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Evidence for a role for the p110-α isoform of PI3K in skeletal function

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ABSTRACT

Signaling through phosphatidylinositol-3 kinases (PI3K) regulates fundamental cellular processes such as survival and growth, and these lipid kinases are currently being investigated as therapeutic targets in several contexts. In skeletal tissue, experiments using pan-specific PI3K inhibitors have suggested that PI3K signaling influences both osteoclast and osteoblast function, but the contributions of specific PI3K isoforms to these effects have not been examined. In the current work, we assessed the effects of pharmacological inhibitors of the class Ia PI3Ks, α, β, and δ, on bone cell growth, differentiation and function in vitro. Each of the class Ia PI3K isoforms is expressed and functionally active in bone cells. No consistent effects of inhibitors of p110-β or p110-δ on bone cells were observed. Inhibitors of p110-α decreased osteoclastogenesis by 60–80% ($p < 0.001$ vs control) by direct actions on osteoclast precursors, and decreased the resorptive activity of mature osteoclasts by 60% ($p < 0.01$ vs control). The p110-α inhibitors also decreased the growth of osteoblastic and stromal cells ($p < 0.001$ vs control), and decreased differentiated osteoblast function by 30% ($p < 0.05$ vs control). These data suggest that signaling through the p110-α isoform of class Ia PI3Ks positively regulates the development and function of both osteoblasts and osteoclasts. Therapeutic agents that target this enzyme have the potential to significantly affect bone homeostasis, and evaluation of skeletal endpoints in clinical trials of such agents is warranted.

Introduction

Phosphatidylinositol-3 kinases (PI3Ks) phosphorylate phosphatidylinositol-containing substrates to generate polar lipid products which activate signaling pathways that regulate fundamental cellular processes such as survival and proliferation [1]. Class Ia PI3Ks are dimeric enzymes, consisting of a catalytic subunit (p110-α, β, and δ, each encoded by a different gene) and a common regulatory subunit, that are activated by tyrosine kinase signaling [2]. Current evidence implicates signaling via p110-β in thrombosis and tumorigenesis [3,4], via p110-δ in inflammation and immunity [5,6], and via p110-α in tumorigenesis [7,8]. These observations have prompted the development of p110 isoform-specific pharmacological inhibitors as potential therapies for related diseases [9,10]. Such compounds also provide a means to explore the contribution of each of the p110 isoforms to the physiological effects of PI3K signaling that have been identified using pan-specific PI3K inhibitors [11,12].

In skeletal tissue, experiments using pan-specific PI3K inhibitors have suggested that PI3K signaling influences both osteoclast and osteoblast function [13]. PI3K signaling is activated by several cytokines and growth factors that regulate osteoclast development and function, including macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB signaling ligand (RANKL), and pan-specific PI3K inhibitors block osteoclast development, osteoclast motility and bone resorption in vitro [13–16]. Similarly, activation of PI3K signaling in osteoblastic cells contributes to the anti-apoptotic and proliferative activity of important skeletal growth factors such as IGF-I [17]. Activation of PI3K signaling in osteoblasts in vivo, by conditional deletion of PTEN, which encodes a lipid phosphatase that inactivates lipid products of PI3K, leads to increased osteoblast differentiation, prolonged osteoblast survival and increased bone mass [18]. However, at present there are no data on the functions of specific class Ia PI3K isoforms in bone. In the current work, we have used isoform-specific PI3K inhibitors to investigate the role of each isoform in bone cell function.

Material and methods

Cell culture. Primary rat osteoblastic cells were prepared as previously described [19]. Murine pre-osteoblastic MC3T3E1 cells,

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murine bone marrow stromal ST2 cells, and RAW264.7 cells were maintained in standard cell culture conditions. Murine bone marrow cultures were established after harvesting marrow cells from the femora and tibiae of 4- to 6-week-old male animals, as previously described [20]. Mature osteoclasts were isolated from long bones of 1-day-old neonatal rats, as previously described [21].

Reagents. PI3K inhibitors were synthesized at the University of Auckland, as previously described [11]. PIK-75 and PI-103 are selective inhibitors of p110-α, TGX221 is a selective inhibitor of p110-β, and IC87114 is a selective inhibitor of p110-δ [11]. Stock solutions of each inhibitor were prepared in DMSO, and control treatments in all experiments included equivalent concentrations of DMSO to those in the inhibitor treatment wells.

The polyclonal antibodies used for p85α were raised against the N-terminal SH2 domain of bovine p85α [22].

Analysis of gene expression. Total cellular RNA was extracted from murine bone cell lines using RNeasy mini kit, genomic DNA removed, and cDNA synthesized. Multiplex PCR was performed in triplicate with FAM-labeled probes specific for the gene of interest and VIC-labeled 18S rRNA probes. The $\Delta\Delta C_t$ method was used to calculate the relative levels of expression.

Lipid kinase assay. Total class Ia PI3Ks were immunoprecipitated from cellular lysates by overnight incubation with a polyclonal antibody to p85α. PI3K activity was measured using a standard lipid kinase assay with phosphatidylinositol as a substrate, in the presence of 100 nM of the relevant isoform-specific inhibitor. The assay was conducted according to Beeton et al. [23], with the exception that 100 μM cold ATP and [γ-^{33}]P-ATP were used instead of [γ-^{32}]P-ATP.

Osteoclastogenesis assays. Assays of osteoclastogenesis in RAW264.7 cells and murine bone marrow cells were performed as previously described [24]. After culture for 4–5 d (RAW264.7 cells) or 7 d (bone marrow), cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Multinucleated cells (containing three or more nuclei) positive for TRAP were counted by an observer blinded to treatment allocation.

Mature osteoclast function. As previously described [21], the osteoclast-rich suspension derived from neonatal rat long bones was seeded onto 25 mm^2 bovine bone slices in 96-well plates, and incubated at 37 °C for 30 min. The bone slices were then washed several times in PBS to remove contaminating non-osteoclastic cells, rinsed in medium, and incubated in 12-well plates with test substances or vehicle for 24 h. After fixing with 2.5% glutaraldehyde/PBS and staining for TRAP, the number of TRAP-positive multinucleated cells on each bone slice was quantified. The cells were then removed by gentle scrubbing, the bone slices stained with toluidine blue, and resorption pits analyzed using reflected light microscopy and metallurgic lenses. The results were expressed as the number of pits/number of osteoclasts per bone slice.

Osteoblast mitogenesis assay. Osteoblast mitogenesis was measured by [^{3}H]-thymidine incorporation, as previously described [19]. In experiments designed to test the effects of PI3K inhibitors on mitogenesis, cells were cultured overnight in 5% FCS, then the medium changed to 1% FCS at the time of addition of the inhibitor.

Osteoblast differentiation. Assessment of osteoblast differentiation was performed as previously described [24]. In brief, MC3T3E1 cells were seeded in 6-well plates, cultured to confluence, then placed in mineralizing medium (15% FCS/MEM supplemented with L-*ascorbic* acid-2-phosphate and 10 mM β-glycerophosphate) in the presence or absence of PI3K inhibitors. After 18–21 d, the cells were fixed and stained for mineral with von Kossa stain. Quantification of mineral content was performed using image analysis (BioQuant, Nashville, Tn).

All experiments involving animals were approved by the institutional ethics committee.

Statistical analysis. Data were analyzed using ANOVA with post hoc Dunnett's tests for significant main effects. A 5% significance level (two tailed) was used throughout. Data are presented as mean ± SEM, unless indicated otherwise. Analyses were performed using Prism version 4.03 (GraphPad, San Diego, Ca).

Results

Expression and activity of PI3K isoforms in bone cells

Quantitative PCR demonstrated expression of genes encoding each of the class Ia isoforms of PI3K (p110-α, p110-β, and p110-δ) in murine bone cells (Fig. 1A). Levels of expression of p110-α were higher than those of the other isoforms in each cell type examined. To determine the functional activity of the individual PI3K isoforms, lipid kinase assays were performed in the presence of the isoform-specific inhibitors. The results indicated that each of the p110 isoforms contributed to total cellular PI3K activity; however the p110-α inhibitors consistently produced the greatest decreases in PI3K activity, ranging from 35% to 85% among the cell types tested (Fig. 1B).

p110-α inhibitors decrease osteoclast development and function

To determine the role of each PI3K isoform in osteoclast development, we first examined the effect of the relevant inhibitor in

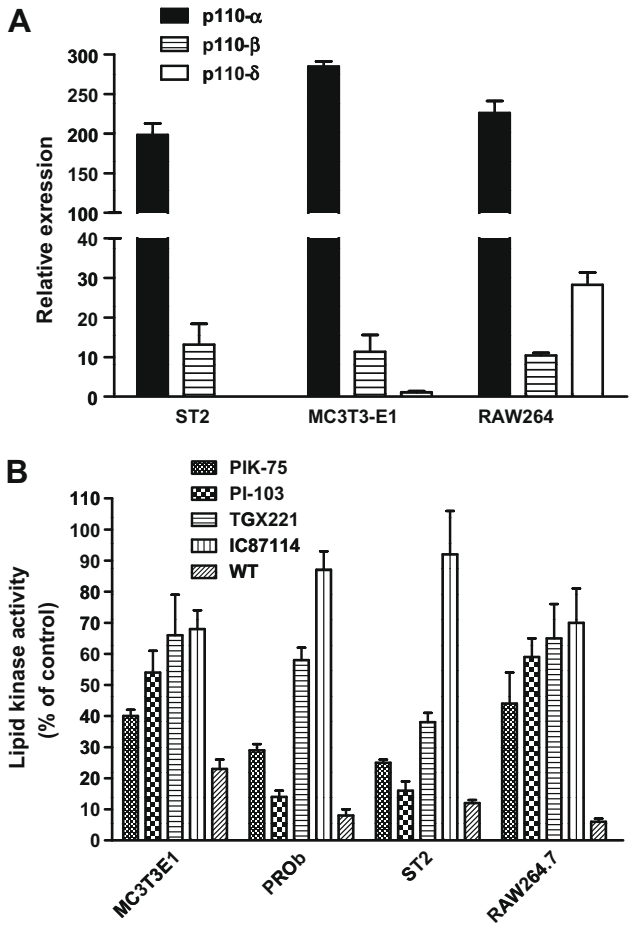

Fig. 1. (A) Expression of genes encoding class Ia PI3K isoforms in bone cells, determined by quantitative PCR. Data are mean (SEM) levels of expression, relative to that of p110-δ in MC3T3E1 cells, and are from a representative experiment. (B) Lipid kinase activity in lysates of bone cells, determined in the presence of 100nM of the indicated inhibitor. Data are mean (SEM) from at least 2 replicates of each experimental condition. WT, wortmannin.

murine bone marrow cultures. In this assay, the pan-specific PI3K inhibitor LY294002 decreased osteoclastogenesis by about 25%. Profound dose-dependent inhibition of osteoclastogenesis was observed in response to treatment with PIK-75, but not in response to treatment with 100 nM PI-103, TGX221, or IC87114 (Fig. 2A). In this assay, stromal cell-derived cytokines play a critical role in driving maturation of osteoclast precursors. Examination of the cultures exposed to PIK-75 demonstrated a substantial decrease in the number of surviving stromal cells, suggesting that the observed effect on osteoclast development was, at least in part, attributable to indirect effects mediated via the stromal cells. This effect was not apparent in cultures exposed to PI-103 (Fig. 2B). In order to clarify whether inhibition of p110-α directly affects osteoclastogenesis, each inhibitor was added to cultures of RANKL-treated RAW264.7 cells. This assay assesses osteoclast development in a stromal cell-free context. Each of the p110-α inhibitors dose-dependently decreased osteoclast development in this assay, the PIK-75 compound demonstrating greater potency (Fig. 2C). No cellular toxicity was observed in these cultures. Finally, we also determined the effect of each of the p110-α inhibitors on the bone

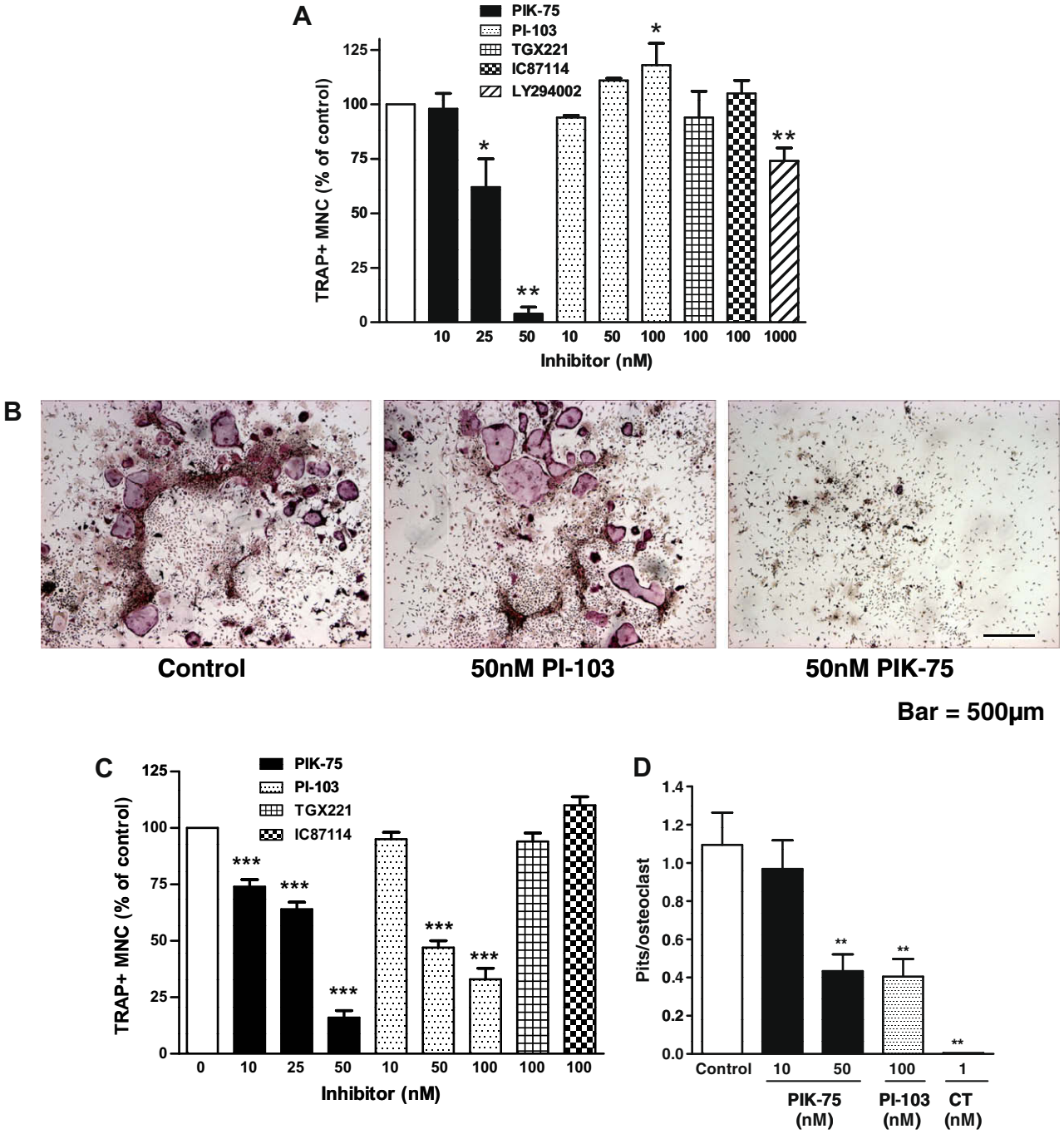

Fig. 2. (A) Effects of PI3K inhibitors on osteoclastogenesis in murine bone marrow cultures. Data are mean (SEM) number of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells per well, expressed as treatment to control ratio. ∗, $p < 0.05$ vs control; ∗∗, $p < 0.01$ vs control. (B) Representative images from day 7 murine bone marrow cultures exposed to vehicle, PI-103 50 nM, and PIK-75 50 nM. (C) Effects of PI3K inhibitors on osteoclastogenesis in RAW264.7 cells. Data are mean (SEM) number of TRAP-positive multinucleated cells per well, expressed as treatment to control ratio. ∗∗∗, $p < 0.001$ vs control. (D) Effects of PI3K inhibitors on bone resorption by mature rat osteoclasts. Data are mean (SEM) number of pits per osteoclast, from a representative experiment. CT, calcitonin. ∗∗, $p < 0.01$ vs control.

resorbing activity of mature osteoclasts isolated from rat long bones. In this assay, each compound reduced bone resorption by about 60% (Fig. 2D).

p110-α inhibitors decrease osteoblast growth

The finding that PIK-75 substantially decreased stromal cell numbers in the murine bone marrow assay (Fig. 2B) suggested that inhibition of p110-α might significantly affect osteoblast growth. To investigate this possibility, we tested the effect of each inhibitor on mitogenesis in primary rat osteoblasts and ST2 stromal cells (Fig. 3A and B). In each cell type, each of the p110-α inhibitors significantly decreased osteoblast growth. However, the PIK-75 compound was considerably more potent in this action than PI-103, and inspection of the cultures revealed significant cellular toxicity in those exposed to PIK-75 (Fig. 3C). In contrast, PI-103 moderately decreased osteoblast mitogenesis in both cell types (mean reductions of 30% and 50% in primary rat osteoblasts and ST2 cells, respectively), without evidence of toxicity (Fig. 3C). Neither the p110-β nor the p110-δ inhibitor affected osteoblast proliferation (Fig. 3A).

Inhibition of p110-α decreases osteoblast differentiation

The p110-α inhibitors produced discrepant effects on osteoblast differentiation (Fig. 4). Thus, PIK-75 increased the amount of mineralized tissue produced in long-term osteoblast cultures, while PI-103 modestly decreased this parameter. The pan-specific PI3K

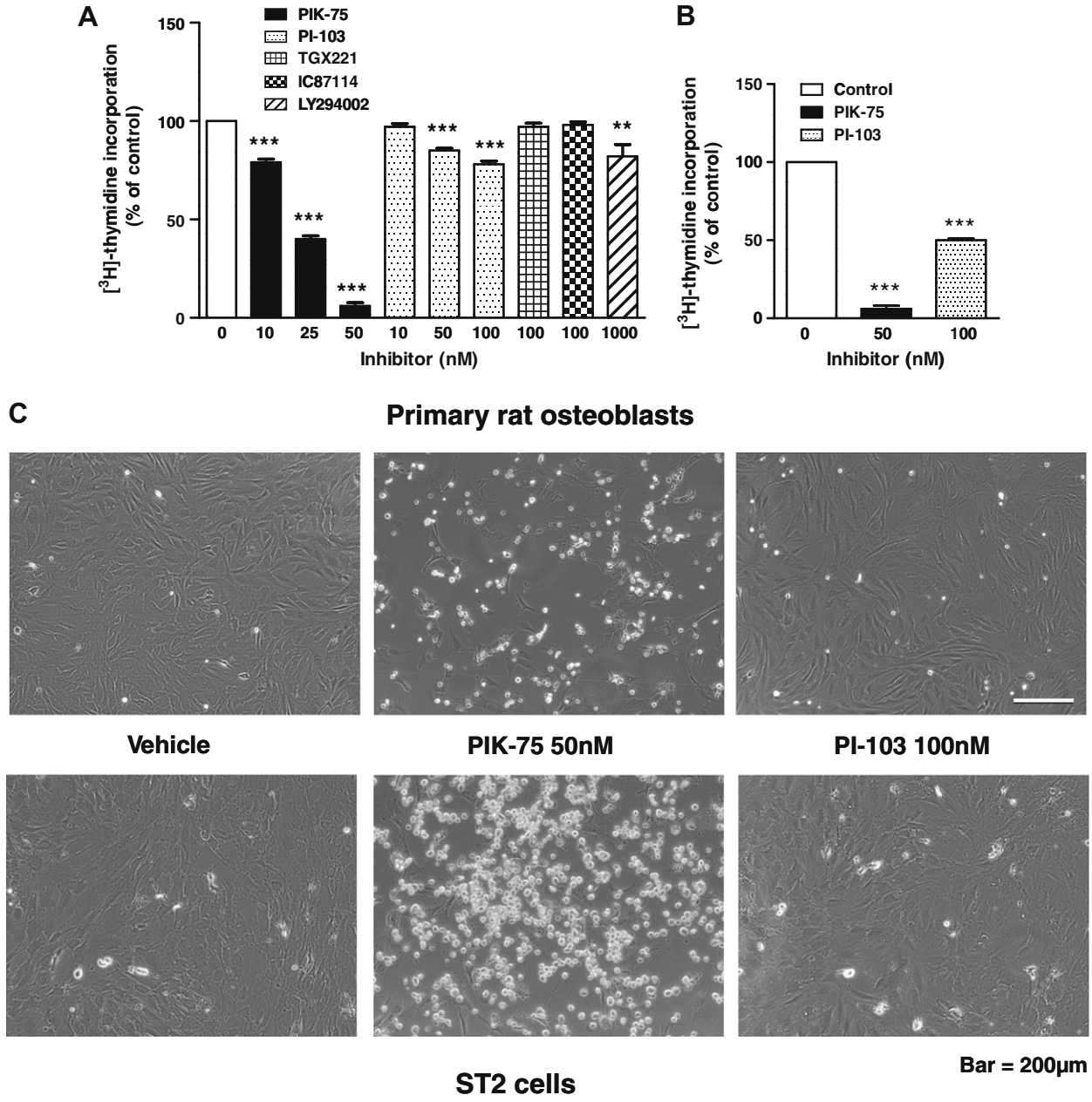

Fig. 3. (A) Effects of PI3K inhibitors on primary rat osteoblast mitogenesis. Data are mean (SEM) [^{3}H]-thymidine incorporation, expressed as a percentage of control values. **, $p < 0.01$ vs control; ***, $p < 0.001$ vs control. (B) Effects of p110-α inhibitors on ST2 cell mitogenesis. Data are mean (SEM) [^{3}H]-thymidine incorporation, expressed as a percentage of control values. ***, $p < 0.01$ vs control. (C) Representative images of cultures of primary rat osteoblasts (*upper*) and ST2 cells (*lower*) exposed to vehicle or p110-α inhibitors.

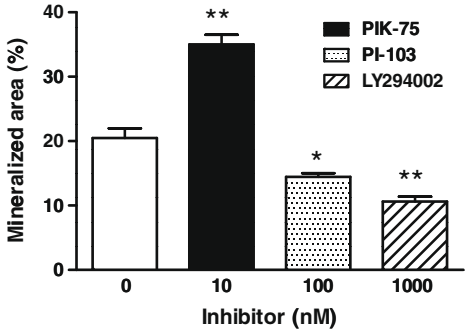

Fig. 4. Effects of PI3K inhibitors on osteoblast differentiation. Data are mean (SEM) percent mineralized area per well, from a representative experiment. $*$, $p < 0.05$ vs control; $**$, $p < 0.01$ vs control.

inhibitor, LY294002, also decreased osteoblast differentiation, suggesting that the stimulatory actions of PIK-75 in this assay may represent an "off-target" action of that compound. Neither the p110-β nor the p110-γ inhibitor affected osteoblast differentiation (data not shown).

Discussion

The present work suggests that p110-α is the predominant class Ia PI3K isoform in bone cells and that, in vitro, inhibition of signaling via the p110-α isoform of PI3K directly inhibits osteoclastogenesis, inhibits the bone resorbing activity of mature osteoclasts, and decreases osteoblast proliferation and differentiation. Since the coupled activities of both cell types are critical to skeletal homeostasis, predicting the integrated in vivo effects of these compounds from the current data is difficult, with potential for overall beneficial skeletal actions (predominance of anti-osteoclastic effects), or adverse skeletal actions (predominance of osteoblast inhibitory effects). We did not find evidence for activity of the p110-β or p110-δ inhibitors in bone cells. Given the growing interest in isoform-specific PI3K inhibitors as pharmaceutical agents [9,10,25], evaluation of the effects of p110-α inhibitors in preclinical and clinical trials should include an assessment of their skeletal actions.

A considerable body of evidence suggests that PI3K signaling plays an important role in skeletal homeostasis [13]. In vitro, pan-specific PI3K inhibitors decrease osteoclastogenesis [15]. In particular, PI3K signaling is required for induction of osteoclast development by the cytokines RANKL and M-CSF [15], each of which is essential for osteoclastic bone resorption in vivo [26,27]. PI3K signaling is also activated by growth factors and cytokines which play important roles in the resorptive function of mature osteoclasts, including M-CSF and integrins [26,28–31], and pan-specific PI3K inhibitors decrease the survival [32,33], impair the cytoskeletal function [16,34,35], and inhibit the resorptive capacity [14] of mature osteoclasts. Our findings extend this body of evidence by demonstrating that it is the p110-α isoform that mediates the effects of PI3K on both osteoclast development and function. In our evaluation of osteoclastogenesis in a stromal cell-independent assay, the RAW264.7 cells, each of the p110-α inhibitors dose-dependently decreased osteoclast formation. This finding is consistent with those reported in response to the pan-PI3K inhibitor LY294002 in stromal cell-independent osteoclastogenesis assays [15], and suggest that p110-α signaling in osteoclast precursors is important for the development of mature, bone resorbing cells. The disparate actions of the p110-α inhibitors on osteoclast formation in the murine bone marrow assay are likely attributable to the potent effects of PIK-75 on stromal cell growth and survival. In addition, the effect of PI-103 to inhibit osteoblast differentiation may have contributed to this finding, as less differentiated osteoblastic cells more efficiently support osteoclastogenesis [36]. Each of the p110-α inhibitors substantially decreased the ability of isolated osteoclasts to resorb bone, indicating that p110-α signaling is also important for the functional activity of mature osteoclasts.

PI3K signaling is also activated in osteoblastic cells by mechanical stimuli [37], and a number of growth factors and cytokines [13,17,38,39]. Pan-specific PI3K inhibitors inhibit osteoblast growth and survival induced by a wide range of extracellular ligands [17,38–43], suggesting that PI3K signaling positively regulates the number of available osteoblast precursors. In addition, conditional deletion in osteoblasts of *Pten*, which encodes a lipid phosphatase that downregulates PI3K signaling, promotes osteoblast proliferation and survival [18]. The current data suggest that the effect of PI3K to promote osteoblast growth and survival is attributable to the p110-α isoform, as each of the p110-α inhibitors significantly decreased osteoblast and stromal cell growth. However, the magnitude and nature of the growth inhibitory effect induced by the p110-α inhibitors differed. The PIK-75 compound induced substantial cell death, while the PI-103 compound decreased cell growth without evidence of toxicity. This difference may be attributable to either greater intracellular concentrations of PIK-75 than PI-103, or an off-target effect of the PIK-75 compound.

The role of PI3K signaling in osteoblast differentiation has been less comprehensively studied than in osteoblast proliferation and survival. Pan-specific PI3K inhibitors have been reported to both augment and abrogate BMP2-induced expression of genetic markers of osteoblast differentiation [44,45], and decrease PTH-induced expression of type I collagen [46]. Constitutive activation of PI3K signaling in osteoblasts by deletion of *Pten* caused increased expression of genetic markers of osteoblast differentiation [18]. Our data are consistent with a role for PI3K in the promotion of differentiated osteoblast function, as the pan-specific PI3K inhibitor, LY294002, decreased the generation of mineralized tissue in long-term cultures of osteoblastic cells. It is likely that this action of PI3K is also mediated by the p110-α isoform, since the PI-103 compound mimicked the effect of LY294002, and neither the p110-β inhibitor nor the p110-δ inhibitor had any effect. A surprising finding was that the PIK-75 compound promoted osteoblast differentiation. This is likely to represent an off-target effect, in view of the congruent PI-103 and LY294002 data in the same assay, and the low concentration (10 nM) of the PIK-75 compound at which it was observed.

Conflict of interest

Peter Shepherd, Claire Chaussade and Gordon Rewcastle are shareholders in, and consultants to, Pathway Therapeutics, a company which is developing PI3K inhibitors.

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