonadal dysfunction or changes in female sex-hormones in Pyk2 KO mice. Unexpectedly, we were unable to detect a difference in circulating estradiol levels in Pyk2 KO and WT sham mice, perhaps due to the limits of sensitivity of our estradiol assay. Nevertheless, when both Pyk2 KO and WT mice were given estrogen supplementation, uterine weight increased in both mice groups, but no statistically significant difference between the WT and Pyk2 KO mice was detected. Consistent with this latter finding, there were no significant differences in circulating estradiol levels between the WT and Pyk2 KO mice. In both Pyk2 KO and WT mice, estrogen supplementation unexpectedly resulted in a supra-physiological level of serum estradiol. Although mice were given extended slow-release estrogen pellets, the high estradiol levels could be attributed to the timing of when the pellet actually released a bolus.

The results of this study suggest a link between estrogen and the regulation of bone mass by Pyk2. In other unpublished studies, we also found that Pyk2 KO female mice have a higher bone mass than Pyk2 KO male mice, when compared to WT mice of the same sex, suggesting that ovarian dysfunction and estrogen levels may in part explain the high bone mass phenotype of Pyk2 KO female mice. However, many questions still remain regarding the role of Pyk2 on estrogen signaling. Future studies are planned to investigate if increasing the duration of time between OVX and necropsy will allow for greater changes in trabecular and cortical bone parameters, uterine weight, and estrodiol levels. In addition, increasing the number of mice used in the study may clarify some results. Though we used estrogen supplementation doses reported in the literature, we

detected supra-physiologic levels of estradiol in OVX+E2 mice at necropsy. Therefore, in future studies, a lower dose of estrogen supplementation will be used and we will measure serum estrodiol levels weekly. Histological analysis of our bone samples is also planned to assess how osteoclast and osteoblast numbers and bone turnover rate are affected by Pyk2 KO and estrogen status. We anticipate estrogen supplementation would increase osteoblast numbers and differentiation and decrease osteoclast numbers by inhibiting osteoclast differentiation. Because Pyk2 is expressed in neural and hematopoietic tissues, and other tissues that impact bone, many endocrine or paracrine factors may be influencing the results of the current study. Future studies will also involve the use of conditional Pyk2 KO mice which lack Pyk2 in either osteoclasts, osteoblasts, or osteocytes. This will allow us to better investigate the bone cell specific effect of Pyk2 in response to high and low levels of estrogen.

In summary, our results demonstrate a strong connection between Pyk2 and the control of bone mass by estrogen in mice. Furthermore, our studies suggest that Pyk2 inhibitors in combination with estrogen supplementation may provide a new potential therapy to prevent the devastating effects of post-menopausal bone loss and osteoporosis.

SUMMARY AND CONCLUSIONS

This study was conducted to determine the role of Pyk2 on estrogen-regulated bone mass. We hypothesized that Pyk2 KO mice would be protected from bone loss associated with ovariectomy and that when supplemented with estrogen would have increased bone density compared to WT ovariectomized mice. Twelve week old WT and Pyk2 KO mice were subject to sham surgery, OVX + placebo, and OVX + E2 pellet implantation. Mice were sacrificed at 16 weeks of age, and micro-CT analysis of the distal femoral metaphysis was performed. Bone was analyzed for BV/TV, trabecular number, trabecular thickness, cortical thickness, cortical cross-sectional bone area, endocortical perimeter, and mean polar moment of inertia. Our results confirmed that Pyk2 KO mice had significantly higher bone volume than WT mice. Pyk2 KO mice had greater bone volume compared to WT mice after ovariectomy and showed a greater increase in bone density after estrogen supplementation than WT mice. Our studies did not show cortical bone changes seen in other published studies [61] however, this could be attributed to an inadequate amount of time between OVX and sacrifice. In summary, our study revealed a connection between estrogen status and its effects on bone morphometrics in Pyk2 KO mice. The clinical implications of these findings are that pharmacological strategies to inhibit Pyk2 may yield new approaches for the management of post-menopausal bone loss.

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APPENDIX

Abbreviation	legend
BV/TV	bone volume to total volume
CSMI	polar cross-sectional moment of intertia
Ct. Ar	cortical area
Ct. Th	cortical thickness
E2	estrogen
ELISA	enzyme-linked immunosorbant assay
ER α	estrogen receptor alpha
ER β	estrogen receptor beta
FAK	focal adhesion kinase
FSH	follicle stimulating hormone
IGF 1	insulin-like growth factor 1
KO	knock out
M-CSF	macrophage colony stimulating factor
micro-CT	micro-computed tomography
OPG	osteoprotegerin
OVX	ovariectomized
RANK L	receptor activator of nuclear factor κβ ligand
SERMS	selective estrogen receptor modulators
Tb.N	trabecular number
Tb.Th	trabecular thickness

ABSTRACT

Pyk2: Potential Regulator of Post-Menopausal Bone Loss

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Osteoporosis is a pathologic condition of bone, commonly found in post-menopausal women, which occurs from an imbalance between bone formation and resorption. Following menopause, the bone resorbing activity of osteoclasts exceeds bone formation by osteoblasts, resulting in decreased trabecular and cortical bone and a subsequent decrease in bone mass. Reduced bone mass increases the risk of pathologic fracture of bones. Due to adverse effects associated with current treatment protocols for bone loss, alternative treatment modalities with reduced adverse effects are needed.

Estrogen plays a role in maintaining balance in the bone remodeling cycle by controlling remodeling activation, osteoblast and osteoclast numbers, and their respective effectiveness in formation and resorption. With declining estrogen levels, this elegantly balanced interaction is altered and bone resorption exceeds bone formation, resulting in bone loss and increased bone fragility.

Pyk2 is a protein tyrosine kinase that plays an important role in regulating bone resorption by osteoclasts, as well as osteoblast proliferation and differentiation. Deletion of the *Pyk2* gene in mice leads to an increase in bone mass, in part due to dysfunctional osteoclast and osteoblast activity. In this study, we examined the role of Pyk2 in the effects of estrogen on bone mass. We used wild type (WT) and Pyk2 knock-out (KO)

mice that had been ovariectomized (OVX) and treated with or without estrogen (E_2)-releasing pellets. Control mice included sham OVX surgery receiving placebo pellet. We found that deletion of Pyk2 conferred increased bone mass in sham, OVX and OVX+E2 mice. In addition, Pyk2 KO mice supplemented with 17β–estradiol exhibited a marked increase in bone volume/trabecular volume, trabecular number, and trabecular thickness, but not cortical bone parameters compared to WT mice. Results of this study provide evidence for the role of Pyk2 in the effects of estrogen on bone mass. Understanding the role of Pyk2 in bone could lead to the development of new pharmaceutical targets for the treatment of bone loss associated with osteoporosis.

CURRICULUM VITAE

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Education

- Orthodontics Residency, July 2011-present, IU School of Dentistry, Indianapolis, IN.
- AEGD: 1999-2000, United States Air Force, Scott AFB, Belleville, IL
- DDS: 1995-1999, Indiana University School of Dentistry, Indianapolis, IN
- BS Biology: 1989-2003, Indiana University, Bloomington, IN

Professional Experience

- Research Volunteer, 2010-2011, Department of Oral Biology, IU School of Dentistry, Indianapolis, IN.
- Independent Contractor, 2008-2011, MiraVista Diagnostics, Indianapolis, IN.
- Temporary Dentist, 2009-2011, Dental Medical Support Services, Indianapolis, IN.
- Dentist, 2004- 2006, Marion County Health Department, Indianapolis, IN.
- Temporary Dentist, 2003- 2004, Dental Support Services, Indianapolis, IN.
- Dentist, 2000- 2003, United States Air Force, RAF Lakenheath AFB, Lakenheath, England.
- AEGD Resident, 1999- 2000, United States Air Force, Scott Air Force Base, Belleville, IL.
- Laboratory Technician, 1993- 1995, Histoplasmosis Reference Laboratory, Indianapolis, IN.

Recognitions and Achievements:

- Dean's Silver Award for National Board Scores
- Dean's List throughout dental school
- Graduated with Distinction (graduated within the top 10)
- Omicron Kappa Upsilon Honor Dental Society
- HPSP Scholarship Recipient, USAF
- Air Force Commendation Medal, Meritorious Service, 2000-2003
- Junior Officer of the Quarter, USAF

Professional Organizations:

- American Association of Orthodontists
- American Dental Association