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Pyk2 and Megakaryocytes Regulate Osteoblast Differentiation and Migration via Distinct and Overlapping Mechanisms

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Abstract

Osteoblast differentiation and migration are necessary for bone formation during bone remodeling. Mice lacking the proline-rich tyrosine kinase Pyk2 (Pyk2-KO) have increased bone mass, in part due to increased osteoblast proliferation. Megakaryocytes (MKs), the platelet-producing cells, also promote osteoblast proliferation *in vitro* and bone-formation *in vivo* via a pathway that involves Pyk2. In the current study, we examined the mechanism of action of Pyk2, and the role of MKs, on osteoblast differentiation and migration. We found that Pyk2-KO osteoblasts express elevated alkaline phosphatase (ALP), type I collagen and osteocalcin mRNA levels as well as increased ALP activity and mineralization, confirming that Pyk2 negatively regulates osteoblast function. Since Pyk2 Y402 phosphorylation is important for its catalytic activity and for its protein-scaffolding functions, we expressed the phosphorylation-mutant ($Pyk2^{Y402F}$) and kinase-mutant ($Pyk2^{K457A}$) in Pyk2-KO osteoblasts. Both $Pyk2^{Y402F}$ and $Pyk2^{K457A}$ reduced ALP activity, whereas only kinase-inactive $Pyk2^{K457A}$ inhibited Pyk2-KO osteoblast migration. Consistent with a role for Pyk2 on ALP activity, co-culture of MKs with osteoblasts led to a decrease in the level of phosphorylated Pyk2 (pY402) as well as a decrease in ALP activity. Although Pyk2-KO osteoblasts exhibited increased migration compared to WT osteoblasts, Pyk2 expression was not required for the ability of MKs to stimulate osteoblast migration. Together, these data suggest that osteoblast differentiation and migration are inversely regulated by MKs via distinct Pyk2-dependent and independent signaling pathways. Novel drugs that distinguish between the kinase-dependent or protein-scaffolding functions of Pyk2 may provide therapeutic specificity for the control of bone-related diseases.

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Keywords

bone formation; alkaline phosphatase; mineralization tyrosine kinase; phosphorylation

INTRODUCTION

Bone remodeling is controlled by the actions of the bone-degrading osteoclasts and bone-forming osteoblasts [Delaisse, 2014; Florencio-Silva et al., 2015]. Bone formation at eroded bone surfaces begins with the recruitment of osteoblast precursors which undergo differentiation and maturation resulting in the secretion of a collagen matrix which is later mineralized by osteoblasts and osteocytes [Atkins et al., 2011; Ducy et al., 2000]. Although several key proteins involved in osteoblast function have been identified, much remains to be understood about the process of osteoblast recruitment, migration and differentiation into mature bone-forming cells.

The proline-rich tyrosine kinase Pyk2 is a member of the family of focal adhesion kinases. Our studies, and those of others, have reported the importance of the Pyk2 in bone remodeling *in vivo* [Avraham et al., 1995; Buckbinder et al., 2007; Cheng et al., 2013] and in bone cell function *in vitro* [Bruzzaniti et al., 2009; Duong et al., 2001; Eleniste and Bruzzaniti, 2012; Lakkakorpi et al., 2003; Wang et al., 2003]. Global deletion of the *Pyk2* gene in mice (Pyk2-KO mice) results in a high bone mass phenotype, which is in due to increased osteoblast activity [Buckbinder et al., 2007; Cheng et al., 2013] as well as decreased osteoclast activity [Gil-Henn et al., 2007]. Studies in osteoclasts reveal that Pyk2 localizes to the actin-rich podosome ring (sealing zone) and promotes osteoclast maturation and bone resorption [Bruzzaniti et al., 2009; Duong et al., 2001; Gil-Henn et al., 2007; Miyazaki et al., 2004; Zhang et al., 2002]. Upon integrin engagement and in response to increased intracellular Ca^{2+} levels, Pyk2 is auto-phosphorylated at tyrosine residue Y402 [Duong et al., 1998; Park et al., 2004; Sanjay et al., 2001]. Tyrosine 402 phosphorylation is necessary for the maximal catalytic activity of Pyk2 [Duong et al., 1998; Park et al., 2004; Sanjay et al., 2001]. In addition, phosphorylation of Pyk2 at Y402 forms a consensus binding site for SH2-containing proteins [Dikic et al., 1996] and is involved in the activation of downstream signaling cascades [Avraham et al., 2000; Dikic et al., 1996; Faccio et al., 2003]. Indeed, Pyk2 autophosphorylation, but not its kinase activity, has been shown to be important for the adhesion-induced association of Pyk2 with c-Src in osteoclasts [Lakkakorpi et al., 2003; Miyazaki et al., 2004]. Thus, Pyk2 cellular function goes beyond its kinase activity.

In osteoblasts, the actions of Pyk2 are not well characterized. We reported that the proliferation of osteoblasts is increased in cultures containing megakaryocytes (MKs), the blood cells responsible for the production of thrombocytes [Ciovacco et al., 2010; Kacena et al., 2012; Kacena et al., 2004]. The increase in osteoblast proliferation by MKs *in vitro* is mediated, in part, by increased expression of the *Pyk2* gene as well as the activation of integrin receptors. Consistent with these findings, MKs did not increase the bone mass of Pyk2−/− mice, suggesting the important role of Pyk2 in the MK-induced increase in osteoblast proliferation and bone volume [Cheng et al., 2013]. In the current study, we

examined further the mechanism of action of Pyk2 in osteoblast differentiation and migration. We focused on the role of Pyk2 kinase activity and Y402 phosphorylation on osteoblast differentiation and migration. In addition, we examined the effect of MKs on Pyk2 Y402 phosphorylation in osteoblasts, and the role of MKs on osteoblast migration in the presence or absence of Pyk2.

MATERIALS AND METHODS

MATERIALS

Antibodies used for Western blot analyses were obtained from Transduction Laboratories (Lexington, KY). An antibody to phosphorylated Pyk2-Y402 was obtained from Biosource International. Anti-hemagglutinin (anti-HA) to detect transfected Pyk2 was from Bethyl Laboratories (Montgomery, TX). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Fisher Scientific (Fairlawn, NJ). Phenylarsine oxide (PAO) was purchased from Sigma-Aldrich (St. Louis, MO).

PREPARATION OF NEONATAL CALVARIAL CELLS

All animals were maintained, bred and handled following the guidelines of the American Association for Laboratory Animal Science using Institutional Animal Care and Use Committee (IACUC) approved protocols and in according with the NIH (Guide for the Care and Use of Laboratory Animals, 1996). Murine calvarial cells from C57BL/6 (WT) and Pyk2−/− (Pyk2-KO) mice were prepared using a modification of a previous protocol [Jilka and Cohn, 1981; Simmons et al., 1982; Wong and Cohn, 1975]. Briefly, calvaria from neonatal mice were incubated with EDTA in PBS for 30 min. The calvaria were subjected to sequential collagenase digestions and fractions 3–5 were collected. These cells were assessed by a variety of criteria to be over 95% enriched for osteoblasts or osteoblast precursors [Boutahar et al., 2004; Guignandon et al., 2006; Richardson and Parsons, 1996]. Osteoblast cultures were expanded and further enriched by passaging 3 times and aliquots were frozen in liquid nitrogen until required. For experimentation, osteoblasts were thawed and allowed to recover for 3 days before being seeded at $2x10^4$ cells per well in 6-well plates.

PREPARATION OF MEGAKARYOCYTES

MKs were prepared as previously described [Kacena et al., 2006; Kacena et al., 2004]. Briefly, embryonic E13–15 fetuses were dissected from pregnant WT mice, fetal livers were removed and single cell suspensions were generated. Cells were washed and then seeded in 100 ml culture dishes in Dulbecco modified eagle medium (DMEM) supplemented with 10% FCS and 1% murine thrombopoietin [Villeval et al., 1997]. MKs were isolated 5–7 days later by separating them from the lymphocytes and other cells using a one-step albumin gradient to obtain a 90–95% pure MK population [Drachman et al., 1997].

GENERATION OF RECOMBINANT ADENOVIRUSES

Recombinant adenoviruses expressing active WT Pyk2 ($Pyk2^{WT}$), kinase-inactive $Pyk2^{K457A}$ and $Pyk2^{Y402F}$ were previously described [Gil-Henn et al., 2007; Sanjay et al., 2001]. Briefly, pShuttle plasmids (Qbiogene) containing Pyk2 were used for infection of

primary osteoblasts. Recombinant adenoviruses expressing WT Pyk2 or its mutants were prepared by recombination of the above plasmids using the Adenovator Vector System (Qbiogene) according to the manufacturer's instructions. Adenoviruses with similar MOIs (multiplicity of infection) were used. Infection was performed for 3 days which showed maximal protein expression. Western blot analysis was used to confirm expression levels of the Pyk2 proteins in osteoblasts after 3 days of infection.

IMMUNOPRECIPITATION ASSAYS

Immunoprecipitation (IP) assays were performed as previously described [Bruzzaniti et al., 2009; Eleniste et al., 2012]. Cells were lysed in buffer containing 50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin). Lysates were sonicated and the supernatant clarified by centrifugation. IPs were performed with protein G-agarose beads for 1 h. After binding and washing, the samples were heated to 100°C in sample buffer and resolved by SDS-PAGE. Western blot analysis was performed using the enhanced chemiluminescence detection kit (Amersham) with multiple exposure times. The phospho-Y402 antibody was specific for Pyk2 as determined by Western blot analysis of immunoprecipitated Pyk2 and did not detect immunoprecipitated mutant $Pyk2^{Y402F}$. All experiments were performed a minimum of three times, and representative blots or images are shown as indicated. The intensity of protein bands in Western blots were analyzed and normalized by densitometric analysis using NIH-ImageJ software.

QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using RNAeasy kit (Qiagen). DNAse (Qiagen)-treated RNA was used to generate cDNA by reverse transcription according to the manufacturer's instructions (SuperScript II kit; Invitrogen). PCR reactions were performed in an MX3000 detection system using SYBR green PCR reagents following the manufacturer's instructions. For each gene, a calibration curve was performed. All oligonucleotide primers were tested to ensure correct specificity and sensitivity. For each sample, arbitrary units obtained using the standard curve and the expression of GAPDH was used to normalize the amount of the investigated transcript. The following primer sequences were used:

Alkaline phosphatase forward primer:	5′ GCTGATCATTCCCACGTTTT
Alkaline phosphatase reverse primer:	5′ CTGGGCCTGGTAGTTGTTGT
Osteocalcin forward primer:	5′ AAGCAGGAGGGCAATAAGGT
Osteocalcin reverse primer:	5′ TTTGTAGGCGGTCTTCAAGC
Type I collagen forward primer:	5′ CAGGGAAGCCTCTTTCTCCT
Type I collagen reverse primer:	5′ ACGTCCTGGTGAAGTTGGTC
GAPDH forward primer:	5′ CGTGGGGCTGCCCAGAACAT
GAPDH reverse primer:	5′ TCTCCAGGCGGCACGTCAGA

ALKALINE PHOSPHATASE ACTIVITY AND STAINING

Osteoblasts were cultured for up to 21 days in osteogenic media containing 10 μM ascorbic acid and 50 μM β-glycerolphosphate. For alkaline phosphatase (ALP) staining, cells were

fixed in 10% formalin and stained as previously detailed [Ciovacco et al., 2009]. Alternatively, osteoblasts were cultured for 14 days in osteogenic media and calcium deposition was quantified using Alizarin Red-S as a functional measure of osteoblast mineralization as previously described [Stanford et al., 1995].

MIGRATION ASSAYS

Osteoblast migration assays were performed using Culture-Insert μ-Dishes as described by the manufacturer (Ibidi). Primary WT or Pyk2-KO osteoblasts were seeded into the inner well of the μ-Dish and incubated at 37°C and 5% CO_2 overnight. Alternatively, prior to plating in migration chambers, primary osteoblasts were infected with adenovirus expression constructs for Pyk2 using multiplicity of infection (MOI) of 300 per virus for 3 days. The migration chamber inserts were removed, unattached cells were rinsed off, and osteoblasts were incubated with alpha-MEM containing 10% serum for 6 hours. The migration of cells into the clear zone outside of the plating area was quantified microscopically. For migration assays involving MKs, $1x10^5$ osteoblasts were first plated into migration chambers and incubated overnight. The following day, chamber inserts were removed and osteoblasts were imaged before adding $1x10^4$ MKs to each well. Osteoblasts plus MK co-cultures were then incubated for an additional 6 hr, after which the cells were fixed in 4% formaldehyde. Osteoblast migration distance was determined by microscopic imaging using a Leica DMI4000B inverted microscope with attached digital camera. Final analyses were performed using Image Pro software (Media Cybernetics, Bethesda, MD).

STATISTICAL ANALYSES

All data was reproduced a minimum of three time. The average plus/minus standard error of the mean is shown as indicated. The statistical significance level was set at 0.05% ($p<0.05$) and data was analyzed using two-tailed distribution and equal variance student t-Test.

RESULTS

OSTEOBLAST DIFFERENTIATION IS INCREASED IN THE ABSENCE OF PYK2

Osteoblast differentiation is characterized by an early increase in alkaline phosphatase (ALP) followed by an increase in collagen type 1 and later osteocalcin. To examine the role of Pyk2 on osteoblast differentiation, calvarial osteoblasts were isolated from WT and Pyk2-KO mice and cultured under osteogenic conditions containing ascorbic acid and β-glycerolphosphate for 7 days. Osteoblasts were then stained for ALP. In addition, osteoblasts were cultured for 21 days and then stained with Alizarin-S red, which stains bound calcium and is used as a measure of the ability of osteoblasts to mineralize the collagen matrix (Fig. 1A). Consistent with published studies [Buckbinder et al., 2007], Pyk2-KO osteoblasts exhibited higher levels of ALP staining and were more active in calcium deposition than wild-type (WT) osteoblasts. Similar effects were observed when bone marrow-derived stromal osteoblasts from WT and Pyk2-KO mice were compared (data not shown). The expression of the osteoblastic genes, ALP, collagen type I and osteocalcin were also analyzed by quantitative PCR (QPCR) and were found to be significantly higher in Pyk2-KO osteoblasts than WT osteoblasts (Fig. 1B) confirming that Pyk2 is a negative regulator of osteoblast differentiation.

ALP ACTIVITY IS INHIBITED BY PYK2 PHOSPHORYLATION AND KINASE MUTANTS

The phosphorylation of Pyk2 at Y402 not only elevates Pyk2's catalytic activity but also promotes its association with its binding partners such as c-Src [Avraham et al., 2000; Dikic et al., 1996; Faccio et al., 2003][Lakkakorpi et al., 2003]. Therefore, to begin to examine the mechanism of action of Pyk2 in osteoblasts, we determined the extent to which Pyk2 was phosphorylated at Y402 (pY402), in osteoblasts under steady-state conditions. However, Pyk2 pY402 levels in WT osteoblasts were generally difficult to detect by Western blotting using a phospho-specific antibody (Fig. 2A, lane 1). Therefore, osteoblasts were treated with phenylarsine oxide (PAO), a broad spectrum chemical inhibitor of tyrosine phosphatases. As anticipated, Pyk2 Y402 phosphorylation was increased in a dose-dependent manner suggesting that Pyk2 phosphorylation was negatively regulated by protein-tyrosine phosphatases in osteoblasts.

The tight regulation of Y402 phosphorylation in osteoblasts indicated that Pyk2 phosphorylation may have effects on osteoblast function that are distinct from its kinase activity. We therefore compared the effects of Pyk2 phosphorylation or kinase activity on osteoblast differentiation. To test this, Pyk2-KO osteoblasts were infected with adenoviruses expressing normal ($Pyk2^{WT}$), an auto-phosphorylation mutant ($Pyk2^{Y402F}$) or a kinase-inactive mutant ($Pyk2^{K457A}$). Note that in the Y402F mutant, tyrosine residue 402 is replaced by phenylalanine which removes the c-Src binding site and previous studies have revealed that targeting Src kinase is critical for the cellular function of Pyk2 [Lakkakorpi et al., 2003; Miyazaki et al., 2004]. In this experiment, the infected osteoblast cells were differentiated for 3 days instead of 7 days which maximized viral titer and viral protein expression. After 3 days in osteogenic media, the cells were stained for ALP (Fig. 2B) or assayed using a quantitative biochemical ALP activity assay *in vitro* (Fig. 2C). Uninfected Pyk2-KO osteoblasts and WT osteoblasts were used as controls. Consistent with an increase in ALP protein staining levels observed in Pyk2-KO after 7 days (Fig. 1), Pyk2-KO exhibited elevated ALP activity compared to WT osteoblasts after 3 days in culture (Fig. 2B). However, re-expression of $Pyk2^{WT}$ in Pyk2-KO osteoblasts had little effect on ALP staining or ALP activity. In contrast, in Pyk2-KO osteoblasts expressing either $Pyk2^{Y402F}$ or $Pyk2^{K457A}$, ALP activity was significantly lower than in control osteoblasts. These findings suggested that inhibiting either the Y402 phosphorylation or the kinase activity of Pyk2 suppressed ALP activity and decreased osteoblast differentiation. Similar to our findings using primary osteoblasts, MC3T3-E1 osteoblastic cells transiently expressing $Pyk2^{Y402F}$ or $Pyk2^{K457A}$ cDNA showed decreased ALP activity compared to cells expressing $Pyk2^{WT}$ cDNA (data not shown). These finding confirmed the reproducibility of our findings in an osteoblastic cell model and suggested our current findings were not due to the use of adenoviruses.

OSTEOBLAST MIGRATION IS REGULATED BY PYK2 Y402 PHOSPHORYLATION

Osteoblast migration is critical for their recruitment to eroded bone surfaces. Therefore, we examined the role of Pyk2 phosphorylation and kinase activity on cell migration. Pyk2-KO osteoblasts were infected with adenoviruses expressing similar amounts of $Pyk2^{WT}$, $Pyk2^{Y402F}$, or $Pyk2^{K457A}$. After 3 days, osteoblasts were replated into migration chambers. Microscopic imaging was performed at 0 and 6 hours and the distance traveled was

quantified (Fig. 3A). Compared to WT osteoblasts, control Pyk2-KO osteoblasts (no virus) displayed an increase in migration distance of approximately 40–60%. Consistent with an inhibitory role for Pyk2 during osteoblast migration, adenovirus-expressed $Pyk2^{WT}$ decreased Pyk2-KO migration by 40% and was similar to WT osteoblast (no virus) levels (Fig. 3B). $Pyk2^{K457A}$ also significantly decreased the migration of Pyk2-KO osteoblasts. However, Western blotting confirmed significantly lower expression levels of $Pyk2^{K457A}$ than $Pyk2^{WT}$. In contrast, $Pyk2^{Y402F}$ had little effect on Pyk2-KO osteoblast migration even when its expression level was allowed to exceed that of $Pyk2^{WT}$ in Pyk2-KO osteoblasts or endogenous Pyk2 in WT osteoblasts (Fig. 3C). Taken together, these data reveal that kinase-inactive $Pyk2^{K457A}$, but not the phosphorylation mutant $Pyk2^{Y402F}$, represses Pyk2-KO osteoblast migration. These findings also suggested that kinase-inactive $Pyk2^{K457A}$ was more effective at reducing Pyk2-KO osteoblast migration than $Pyk2^{WT}$ which retains catalytic activity and the ability to be phosphorylated.

MEGAKARYOCYTES REDUCE PYK2 PHOSPHORYLATION AND ALP ACTIVITY

We previously reported that Pyk2 plays a role in the mechanism by which megakaryocytes (MK) increase osteoblast number *in vitro* and bone mass *in vivo* [Cheng et al., 2013]. A role for Pyk2 in the actions of MKs is also supported by the finding that Pyk2 tyrosine phosphorylation in platelets is stimulated by phorbol 12-myristate 13-acetate (PMA), a known protein kinase C (PKC) agonist [Ohmori et al., 2000]. Therefore, we examined if MKs regulated Pyk2 phosphorylation in osteoblasts. We also determined the extent to which MKs regulated Pyk2 phosphorylation in the presence or absence of PMA (Fig. 4). For these studies, osteoblasts were co-cultured with MKs for up to 24 hours and then MKs were removed by extensive washing. Osteoblast cultures (devoid of MKs) were treated with PMA or vehicle for 1 hour prior to harvesting and Western blotting. Phosphorylated Pyk2 was identified by Western blotting using an antibody to phosphorylated Y402. Densitometry was used to determine changes in the ratio of phosphorylated Pyk2 (pY402) to total Pyk2 (pY402/Pyk2).

Similar to previous studies [Cheng et al., 2013], total Pyk2 levels were increased when osteoblasts were cultured in the presence of MKs for 24 hours. However, in osteoblasts co-cultured with MKs, the ratio of pY402 to total Pyk2 (pY402/Pyk2) was consistently lower than in control osteoblasts without MKs for all incubation periods (1–24 hr). These findings suggested that MKs inhibit Pyk2 phosphorylation in osteoblasts. In osteoblasts treated with PMA, we observed a time-dependent increase in Pyk2 phosphorylation levels compared to control osteoblasts, especially at 24 hr when a 5-fold increase in the pY402/Pyk2 ratio was observed. However, PMA failed to significantly increase the pY402/Pyk2 ratio in osteoblasts pre-cultured with MKs. Taken together, these findings suggest that Pyk2 phosphorylation is activated by PMA, most likely via activation of PKC, and that MKs prevent the PMA-stimulated increase in Pyk2 phosphorylation in osteoblasts.

Given that MKs decreased Pyk2 phosphorylation (lowered the pY402/Pyk2 ratio) in the presence or absence of PMA and that $Pyk2^{Y402F}$ expression in Pyk2-KO osteoblasts had an inhibitory effect on ALP activity (Fig. 2), we examined the effect of MKs on ALP activity in osteoblasts. MKs were cultured with osteoblasts for ten days and then removed by washing

prior to assaying ALP activity. As shown in Fig. 4B, OBs co-cultured with MKs showed a robust 3.5-fold reduction in ALP activity compared to OBs cultured alone, which reproduced our previously published studies [Ciovacco et al., 2009]. Thus, our findings suggest that the MKs act in part to decrease Pyk2 phosphorylation which decreases ALP activity and osteoblast differentiation.

MEGAKARYOCYTES PROMOTE OSTEOBLAST MIGRATION INDEPENDENT OF PYK2

Given that Pyk2 phosphorylation was found to be important for regulating ALP activity but was not required for osteoblast migration, we examined the extent to which MKs regulate osteoblast migration and whether Pyk2 was required for this process. Osteoblasts from WT or Pyk2-KO mice were plated in migration chambers overnight. The next morning osteoblasts were co-cultured in the absence or presence of MKs for 6 hours. As shown in Fig. 5, MKs stimulated the migration of WT osteoblasts by 24±5 percent, compared to osteoblasts cultured alone. The basal migration of Pyk2-KO is higher than that of WT osteoblasts as shown in Fig 3. In the presence of MKs, the migration of Pyk2-KO osteoblasts is increased by 31±12 percent. However, no significant difference was observed between the effects of MKs on the migration of WT or Pyk2-KO osteoblasts, suggesting that the stimulatory effect of MKs on osteoblast migration is independent of Pyk2 expression.

DISCUSSION

Bone formation by osteoblasts is crucial for bone modeling and remodeling as well as fracture repair. We and others have demonstrated that Pyk2-KO mice exhibit higher bone mass than WT mice which is due to increased osteoblast activity and decreased osteoclast bone resorbing activity [Buckbinder et al., 2007; Gil-Henn et al., 2007]. We also reported that Pyk2 regulates the stimulatory effects of MKs on bone formation and osteoblast number [Cheng et al., 2013]. Pyk2 phosphorylation at Y402 is necessary for the activation of downstream signaling cascades [Avraham et al., 2000; Dikic et al., 1996; Faccio et al., 2003], as well as for maximal catalytic activity against its substrates [Duong et al., 1998; Park et al., 2004; Sanjay et al., 2001]. In support of this, we demonstrated that bone resorption was inhibited in osteoclasts over-expressing $Pyk2^{Y402F}$ or kinase-inactive $Pyk2^{K457A}$ [Gil-Henn et al., 2007; Lakkakorpi et al., 2003]. Of interest, Lakkakorpi et al. (2003) also showed that Pyk2 autophosphorylation, but not Pyk2 kinase activity was necessary for the association of Pyk2 with c-Src in osteoclasts [Lakkakorpi et al., 2003; Miyazaki et al., 2004]. Thus, cellular function of Pyk2 extends beyond simply its kinase activity and is likely to involve its non-enzymatic protein subdomains, including two protein-rich domains which bind SH3-containing proteins.

In the current study, we examined the mechanism of action of Pyk2 in osteoblasts. We focused on elucidating the role of Pyk2 phosphorylation and kinase activity in the function of osteoblasts. We demonstrated that osteoblasts lacking Pyk2 show a significant increase in ALP mRNA levels, ALP catalytic activity, and mineralizing activity (Ca^{2+} deposits). In addition, type I collagen and osteocalcin mRNA expression, which are markers of mature osteoblast activity, were increased, supporting the role for Pyk2 as a negative regulator of osteoblast differentiation. Furthermore, our studies reveal that Pyk2 phosphorylation and

activity have distinct effects on the differentiation and migration of osteoblasts. Finally, we demonstrated that although Pyk2 is important for osteoblast migration, MKs can also promote osteoblast migration by a process that is independent of *Pyk2* gene expression.

Based on our finding that basal Pyk2 phosphorylation is low in WT osteoblasts, and the fact that over-expression of $Pyk2^{Y402F}$ decreases ALP activity in Pyk2-KO osteoblasts, we speculate that the ratio of pY402/total Pyk2 regulates ALP activity. Consistent with this, cellular events that inhibit Pyk2 phosphorylation, such as co-culture of osteoblasts with MKs, decrease Pyk2 phosphorylation and correspondingly decrease ALP activity. We also found that the kinase-inactive mutant of Pyk2 ($Pyk2^{K457A}$) had a negative effect on ALP mRNA in Pyk2-KO osteoblasts whereas $Pyk2^{WT}$ had little effect on ALP activity. These findings are consistent with published findings showing that kinase-inactive Pyk2 fails to enhance ALP activity in human mesenchymal stem cells [Buckbinder et al., 2007]. It is important to point out that ALP is itself a phosphatase and we previously showed that the protein phosphatase PTP-PEST negatively regulates Pyk2 activity [Eleniste et al., 2012]. Whether ALP can inhibit Pyk2 (directly or indirectly via other proteins) or whether $Pyk2^{K457A}$ can act as dominant-negative inhibitor of the catalytic activity of ALP is unknown. It is also possible that Pyk2-KO osteoblasts expressed maximal ALP enzymatic activity preventing a further increase in ALP activity. In contrast to our results which showed that $Pyk2^{Y402F}$ inhibited ALP activity, others have reported that a chemical inhibitor of Pyk2, which can block Pyk2-Y402 phosphorylation, increases ALP levels in MC3T3 cells, an immortalized pre-osteoblastic cell line [Allen et al., 2009]. This discrepancy may be due to use of an immortalized cell by Allen et al. (2009) or that MC3T3 cells were cultured for two-three weeks prior to examining ALP activity. Furthermore, the authors did not report if long-term culture of MC3T3 cells with the Pyk2 inhibitors affected Pyk2 mRNA or protein levels.

Pyk2 is activated following engagement of integrins, resulting in its autophosphorylation at Y402 [Duong et al., 1998; Park et al., 2004; Pfaff and Jurdic, 2001; Sanjay et al., 2001]. Although $[Ca^{2+}]_i$ is required for Pyk2 activity, the induction of Pyk2's full catalytic activity also requires the binding of the Src SH2 domain to phosphorylated Pyk2-Y402 and the subsequent phosphorylation of additional C-terminal tyrosine residues [Duong et al., 1998; Park et al., 2004; Sanjay et al., 2001]. We found that in the absence of Pyk2, osteoblast migration is significantly enhanced. Although $Pyk2^{Y402F}$ (auto-phosphorylation mutant) inhibited ALP activity, it did not affect the migration of Pyk2-KO osteoblasts. In contrast, kinase-dead $Pyk2^{K457A}$ inhibited both ALP activity and the migration of Pyk2-KO osteoblasts. These findings suggest that eliminating the Src binding site on Pyk2 (Y402F) likely affects its ability to recruit Src or other downstream scaffolding proteins such as vinculin and paxillin which are also required for cellular adhesion and migration [Duong and Rodan, 2000; Eleniste and Bruzzaniti, 2012; Kintscher et al., 2001]. Of interest, recent evidence suggests that the activation of Pyk2 in response to fibroblast growth factor receptor 3 (FGFR3) signaling is independent of Y402 phosphorylation, obviating the requirement for Src recruitment to Y402 in some instances [Meyer et al., 2004].

We previously reported that Pyk2 dephosphorylation in osteoclasts occurs through the actions of the protein tyrosine phosphatase PTP-PEST [Eleniste et al., 2012]. Unlike

osteoclasts in which phosphorylated Pyk2 is readily detectable, we detected only low levels of phosphorylated Pyk2 in wild-type osteoblasts. However, similar to osteoclasts, Pyk2 phosphorylation in osteoblasts was induced by treatment with phenylarsine oxide (PAO) (Fig. 2) and orthovanadate (data not shown), two broad specificity phosphatase inhibitors. In addition, we found that Pyk2-Y402 phosphorylation was stimulated by PMA (Fig. 4), a known protein kinase C agonist [Ohmori et al., 2000]. Moreover, we found that co-culture of osteoblasts with MKs leads to an increase in Pyk2 levels and a corresponding decrease in the ratio of pY402/Pyk2. Taken together, these findings suggest that the Pyk2 phosphorylation/dephosphorylation cycle is tightly regulated by upstream and downstream signaling cascades and that sustained Pyk2 phosphorylation is detrimental to osteoblast function.

We recently reported that osteoblasts express two isoforms of Pyk2; full-length Pyk2 and a shorter splice variant known as Pyk2-S which lacks a 42 amino acid mid-molecule domain [Kacena et al., 2012]. Our studies also reveal that co-culture of MK with osteoblasts promotes Pyk2-S mRNA expression but decreases Pyk2 mRNA in osteoblasts [Kacena et al., 2012]. In the current study, we found that MK-stimulation of osteoblasts increases total Pyk2 levels, mostly likely Pyk2-S, which is largely non-phosphorylated as shown by the low pY402/Pyk2 ratio. Although PMA increased the pY402/Pyk2 ratio in untreated osteoblasts, pY402 levels were low after PMA stimulation of osteoblasts co-cultured with MKs. This was particularly evident in the 16 and 24 hours co-cultures. Given that the MKs increase the expression of the shorter Pyk2-S isoform (our previous publication), these findings may suggest that Pyk2-S is not responsive to a PMA/PKC-mediated increase in phosphorylation. Additional studies will be required to determine if MKs act to prevent Pyk2 phosphorylation or enhance Pyk2 dephosphorylation. In addition, it remains to be determined whether Pyk2 or Pyk2-S exhibit distinct functional roles in osteoblasts in response to MK stimulation.

MKs promote osteoblast proliferation (our previous studies) and cell growth but inhibit ALP activity *in vitro* (Fig. 4). We demonstrated that mice deficient in the MK transcription factor, GATA-1, exhibit a profound increase in bone marrow MKs and exhibit a marked increase in osteoblast number and bone mass [Drachman et al., 1997; Kacena et al., 2005; Kacena et al., 2006; Kacena et al., 2004; Villeval et al., 1997]. Our current studies provide a potential Pyk2-regulated mechanism for the MK-mediated decrease in ALP. That is, our findings suggest that MKs inhibit Pyk2 phosphorylation which decreases ALP activity and inhibits osteoblast differentiation, favoring osteoblast proliferation.

The process of bone formation is preceded by the recruitment of osteoblasts to eroded bone surfaces. However, the role of Pyk2 phosphorylation and/or kinase activity and the role of MKs in osteoblast migration had not previously been examined. To this end, we examined if MKs regulated osteoblast migration and the role of Pyk2 in this process. Our findings demonstrate that $Pyk2^{Y402F}$ had little effect on Pyk2-KO osteoblasts migration, although basal Pyk2-KO osteoblast migration was higher than WT osteoblasts. In contrast to $Pyk2^{Y402F}$, kinase-dead $Pyk2^{K457A}$ repressed osteoblast migration, which is consistent with published reports that pharmacologic Pyk2 inhibitors inhibit cancer cell migration and metastasis [Han et al., 2009; Tse et al., 2012; Walker et al., 2009]. We also report for the first time that MKs promote osteoblast migration in a process that is independent of Pyk2 expression. Together, these findings suggest that the ability of MKs to promote bone

formation and osteoblast proliferation (our previous studies), while conversely inhibiting osteoblast differentiation *in vitro* (this study and Ciovacco et al., 2009) may in part be explained by the sum effect of Pyk2-dependent and independent actions of MKs. That is, MKs decrease osteoblast differentiation by decreasing Pyk2-phosphorylation (favoring osteoblast proliferation) and MKs promote osteoblast migration to eroded bone surfaces via a process that is independent of Pyk2. Since MKs are short lived, the net increase in osteoblast number may ultimately lead to an increase in bone volume.

In summary, our findings indicate the presence of unique and overlapping Pyk2 signaling pathways in osteoblasts, which may be controlled by regulating the phosphorylation, kinase activity and scaffolding functions of Pyk2. Our data demonstrate that osteoblast migration and bone-forming activity are functionally enhanced in the absence of Pyk2, and that inhibition of Pyk2 Y402 phosphorylation plays a significant role in osteoblast differentiation (decreased ALP activity) but not migration. Furthermore, MKs promote osteoblast differentiation and migration via distinct Pyk2-dependent and independent pathways, respectively. Therefore, new therapeutic approaches that selectively target Pyk2 phosphorylation or MK-induced pathways in osteoblasts may provide specificity for the treatment of different bone-related diseases such as osteoporosis, bone regeneration or tumor metastasis.

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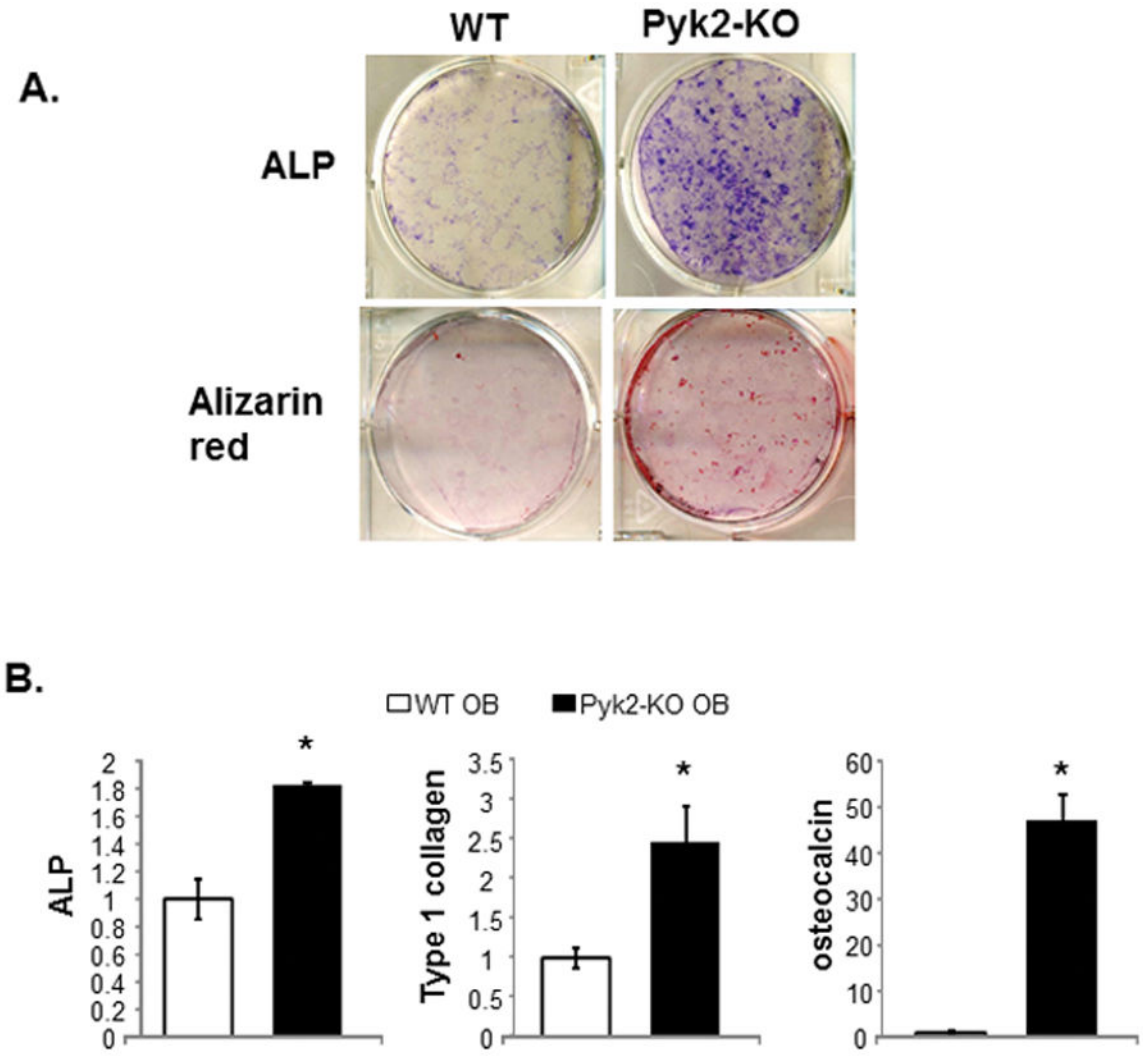

Fig. 1. Osteoblast differentiation is impaired in the absence of Pyk2

Two day calvarial osteoblasts (OB) were generated from WT and Pyk2-KO mice. (A) Osteoblasts were cultured under osteogenic conditions containing ascorbic acid and β-glycerolphosphate for 7 days and then stained for alkaline phosphatase expression or cultured for 21 days and stained with Alizarin-S red as a measure of osteoblast mineralization. (B) QPCR analysis was used to determine mRNA expression of osteoblastic genes; ALP, type I collagen, and osteocalcin. GAPDH was used as the control to normalize the amount of the mRNA transcript under investigation. The mean of duplicate samples is shown for each transcript ± standard error of the mean (SEM). Experiments were performed a minimum of 3 times and representative data are shown. The asterisks (*) indicate statistical significance ($p<0.05$) relative to control WT osteoblasts set at RQ 1.0.

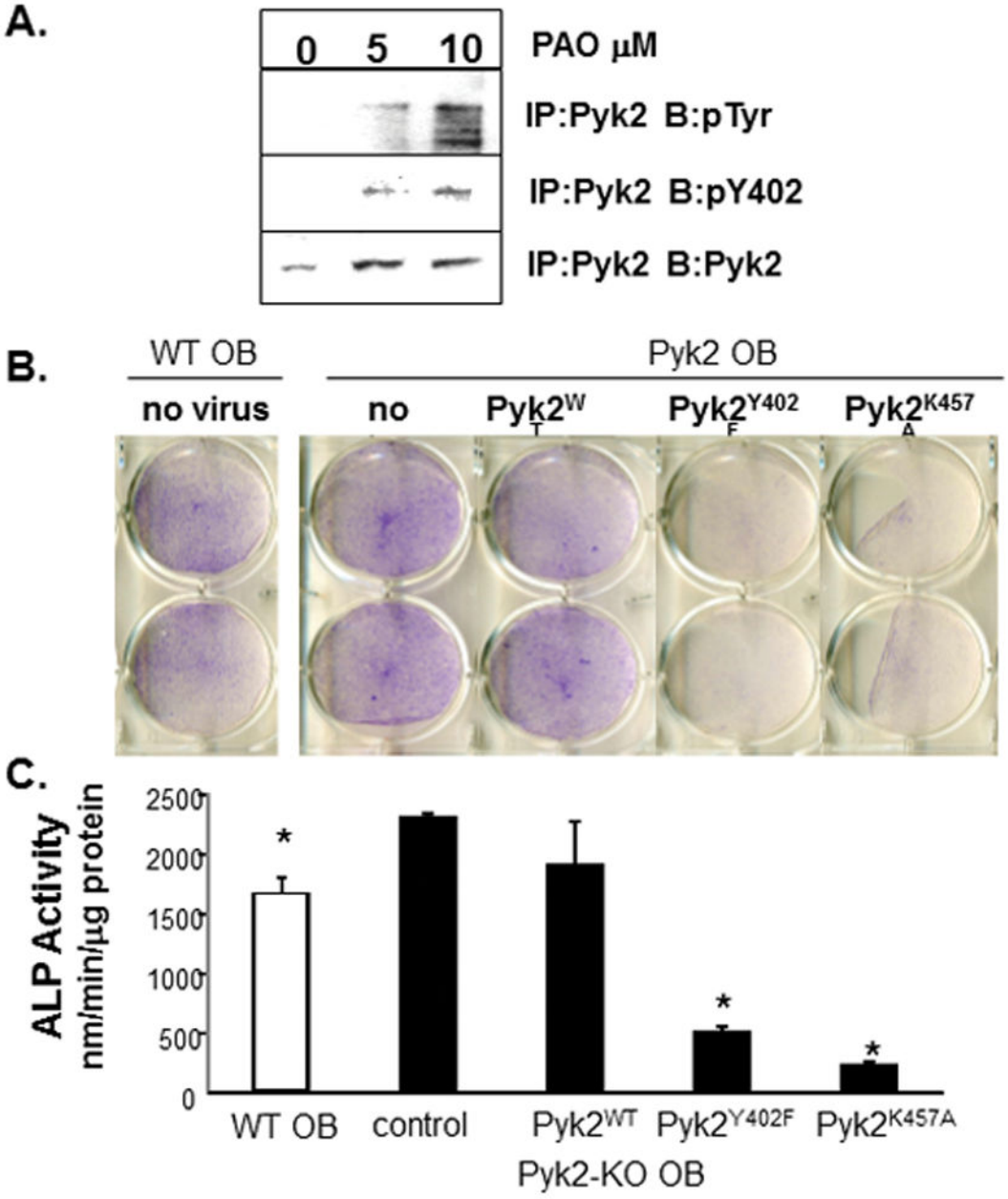

Fig. 2. Pyk2-KO osteoblast differentiation is inhibited by Pyk2Y402F and Pyk2K457A, but not Pyk2WT

(A) WT osteoblasts were treated with 5 or 10 μm PAO for 1 hr. Lysates were immunoprecipitated with anti-Pyk2 and blotted for phosphorylated tyrosine (p-Tyr) or using a phosho-specific p-Y402 antibody. Membranes were reblotted for total Pyk2 as a control. The Pyk2 antibody detects Pyk2WT, Pyk2Y402F and Pyk2K457A. (B–C) Pyk2-KO osteoblasts were infected with adenoviruses expressing similar amounts of Pyk2WT (active), Pyk2Y402F (phosphorylation mutant), or Pyk2K457A (kinase-inactive). Osteoblasts were cultured for 3 days in osteogenic media and (B) stained for ALP or (C) assayed for ALP activity. Experiments were reproduced 4 times using different amounts of viruses, with

consistent results. Representative data are shown. The asterisk (*) indicates statistical significance relative to controls.

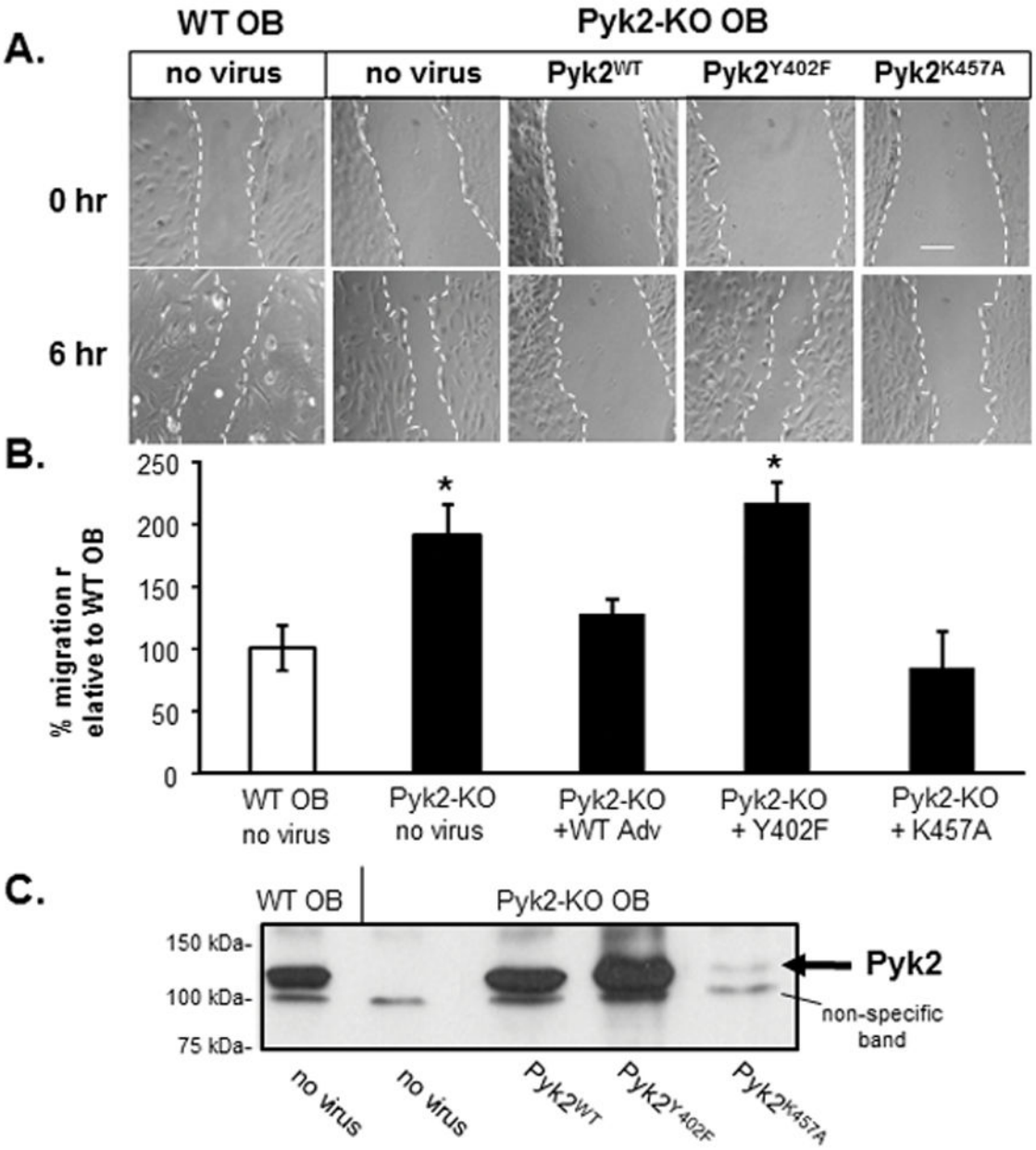

Fig. 3. Pyk2Y402F does not inhibit migration in Pyk2-KO osteoblasts

Pyk2-KO osteoblasts were infected with adenoviruses expressing Pyk2WT, Pyk2Y402F or Pyk2K457A. After 3 days, cells were counted and added to migration chambers. The following day, the distance migrated at 0 and 6 hr was calculated. A) Representative images are shown. Dashed lines indicates leading cell edge. Solid bar indicates scale bar at 100 μm. B) The distance migrated as a percentage of WT osteoblasts is shown. The data shown are average % migration ± SEM of 4 experiments. The asterisks indicate statistical significance compared to WT ($p<0.05$). C) Western blotting was used to confirm virus-expressed Pyk2 relative to WT controls. A non-specific protein band seen by the Pyk2 antibody is indicated. Experiments were replicated more than 5 times. A representative blot is shown which

demonstrates higher Pyk2Y402F and lower Pyk2K457A levels compared to Pyk2-KO + Pyk2WT virus or WT osteoblasts.

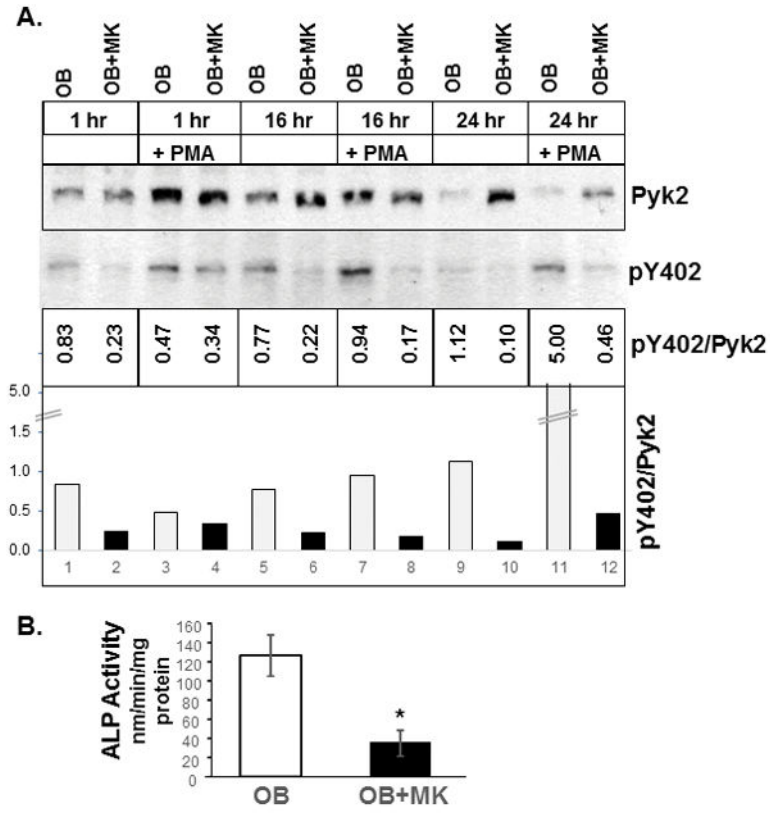

Fig. 4. Differential regulation of Pyk2 phosphorylation in osteoblasts by MKs and PMA

(A) Osteoblasts were cultured with MKs for 1, 16 or 24 hours. MKs were removed by extensive washing and osteoblasts were treated with 200 nM PMA or vehicle for 1 hour. Western blotting was performed for total Pyk2 or phosphorylated Pyk2-Y402 (pY402) as indicated. Densitometry was performed using ImageJ software and used to calculate the ratio of pY402/Pyk2. Studies were replicated 3 times and representative data are shown. (B) Osteoblasts were co-cultured with MKs for 10 days and assayed for ALP activity. The average ± standard deviation from 4 separate osteoblast experiments and 8 separate osteoblast + MKs experiments are shown. In each experiment triplicate cultures were assayed. The asterisk (*) indicates statistical significance for ALP activity in OB+MK compared to the OB control.

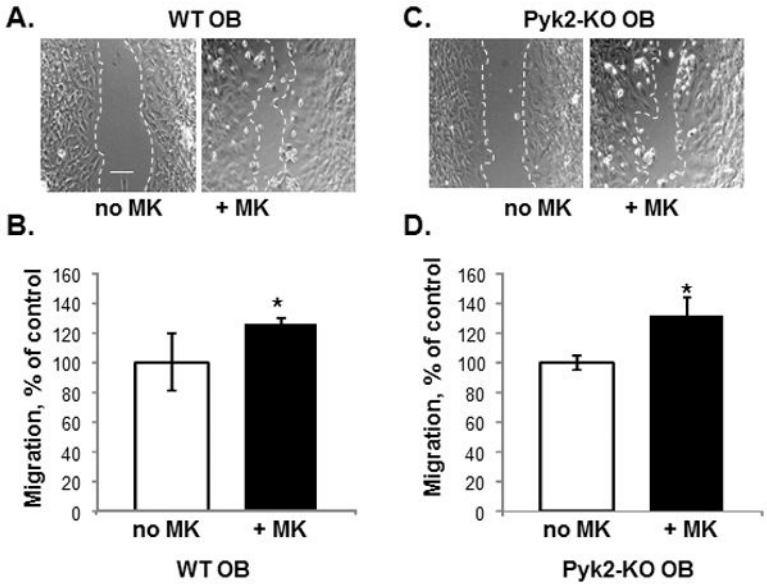

Fig. 5. MKs promote osteoblast migration in the absence of Pyk2

Calvarial osteoblasts from WT mice or Pyk2-KO mice were plated at a similar density in migration chambers. The next morning, MKs were added to chamber slides (0 hour) and osteoblasts were imaged. After 6 hours, the wells were re-imaged and the distance traveled in the presence or absence of MKs was calculated and expressed as a percentage of the 0 hour control. (A; C) Representative cell images at 0 and 6 hours for WT and Pyk2-KO osteoblasts, respectively. The dashed line indicates the leading edge of osteoblasts and solid white bar indicates scale bar (100 μm). (B; D) Data represent the average of 3 independent studies expressed as a percentage of the genotype-matched control. Although the basal migration of Pyk2-KO is elevated compared to WT osteoblasts (see Fig. 3). MKs significantly increase the migration of both (B) WT osteoblasts and (D) Pyk2-KO osteoblasts to a similar extent. Asterisks (*) indicate statistical significance for the effects of MKs on WT or Pyk2-KO osteoblasts compared no MKs ($p<0.05$).