Expression of PGK1 by Prostate Cancer Cells Induces Bone Formation


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Abstract
Prostate cancer (PCa) is one of the solid tumors that metastasize to the bone. Once there, the phenotype of the bone lesions is dependent upon the balance between osteoblastogenesis and osteoclastogenesis. We previously reported that overexpression of phosphoglycerate kinase 1 (PGK1) in PCa cell lines enhanced bone formation at the metastatic site in vivo. Here, the role of PGK1 in the bone formation was further explored. We show that PCa-derived PGK1 induces osteoblastic differentiation of bone marrow stromal cells. We also found that PGK1 secreted by PCa inhibits osteoclastogenesis. Finally, the expression levels of the bone-specific markers in PCa cells were higher in cells overexpressing PGK1 than controls. Together, these data suggest that PGK1 secreted by PCa regulates bone formation at the metastatic site by increasing osteoblastic activity, decreasing osteoclastic function, and expressing an osteoblastic phenotype by PCa cells. (Mol Cancer Res 2009; 7(10):OF1–10)

Introduction
Prostate cancer (PCa) is a common neoplasm and the second leading cause of cancer deaths in American males. Almost all men who die from PCa, have hormone-refractory disease with skeletal metastases (1, 2). Due to progress in systemic chemotherapy and radiotherapy, the prognosis of individuals with PCa is improving (3). However, the symptoms of the skeletal metastases, such as pathologic fractures, bone pain, and spinal cord compression, remain incurable (4, 5).

We recently showed that SDF-1/CXCL12 and its receptors (CXCR4 and RDC1/CXCR7) play important roles in PCa bone metastases and growth in bone (6-12). One part of the mechanism activated by CXCL12 signaling is an increase in angiogenesis (9). CXCL12 signaling reduces the expression and secretion of phosphoglycerate kinase 1 (PGK1) expression (11). PGK1 is an ATP-generating glycolytic enzyme that forms part of the glycolytic pathway that is often highly expressed in PCa (13) and is regulated by hypoxia-inducible factor-1α (14). Extracellular PGK1 facilitates the cleavage of plasminogen generating the vascular inhibitor angiostatin (15-18), which is known as an important regulator of an “angiogenic switch” (11). Thus, CXCL12 signaling downregulates PGK1 secretion, suggesting a mechanism for metastatic PCa to grow in tissues with high CXCL12 levels (11).

One of the most intriguing aspects of our previous study was that when PCa cells were induced to overexpress PGK1 in the bone microenvironment, extracortical bone formation was observed adjacent to the growing tumor (11). In most animal models of human PCa disease, an osteolytic bone phenotype predominates. Yet, in patients with skeletal metastases, a predominantly osteosclerotic bone phenotype is typically observed; osteolytic and mixed lesions are also common. Possible mechanisms for the osteoclastic bone phenotype include factors produced by PCa, such as bone morphogenetic protein (BMP), transforming growth factor-β, insulin-like growth factor, fibroblast growth factor, and platelet-derived growth factor (19). Other possibilities include alterations in the local levels of vascular endothelial growth factor (VEGF) secreted by PCa regulates the bone formation indirectly by supporting angiogenesis (20). At present, however, the molecular mechanisms of the osteoblastic progression at the skeletal metastatic site in PCa remain unclear (4, 21).

In this study, we explored the role of PGK1 secreted by PCa in the development of an osteosclerotic bone phenotype. We show that PCa-derived PGK1 induces osteoblastic commitment from bone marrow stromal cells (BMSC) and inhibits osteoclast formation. Moreover, overexpression of PGK1 in PCa cells induces expression of several osteoblastic markers. These results suggest that PGK1 may serve as a key moderator of bone remodeling at metastatic bone sites.
Results

Local Expression of PGK1 by PCa Induce Bone Formation In vivo

Previously, we showed that the secretion of the glycolytic enzyme PGK1 by PCa cells enhanced the formation of extra-cortical-woven bone in vivo (11). To determine whether PGK1 secreted by PCa regulates bone formation, PCa cell lines overexpressing PGK1 (PC3\textsuperscript{PGK1}) or control vector (PC3\textsuperscript{Control}) were injected intratibially into immune-deficient mice. After 4 weeks, the animals were euthanized and the skeletal lesions were evaluated. Significantly more osteoblastic bone formation (Fig. 1A and C) and less osteoclastic bone resorption (Fig. 1B and C) were found in the PC3\textsuperscript{PGK1} cell–bearing animals than the PC3\textsuperscript{Control} cell–injected animals. When the bones of the PC3\textsuperscript{PGK1} cell–injected animals were evaluated for the expression of the osteoblast-specific transcription factor Runx2, higher levels of expression were noted compared with animals injected with PC3\textsuperscript{Control} cells (Fig. 1D and E). Moreover, the levels of bone-specific alkaline phosphatase and osteocalcin in the serum recovered from animals injected with the PC3\textsuperscript{PGK1} cells were increased compared with animals bearing PC3\textsuperscript{Control} cells (Fig. 1F). These data suggest that PGK1 is secreted by PCa induces bone formation by increasing osteoblastic activities and/or decreasing osteoclastic functions.

To further explore the bone formation effects of PCa cells that secrete different levels of PGK1, and to verify that the aforementioned results were not cell line specific, additional cell lines overexpressing PGK1 were established and used in conjunction with an in vivo model of bone formation that was recently developed by our group, which uses transplantation of vertebral bodies (22). First, to evaluate the efficiency of transfections, ELISA for PGK1 that is secreted by PCa cell lines (PC3\textsuperscript{Control}, PC3\textsuperscript{PGK1}, C4-2B\textsuperscript{Control}, and C4-2B\textsuperscript{PGK1}) were done to show that enhanced secreted PGK1 levels were achieved (Fig. 2A). Next, PCa cells were injected directly into the vertebral bodies derived from 4- to 7-day-old animals, and transplanted into immunodeficient hosts (Fig. 2B). Microcomputed tomography (micro-CT) was done at 4 weeks to evaluate the bone growth of the vertebral bodies (Fig. 2C–E). As expected, the bone growth was greater in the vertebral bodies.
transplanted with PGK1-overexpressed PCa, compared with controls (Fig. 2C-E). Interestingly, significantly more osteoblastic bone formation occurred (Fig. 2F and G), whereas less vascularization was found in the vertebral bodies that were implanted with PGK1-overexpressed PCa than controls (Fig. 2H and I). These data further suggest that PGK1 secreted by PCa regulates bone formation.

**Figure 2.** PGK1-derived from PCa is involved in the bone formation in vivo. A, PGK1 levels in CM from PCa cells (PC3Control, PC3PGK1, C4-2BControl, and C4-2BPISK1) were evaluated by ELISA. Columns, mean; bars, SD. Significant difference is from controls. B, Experimental schema. PCa cells (1 x 10^6 cells; n = 4) were injected into vertebral bodies and then vertebral bodies were implanted into severe combined immunodeficient (SCID) mice. At 4 wk, C, three-dimensional micro-CT measurements of the vertebral bodies were done. Bone parameters including D bone mineral density and E bone volume fractions were calculated. Columns, mean; bars, SD. Significant difference is from controls. F, Masson’s trichrome stain of the bone/tumor interface. Original magnification, x40. Bar, 100 μm. G, The numbers of the osteoblasts on the bone surface in F. Columns, mean; bars, SD. Significant difference is from controls. H, The histologic evaluation of microvessel growth (factor VIII) in tumors with different levels of PGK1. Original magnification, x40. Arrows, microvessels. Bar, 100 μm. I, Factor VIII-positive area from D. Columns, mean; bars, SD. Significant difference is from controls. These data showed that PCa-derived PGK1 is involved in the bone formation in vivo.

**PGK1 by PCa Regulates Osteoblastic Differentiation of BMSCs**

To determine if PGK1 secreted by PCa is able to induce osteoblastic induction of mixed BMSCs, BMSCs were treated with conditioned medium (CM) derived from the various PCa cell lines. BMSCs treated with PC3Control CM expressed less Runx2 mRNA compared with BMSCs treated with PC3PGK1 CM, whereas CM from C4-2BPISK1 did not have an osteoblastic effect on BMSCs (Fig. 3A). To determine if PGK1 is directly able to induce BMSCs toward an osteoblastic lineage, BMSCs were treated in vitro with rhPGK1. As positive controls for factors that induce a bone phenotype, BMSCs were also treated with BMP2 and BMP6 (Fig. 3B and C). At 21 days, BMSCs were examined for Runx2 expression and mineralization of their extracellular matrix by staining for Alizarin Red. The data show that PGK1-treated BMSCs express more Runx2 mRNA (Fig. 3B) and mineralized their extracellular matrix to a greater extent than did vehicle-treated BMSCs (Fig. 3C). To further determine the role of PGK1 in bone formation, PGK1 was overexpressed in BMSCs (BMSCPGK1, Fig. 3D and E). The expression of Runx2 mRNA (Fig. 3F) and alkaline phosphatase levels (Fig. 3G) of the BMSCPGK1
were considerably higher than the levels expressed by BMSC<sup>Control</sup> cells. To validate the *in vitro* findings, $2 \times 10^6$ BMSC<sup>Control</sup> and BMSC<sup>PGK1</sup> cells were implanted into immune competent mice. At 5 weeks, the resulting bony ossicles were evaluated by micro-CT and histologic analyses. A round dense bony ossicle was visualized by micro-CT in the implants generated with BMSC<sup>PGK1</sup> cells, whereas little or no signal was found in the implants derived from BMSC<sup>Control</sup> cells (Fig. 3H). Subsequent histologic analyses showed less bone was formed in the implants established with BMSC<sup>Control</sup> cells compared with implants containing BMSC<sup>PGK1</sup> cells (Fig. 3I). These data suggest that PGK1 secreted by PCa regulates osteoblastic commitment of BMSCs, and further suggest that PGK1 plays an important role in the bone formation *in vivo*.

**FIGURE 3.** PGK1 induces osteoblastic differentiation. To determine if PGK1 induces mesenchymal osteoblastic differentiation, the BMSCs ($2 \times 10^5$ cells per well) were plated onto a 24-well plate with osteogenic media. A. The BMSCs were treated with 10% (v/v) CM derived from PCa cells (PC3<sup>Control</sup>, PC3<sup>PGK1</sup>, C4-2B<sup>Control</sup>, and C4-2B<sup>PGK1</sup>). At 21 d, the expression of Runx2 mRNA in BMSCs was determined by real-time RT-PCR. Significant difference is from control. N.S., no significant. B. BMSCs were treated with rhBMP2, rhBMP6, and rhPGK1. At 21 d, the expressions of Runx2 mRNA in BMSCs were determined by real-time RT-PCR. Significant difference is from vehicle treatment. C. Representative Alizarin Red staining from B. Next, to determine the role of PGK1 in bone formation, BMSCs were overexpressed PGK1 or control vectors. D. Western blot analysis of PGK1 expression in BMSC<sup>Control</sup> and BMSC<sup>PGK1</sup> cells. E. The quantitative data of D. F. The expression of Runx2 mRNA was compared by real-time RT-PCR and normalized to β-actin as a loading control. G. Alkaline phosphatase activity in the cell lysates was evaluated by ELISA. Significant difference is from BMSC<sup>Control</sup>. In H and I, BMSC<sup>Control</sup> or BMSC<sup>PGK1</sup> ($2 \times 10^6$ cells; $n = 3$) was implanted in gelatin sponges into C57BL/6 mice. At 5 wk, (H) three-dimensional micro-CT measurements of the bony ossicles were done. I. Representative H&E staining of the bony ossicles. Columns, mean; bars, SD. These data showed that PGK1 secreted by PCa cells induces osteoblastic commitment from BMSCs. Arrows, positive areas. Bar, 100 μm.

**PGK1 by PCa Inhibits Osteoclastogenesis**

Next, we explored if PGK1 directly regulates osteoclastogenesis. The rationale for these studies was that the histologic analyses suggested that reductions in osteoclast numbers were associated with increased bone formation (Fig. 1A-E). For these studies, mixed marrow mononuclear cells (MMC) and osteoclast precursor cell line RAW264.7 cells were treated with RANKL, rhPGK1, and CM derived from PCa cells (PC3<sup>Control</sup>, PC3<sup>PGK1</sup>, C4-2B<sup>Control</sup>, and C4-2B<sup>PGK1</sup>) for 7 days. Thereafter, TRAP staining was done and multinucleated TRAP-positive cells in the cultures were counted. As expected, the RANKL enhanced osteoclastogenesis in both MMCs and RAW264.7 cells (Fig. 4A-D). Interestingly, compared with the control cells, CM from PC3<sup>PGK1</sup> and C4-2B<sup>PGK1</sup> prevented osteoclast formation by both MMCs and RAW264.7 cells (Fig. 4A-D).
Moreover, rhPGK1 itself had no direct effects on the generation of osteoclasts (Fig. 4A-D). To address whether PGK1 itself affects osteoclastogenesis, MMCs and RAW264.7 cells were treated with either RANKL (50 ng/mL), rhPGK1 (10-50 ng/mL), or 10% (v/v) CM derived from PCa cells (PC3 Control, PC3PGK1, C4-2B Control, and C4-2BPGK1). At 7 d, TRAP staining was done. A. Representative TRAP-positive cells of the MMC cultures. Original magnification, ×20. Bar, 50 μm. B. The numbers of TRAP-positive cells from A. C. Representative TRAP-positive cells of the RAW264.7 cells. Original magnification, ×20. Bar, 50 μm. D. The numbers of TRAP-positive cells from C. Columns, mean; bars, SD. Significant difference is from control. To evaluate if PGK1 inhibits osteoclastogenesis, (E) MMCs (1 x 10^5 cells per well) or (F) RAW264.7 cells (3 x 10^4 cells per well) were plated onto a 96-well plate. The cells were treated with RANKL (50 ng/mL) and/or rhPGK1 (10-50 ng/mL). At 7 d, TRAP staining was done. Columns, mean; bars, SD. Significant difference is from RANKL treatment. These data showed that PGK1 inhibits the osteoclast formation.

Expression of PGK1 by PCa Induces an Osteoblastic Phenotype

Another explanation of the bone formation in the tumor metastatic sites may be that PCa cells themselves express an osteoblastic phenotype. To address this possibility, we compared the expression of the osteoblastic markers between PGK1-overexpressed PCa, PGK1-knocked down PCa, and controls. Significantly more Runx2 expression in PC3PGK1 and C4-2BPGK1 was detected by Western blot (Fig. 5A, B, and D) and by immunohistochemistry (Supplementary Fig. S1A and B; Fig. 5F and G), whereas significantly less Runx2 expression in PC3PGK1 and C4-2BPGK1 was detected by Western blot (Fig. 5A, B, and D) and by immunohistochemistry (Supplementary Fig. S1A and B; Fig. 5F and G), as shown in Fig. 5H and I, alkaline phosphatase activities in the cell lysates of the PC3PGK1 and C4-2BPGK1 cells were also elevated, compared with the controls. In comparison, alkaline phosphatase activities in the cell lysates of the PC3PGK1 and C4-2BPGK1 cells declined, compared with the controls (Fig. 5H and I). These data suggest that tumor-derived PGK1 regulates the expression of bone-related phenotypes.

Discussion

Skeletal metastases resulting from PCa typically result in osteosclerotic bone lesions. Yet, in most animal models of human
disease, osteolytic lesions are most commonly observed (19). The molecular mechanisms that account for the predominantly osteoblastic skeletal phenotype in humans of PCa disease remain unclear (4, 21). Recently, we observed CXCL12 signaling plays a major role in localizing PCa to the marrow (6-12). One of the major down-stream targets of CXCL12 signaling is the inhibition of PGK1 expression (11). Secreted PGK1 acts as a disulfide reductase that cleaves plasminogen generating the vascular inhibitor angiostatin (15-18). Limiting PGK1 secretion provides a mechanism for metastatic PCa to grow in tissues with high CXCL12 levels by promoting angiogenesis (11). However, during the course of these studies, we observed that overexpression of PGK1 in PC3 cells resulted in excessive formation of woven bone reminiscent of the osteoblastic lesions typically observed in PCa disease. Here, we determined that PCa cell lines that overexpress PGK1 have enhanced expression of several osteoblastic markers. In addition, PGK1 expression in PCa induced osteoblastic commitment from mixed bone marrow progenitor cells. Moreover, overexpression of PGK1 by PCa inhibited osteoclast bone formation. These results suggest that PGK1 plays a central role in regulating the induction of expression of osteoblast-specific phenotypes by three potential mechanisms that are inhibiting osteoclastic activity, enhancing osteoblast formation, and possibly altering the phenotype of the cancer cell itself into osteomimetic cells.

One mechanism whereby PCa expression of PGK1 is likely to play a role in the expression of an osteoblastic bone phenotype is to regulate osteoblastic commitment of mesenchymal stem or progenitor cells. In this study, we observed that addition of PGK1 directly to BMSCs, or CM derived from the PCa cells, had a stimulating effect on the formation of mineralized bone nodules. However, how PGK1 regulates mineralization is

FIGURE 5. PGK1 expression in PCa cells regulates expression of an osteoblastic phenotype. To determine if PGK1 induces an osteoblastic phenotype in PCa cells, PGK1 was overexpressed and knocked down in PC3 and C4-2B cells. A, Western blot analysis for Runx2 and PGK1 expression in the PCa cells (PC3Control, PC3PGK1, PC3PGK1, C4-2BControl, C4-2BPAGK1, C4-2BPAGK1, and C4-2BPGK1). Quantitative assessment of data from (B and C) PC3Control, PC3PGK1, PC3PGK1, and C4-2BPGK1 cells and (D and E) C4-2BControl, C4-2BPAGK1, C4-2BPAGK1, and C4-2BPGK1 cells. Runx2-positive cells from immunohistochemistry for Runx2 expressed by (F) PC3Control, PC3PGK1, PC3PGK1, and C4-2BPGK1 cells and (G) C4-2BControl, C4-2BPAGK1, C4-2BPAGK1, and C4-2BPGK1 cells using monoclonal antibodies. H and I, Alkaline phosphatase activity (ALP) in the cell lysates of the PCa cells was elevated by ELISA. Columns, mean; bars, SD. Significant difference is from controls. These data showed that PCa-derived PGK1 regulates their own expression of osteoblastic phenotypes.
not clear. One possible scenario is that PGK1 may play a role in the selective survival of MSCs during hypoxic events. Recent work by Balduino et al. (23) have shown that MSC populations seem to localize to the subendosteal region, which in other studies have been shown to represent areas of hypoxia in the marrow (24, 25). Alternatively, PGK1 may be directly involved in promoting the expansion of osteoblastic progenitor cells or the commitment of preosteoblasts to a mature phenotype. It is also conceivable that PGK1 functions by an alternative mechanism in that several reports have shown that mineralization in vivo during development occurs most efficiently following vascular invasion of the bone analogue. In fact, it seems just as likely that PGK1 could function to promote osteogenesis independent of its effects on the vasculature (26-28). However, the expression of PGK1 was previously noted to be exceptionally strong in odontogenic epithelial cells and the surrounding mesenchymal cells of the tooth germ from embryonic day (E) 10.5 to E18.0, suggesting that PGK1 expression may in fact be a feature of a mineralizing phenotype (29).

Normal bone remodeling is a dynamic balance of bone forming and bone degrading activity. One mechanism that may lead to an overall increase in bone formation when PGK1 is overexpressed in the context of PCa is to either reduce osteoclast numbers or activities. Our data suggest that PCA-derived PGK1 inhibits osteoclast formation, which would serve to uncouple bone formation from resorption. How this occurs is also not clear. Hypoxia and hypoxia-inducible factor-1α develop the osteolytic bone metastases by suppressing osteoblast differentiation and promoting osteoclastogenesis (30). One clue as to the mechanism comes from our previous work that shows that overexpression of PGK1 in PCa cells results in the reduction of interleukin 8 and VEGF synthesis (11). Although VEGF is involved in the vascular formation, VEGF also plays an important role in the osteoclast activity (31-33). In osteopetrotic (op/op) mice that have lack of the functional macrophage colony-stimulating factor, known to activate the osteoclast formation, a single injection of VEGF rescued their osteoclast recruitment (31, 32). The osteoclast differentiation is also regulated by receptor activator of NF-κB ligand (RANKL; ref. 31). However, we recently found that the changing PGK1 expression in PCa did not alter the RANKL levels in PCa (data not shown). Thus, PGK1 may prevent the osteoclastogenesis by suppressing VEGF secretion.

That PGK1 could regulate the expression of an osteomimetic phenotype by PCa cells themselves is a real possibility in that we observed that PGK1 induces the expression of Runx2. Runx2 is a transcriptional factor known to induce osteoblastic differentiation (34, 35). We detected more Runx2 mRNA and protein when PGK1 was expressed in PCa cells or in lesions generated by PGK1 overexpressing PCa cells. In addition, overexpression of PGK1 also resulted in enhanced expression of osteocalcin and alkaline phosphatase by PCa cells. Each of which are critical components of the osteoblast phenotype. In other studies, osteoblastic PCa cells (MDA PCa 2a and MDA PCa 2b) have been shown to participate in the generation of osseous tissues through Runx2 signaling pathway; whereas little osteoblastic reaction are typically found in the osteolytic PCa cells (PC3; ref. 34). Previous work by our group has shown that Wnt/BMPs signaling pathway plays a crucial role in determining the phenotype of the bone metastatic lesion in PCa (36, 37). Although the detailed molecular mechanisms remain unclear, PGK1 may be involved in the mineralization by PCa themselves. However, a large screen of Wnt/BMPs signaling cascades were unable to identify which if any members of the pathway were direct PGK1 targets (data not shown).

Our previous data show that high levels of PGK1 are essential for tumor growth, but limit angiogenesis when secreted extracellularly. However, at sites of high CXCL12 production such as bone, lymph node, and liver, however, PGK1 secretion is likely to be inhibited. Thus, CXCL12 signaling through CXCR4 generates an angiogenic switch that may be necessary for metastatic growth (11). Together, these data show that the chemokine axis and PGK1 represent at least one of the critical determinants for metastasis of PCa as well as a mechanism for a proangiogenic switch that promotes tumor growth. Yet, it is difficult to reconcile our previous report with our current knowledge of the role of PGK1 in PCa bone disease. From a therapeutic standpoint, stimulating PGK1 expression would seem to represent a reasonable strategy if indeed its major effect would be to limit tumor growth at metastatic sites such as the marrow. On the other hand, enhanced PGK1 expression would seem to promote the osteosclerotic phenotype of PCa disease associated with bone metastases. For PGK1 to become an effective therapeutic target, further knowledge as to how to shift the balance from tumor growth without negatively impacting bone health will be required.

In summary, our work suggests that there are three possible mechanisms whereby PGK1 secreted by PCa cells may regulate mineral deposition. PGK1 expression may drive mesenchymal stem or progenitor cells down the osteoblastic lineage providing more mature osteoblasts relative to osteoclasts, while at the same time suppressing osteoclast formation. PGK1 may also alter the ability of PCa cells to participate in the mineralization process. Although further investigation is needed, these results suggest that PGK1 serves as a key modulator of the bone remodeling at the tumor metastatic site.

Materials and Methods

Cell Cultures

PC3 was obtained from the American Type Culture Collection. C4-2B was derived from the parental LNCaP cell lines that were serially passaged in mice to obtain a more metastatic cell line (38). RAW 264.7, a murine osteoclast precursor, was also obtained from the American Type Culture Collection. PC3 and C4-2B were cultured in RPMI 1640 (Invitrogen) and RAW 264.7 was cultured in α-MEM (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 1% (v/v) penicillin-streptomycin (Invitrogen), and 1% (v/v) l-glutamine (Invitrogen). All cultures were maintained at 37°C, 5% CO2, and 100% humidity.

Bone Marrow Cell Preparations

The marrow was flushed from the femur, tibia, and humeri with Hank’s buffer salt solution (Life Technologies) using a 5-mL syringe fitted with a 23-gauge needle. A single-cell suspension was obtained by gentle agitation through the syringe. Debris and remaining cellular aggregates were removed by passing the cell suspension over a 40-μm mesh nylon cell
strainer (BD Biosciences). The cells were plated in α-MEM with 10% fetal bovine serum and 1% penicillin and streptomycin. After 3 d, nonadherent cells were removed and fresh media were replaced.

PGK Expression Constructs and Small Interfering RNA Knockdown of PGK1

A 1.33-kb human PGK1 cDNA was isolated by reverse transcription-PCR from total RNA extracted from PC3 cells (11). The forward and reverse primers were 5′-AGTACA-TATGTGCTTTCTTAACAGTCT-3′ (positions, 80-100) and 5′-AGTAGATCCCTAATGCAATGGAATGCA-3′ (positions, 1409-1389), respectively. For small interfering RNAs knockdown of PGK1, two groups of primers corresponding to nucleotide sequences in open reading frame were synthesized [position, 186-203; Si1. 5′-gatcccaAAAAACCGACGGCTATCTCTTaaaggaAGAGAGTTTtttta-3′ (forward oligonucleotide) and 5′-gatcccaAAAGCTTGCTGGTTCttctcaagagaACAGCACCTTAATCCTTttttta-3′ (forward oligonucleotide) and 5′-gatcccaAAAGCTTGCTGGTTTGTTttctcaagagaACAGCACCTTAATCCTTttttta-3′ (forward oligonucleotide)] and 5′-gatcccaAAAAACCGACGGCTATCTCTTaaaggaAGAGAGTTTtttta-3′ (forward oligonucleotide) and 5′-gatcccaAAAGCTTGCTGGTTTGTTttctcaagagaACAGCACCTTAATCCTTttttta-3′ (forward oligonucleotide)]. As scrambled primers, [5′-gatcccaAAAAACCGACGGCTATCTCTTaaaggaAGAGAGTTTtttta-3′ (forward oligonucleotide) and 5′-gatcccaAAAAACCGACGGCTATCTCTTaaaggaAGAGAGTTTtttta-3′ (forward oligonucleotide)] were used. PCa cells were transfected by lipofection (PC3Control , PC3PGK1, PC3SiControl , PC3SiPGK1, C4-2BControl, C4-2BPGK1, C4-2BSiControl , and C4-2BSiPGk1; ref. 11).

For construction of full-length PGK1-expressing lentiviral vector, infectious viral particles were prepared by cotransfection of CM into the packaging cells. BMSC were transfected with human PGK1-expressing vector (BMSCPGK1) or scrambled control vector (BMSCControl).

Generation of CM

PCa cells were seeded on 100-mm tissue culture dishes at a concentration of 2 × 10⁶ cells per well in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum. After 48 h, supernatant was collected, cleared from any free-floating cells by centrifugation for 5 min at 1,000 g, filtered through a 0.2-mm sterilizing filter, and then stored at −80°C. The concentrations of CM were normalized to total protein.

Animals

All experimental procedures were approved by the University of Michigan Committee for the Use and Care of Animals. C57BL/6 mice (4- to 6-wk-old) and male severe combined immunodeficient mice (4- to 6-wk-old) were obtained from Charles River Laboratories. Male athymic (nude) mice (4- to 5-wk-old) were purchased from Harlan Bioscience. Animals were caged under standard conditions and fed a laboratory diet and tap water ad libitum.

RNA Extraction and Real-time Reverse Transcription-PCR

Total RNA was isolated using RNeasy Mini kit (QIAGEN), and first-strand cDNA was synthesized in a 20-μL reaction volume using 0.4 μg of total RNA. Reverse transcription products were analyzed by real-time PCR in TaqMan Gene Expression Assays of several target genes: mouse BMP2, BMP6, Runx2, osteocalcin, and β-actin (Applied Biosystems). Real-time PCR analysis was done using 15.0 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 1.5 μL of TaqMan Gene Expression Assay (forward and reverse primers at 18 μmol/L and Taqman probe at 5 μmol/L), 1 μL of the cDNA, and 12.5 μL of RNase/DNase-free water in a total volume of 30 μL. Reactions without template and/or enzyme were used as negative controls. The 2nd step PCR reaction was run for 40 cycles (95°C for 15 min) after an initial single cycle of 95°C for 30 s and 60°C for 5 min to activate the Taq polymerase. The PCR product was detected as an increase in fluorescence using an ABI PRISM 7700 instrument (Applied Biosystems). RNA quantity (Cq) was normalized to the housekeeping gene β-actin control by using the formula Cq = 2^((Ct - Ct control) / Ct control).

Immunohistochemistry

The PCa cell–injected tissues were fixed in 10% formalin at 4°C and decalcified in 10% EDTA (pH 7.4) for paraffin embedding. Seven-micrometer sections of the decalcified long bones were then cut with a microtome (Leica RM2125 RT; Leica Microsystems). Masson's trichrome staining (Sigma-Aldrich) and TRAP staining (Sigma-Aldrich) were done and data were expressed as numbers of osteoblasts and osteoclasts per millimeter of bone surface. Sections were also stained with anti-Runx2 antibody (1:250, rabbit polyclonal; Abcam), anti–von Willebrand factor (factor VIII-related antigen) antibody (1:250, rabbit polyclonal; Dako North America), or an IgG isotype–matched control (Sigma-Aldrich).

PCa cells (PC3Control, PC3PGK1, PC3SiControl, PC3SiPGK1, C4-2BControl, C4-2BPGK1, C4-2BSiControl, and C4-2BSiPGK1) were cultured in Lab-Tek II 4-chamber slides (Nalge Nunc International)) at 5 × 10⁴ cells per chamber. After 24 h, the cells were fixed in 4% paraformaldehyde for 25 min at room temperature, and endogenous peroxidase activity quenched with 75 mmol/L NH₄Cl and 20 mmol/L Glycine in PBS at room temperature for 10 min. Thereafter, the cells were incubated with anti–Runx2 antibody or an IgG isotype–matched control. Antibody detection was done by using a rabbit HRP-AEC staining kit (R&D Systems) for 1 h at room temperature. Images were acquired on a Zeiss LSM 510 microscope. The staining intensities of the slides were quantified with Image J software (version 1.4; NIH), and data were normalized to the total cell numbers.

ELISAs

Alkaline phosphatase activity (Sigma-Aldrich) and osteocalcin levels (Biomedical Technologies) of the cell lysates and animal serum were determined by double-antibody sandwich method assembled with commercially available components, according to the directions of the manufacturer. PGK1 levels were determined by direct ELISA using 100 μL of 5 μg/mL.
In vitro Osteoblastogenesis

The cells were lysed by freeze thawing in ice-cold lysis buffer [50 mmol/L Tris-HCl, 1% NP40, 120 mmol/L NaCl, 1 mmol/L EDTA, 25 mmol/L NaF, 40 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1.0% mammalian protease inhibitor cocktail (Sigma-Aldrich)]. The nuclei and cellular debris were removed by centrifugation at 16,000 × g for 15 min at 4°C. SDS-PAGE was done in 10% polyacrylamide gels. Each lane contained 30 μg of cell (lysate) protein. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). Residual protein binding sites on the membrane were blocked with TBS with Tween 20 buffer containing 5% nonfat dry milk. The membranes were then incubated with the primary antibody [a monoclonal anti-PGK1 IgM antibody (39) and an anti-Runx2 antibody] over-night. After washing with TBS with Tween 20, the secondary antibody (antispecies-specific horseradish peroxidase) was added. Finally, the proteins were visualized by autoradiography using an enhanced chemiluminescence detection system (Amersham Pharma). The densities of the bands were quantified with Image J software (version 1.40; NIH).

Western Blot Analyses

The cells were lysed by freeze thawing in ice-cold lysis buffer [50 mmol/L Tris-HCl, 1% NP40, 120 mmol/L NaCl, 1 mmol/L EDTA, 25 mmol/L NaF, 40 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1.0% mammalian protease inhibitor cocktail (Sigma-Aldrich)]. The nuclei and cellular debris were removed by centrifugation at 16,000 × g for 15 min at 4°C. SDS-PAGE was done in 10% polyacrylamide gels. Each lane contained 30 μg of cell (lysate) protein. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). Residual protein binding sites on the membrane were blocked with TBS with Tween 20 buffer containing 5% nonfat dry milk. The membranes were then incubated with the primary antibody [a monoclonal anti-PGK1 IgM antibody (39) and an anti-Runx2 antibody] overnight. After washing with TBS with Tween 20, the secondary antibody (antispecies-specific horseradish peroxidase) was added. Finally, the proteins were visualized by autoradiography using an enhanced chemiluminescence detection system (Amersham Pharma). The densities of the bands were quantified with Image J software (version 1.40; NIH).

In vitro Osteoblastogenesis

BMSCs (1 × 10⁵ cells per well) were plated onto a 24-well culture plate with osteogenic media containing 50 μg/mL ascorbic acid (Sigma-Aldrich) and 10 mmol/L β-glycerophosphate (Sigma-Aldrich). BMSCs were treated with either vehicle, rhBMP2 (200 ng/mL; R&D Systems), rhBMP6 (200 ng/mL; R&D Systems), or rhPGK1 (50 ng/mL). In some case, 10% (v/v) CM derived from PCa cells (PC3Control, PC3PGK1, C4-2BControl, and C4-2BPGK1) were added to the culture. At 21 d, osteoblastogenesis from BMSCs were evaluated by real-time reverse transcription-PCR (RT-PCR) and Alizarin Red staining (Sigma-Aldrich).

In vitro Osteoclastogenesis

MNCs (1 × 10⁵ cells per well) or RAW 264.7 cells (3 × 10⁴ cells per well) were plated onto 96-well culture plates. Cells were treated with RANKL (50 ng/mL; R&D Systems) and/or rhPGK1 (10-50 ng/mL) every other day for 7 d. In some case, 10% (v/v) CM derived from PCa cells (PC3Control, PC3PGK1, C4-2BControl, and C4-2BPGK1) were added to the culture. Thereafter, osteoclastogenesis was evaluated by TRAP staining (Sigma-Aldrich).

Intratibial Injections

PC3Control and PC3PGK1 cells were inoculated intratibially to measure the effect of PGK1 on bone formation. The animals were anesthetized, and both legs were cleaned with betadine and 70% ethanol. Thereafter, the cells (1 × 10⁵ cells/10 μL) were injected into the cortex of the anterior tuberosity of the tibia with a drill-like motion to prevent cortical fracture using a 25-μL syringe fitted with a 25-gauge needle. After 4 wk, animals were euthanized and tibias were fixed in 10% formalin at 4°C. Tibias were further decalcified in 10% EDTA (pH 7.4) for 10 d and embedded in paraffin.

Vertebral Body Transplants

Vertebral body transplants were done, as previously described (22). Lumbar vertebrae were isolated from mice 4 to 7 d after birth. The vertebrae were sectioned into single vertebral bodies. Severe combined immunodeficient mice were used as transplant recipients. Four vertebral bodies per mouse were implanted into subcutaneous pouches. Before implantations, PCa cells (PC3Control, PC3PGK1, C4-2BControl, and C4-2BPGK1) were introduced into vertebral bodies (1 × 10⁵ cells/10 μL of PBS). Vertebral bodies were collected at 4 wk.

Bony Ossicles Transplants

BMSCControl and BMSCPGK1 were assessed for their potential to form bony ossicles in vivo. Cells (2 × 10⁵ cells) were incorporated into a gelatin sponge (Gel-foam; Pharmacia and Upjohn). These cell/scaffold constructs were transplanted s.c. into 4- to 5-wk-old male C57BL/6 mice. At 5 wk, the bony ossicles were harvested and fixed in aqueous buffered zinc formalin for 24 h at 4°C.

In vivo Assessment of Bone Formation

For micro-CT analysis, specimens were scanned at 8.93 μm voxel resolution on a micro-CT scanner (EVS Corporation), with a total of 667 slices per scan. GEMS MicroView software (GE Healthcare Bio-sciences) was used to make a three-dimensional reconstruction from the set of scans. A fixed threshold (1, 500) was used to extract the mineralized bone phase, and actual bone volume fraction and bone mineral density (BMD) were calculated. For histomorphometry, specimens were paraffin embedded, sectioned, stained for H&E.

References

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