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PEDF regulates osteoclasts *via* osteoprotegerin and RANKL

Toru Akiyama [a,b], Crispin R. Dass [a,*], Yusuke Shinoda [b], Hirotaka Kawano [b], Sakae Tanaka [b], Peter F.M. Choong [a,c]

[a] Departments of Orthopaedics and Surgery, University of Melbourne, St. Vincent's Health, Melbourne, Australia
[b] Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan
[c] Bone and Soft Tissue Sarcoma Service, Peter MacCallum Cancer Centre, Melbourne, Australia

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ABSTRACT

Bone homeostasis is maintained through a balance between bone formation and resorption. Bone resorption is mainly carried out by a specific type of cell called the osteoclast (OCL). Previously, expression of pigment epithelium-derived factor (PEDF), the most potent endogenous inhibitor of angiogenesis, has been demonstrated in bone tissue and it known to induce differentiation in osteoblastic cells. Furthermore, therapeutic effects of PEDF on osteosarcoma, a prevalent primary bone tumor, with inhibition of bone destruction has been shown. Thus, PEDF is possibly involved in bone homeostasis as an inhibitor of bone resorption. To address this involvement, we studied the effect of PEDF on OCL function. OCL differentiation, RANKL-mediated survival and bone resorption activity were inhibited by PEDF in a dose-dependent manner. PEDF upregulated osteoprotegerin (OPG), which naturally blocks OCL maturation, in primary osteoblasts and OCL precursor cells. These results suggest that PEDF inhibits OCL function *via* regulating OPG expression, and thereby contributes to the maintenance of bone homeostasis.

Introduction

Pigment epithelium-derived factor (PEDF) is a pluripotent molecule, with neurotrophic, anti-angiogenic, anti-proliferative and pro-differentiation functions [1]. The anti-angiogenic effect of PEDF is far greater than any other known endogenously produced factor. Malignant bone tumor progression requires a multistep process including uncontrolled proliferation, angiogenesis, tissue invasion, bone destruction and acclimation to the secondary tumor microenvironment. Crucially, PEDF is active at all these steps of tumorigenesis. PEDF is localized in regions of active bone formation in the mid-gestation mouse embryo and there are high levels of secretion by osteoblasts [2]. A more focused study demonstrated that PEDF was expressed by osteoblasts lining the bone spicules in the ossification zone of metaphyseal bone, as well as by osteoblasts lining cortical periosteum [3].

Previously, we demonstrated a therapeutic effect of PEDF on osteosarcoma, which is the most common primary malignant bone tumor [4–8]. Interestingly, the activity of PEDF against osteosarcoma is strictly limited to a bone milieu [4]. PEDF inhibited not only tumor growth but also bone destruction (osteolysis) by tumor. PEDF also enhances differentiation in pre-osteoblasts, and also forces osteosarcoma cells to assume a more differentiated status [4]. Collectively, these data suggest that PEDF activity is somehow dependent on the presence of the skeletal system.

The homeostasis of skeletal tissue is maintained by a balance of bone resorption and bone formation. OCL cells are primarily involved in not only physiological but also pathological bone resorption including osteoporosis, rheumatoid arthritis and tumor-induced bone disease [9,10]. In osteolytic lesions of metastatic bone tumor, bone destruction is not caused by the direct effects of cancer cells on bone. It is in fact caused by the OCLs induced by the tumor, that by their action on bone, cause not only a pathway for tumor cells to invade but also release critical growth factors that allow tumor cells to survive [11].

Although the involvement of PEDF in bone homeostasis has not been suggested, previously, it was reported that a relationship between bone resorption and angiogenesis exists in erosive bone pathologies such as bone metastasis and rheumatoid arthritis. TNP-470, an angiogenesis inhibitor decreased osteoclastic bone resorption at the interface between tumor and bone and inhibited the formation of osteolytic bone metastasis *in vivo* [1]. Vascular endothelial growth factor (VEGF), a potent pro-angiogenic factor, facilitates OCL bone resorption activity and elongates OCL survival [2]. Moreover, VEGF receptor-1 signaling is essential for OCL development in macrophage colony-stimulating factor (M-CSF) deficient mice. OCL formation is mediated by the receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [3]. Hence, angiogenesis correlates with osteolytic lesion formation through OCL induction. However, the

* Corresponding author. Address: Department of Orthopaedics, St. Vincent's Health, P.O. Box 2900, Fitzroy 3065, Melbourne, Australia. Fax: +61 3 9416 3610.
E-mail address: crispin.dass@svhm.org.au (C.R. Dass).

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relationship between intrinsic angiogenic inhibitors and OCL function has not been reported yet. Our study is the first report of the linkage between bone biology and an intrinsic anti-angiogenic factor.

Materials and methods

Chemicals. Recombinant human M-CSF and soluble RANKL were from Sigma (St. Louis, MO, USA). α-Modified minimum essential medium (α-MEM), fetal bovine serum (FBS) and dispase were purchased from Invitrogen (Rockville, MD, USA). Bacterial collagenase, $1\alpha,25(OH)_2D_3$, and prostaglandin E2 were purchased from Sigma (St. Louis, MO, USA). Recombinant PEDF and PEDF antibody for neutralizing PEDF were purchased from MD BioProducts (Maryland, Middletown, MD, USA). PEDF and osteopontin antibodies for immunohistochemistry were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Type-I collagen antibody was purchased from Southern Biotechnology (Birmingham, AL, USA).

Cell culture. Ethics for animal use for this study was prior obtained from the St. Vincent's Health Animal Ethics Committee. Newborn and 5-week-old male Balb/c mice were used to obtain large numbers of OCLs for biochemical analyses, and a co-culture system [4] was used. Mouse bone marrow cells (2×10^5/well) were co-cultured with osteoblastic cells (1×10^4/well) for 5 days in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 1 μM prostaglandin E2 in 48-well plates. The cells were then stained for TRAP, and the number of OCL-like $TRAP^+$ multinucleated (>3 nuclei) cells was counted. The crude OCL preparation was placed on 48-well plastic plates, washed with PBS, and treated with 200 μL of PBS containing 0.1% collagenase and 0.2% dispase for 10 min to remove osteoblastic cells [5].

Derivation and culture of OCLs, primary osteoblasts and primary embryonic chondrocytes. For the OCL differentiation assay, cells from mouse bone marrow were cultured in α-MEM containing 10% FBS with 50 ng/ml M-CSF for 24 h. Adherent cells were harvested and cultured with 10 ng/ml M-CSF and 200 ng/ml sRANKL. After 3–5 days of culture, cells were fixed and stained for TRAP. $TRAP^+$ cells containing more than three nuclei were considered to be multinucleated OCLs. For osteoblast cultures, calvariae of neonatal mice were digested by 0.1% collagenase and 0.2% dispase five times, and cells isolated by the last three digestions were combined and cultured in α-MEM containing 10% FBS, 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate at a density of 1×10^5 cells/well in a 12-multiwell plate [6]. For chondrocyte cultures, primary chondrocytes were prepared from ventral rib cages of E18.5 embryonic mice. Chondrocytes were isolated from rib cages by digestion of cartilage with 0.1% collagenase and 0.2% dispase. Cells were then plated at a density of 5×10^4 cells/cm^2 and cultured in DMEM containing 10% FCS and 1% L-glutamine for 10 days. Inorganic phosphate (Pi) was added 24 h after incubation in medium containing low-serum (0.5%) [7].

Gene transcription. mRNA from cultured cells was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Sydney, Australia). Mouse RT-PCR primers were as follows:

gapdh, 5′-GTATGTCGTGGAGTCTACTGGTGT and
3′-TACTCCTTGGAGGCCATGTAGGCC,
nfatC1, 5′-CAACGCCCTGACCACCGATAG and
3′-GGCTGCCTTCCGTCTCATAGT,
f4/80, 5′-GAATCTTGGCCAAGAAGAGAC and
3′-GAATTCTCCTTGTATATCATCAGC,
col II, 5′-GCCTCGCGGTGAGCCATGATC and
3′-CTCCATCTCTGCCACGGGGT,
colX, 5′-GCAACTAAGGGCCTCAATGG
and 3′-GAGCCACTAGGAATCCTGAG,
opg, 5′-CCAAAGTGAATGCCGAGAGT and
3′-TTGTGAAGCTGTGCAGGAAC,
rankl, 5′-AGCCGAGACTACGGCAAGTA and
3′-ATTGATGGTGAGGTGTGCAA.

Osteopontin and osteocalcin primers were designed based on a previous report [8]. An Eppendorf MasterCycler (Australia) and 36 cycles were used at the respective or relatively close theoretical T_{ann}, T_{ext} = 72 °C, T_{den} = 94 °C. The Expand Reverse Transcriptase-PCR and PCR Master (Roche Diagnostics, Australia) Kits were utilized according to the manufacturer's instructions. A water control (no cDNA template) was always included in each series of PCRs. PCR products were run on a 1% agarose gel electrophoresis and sizes confirmed using a 100 bp ladder marker set (Invitrogen, Sydney, Australia).

Survival of OCLs. OCLs were purified 24 h after treatment and some of the cultures were subjected to TRAP staining. OCLs adhere to the bone surface through specialized discrete structures called "podosomes", which consist mainly of dots containing F-actin [5]. The rounded and sporadic appearance with ringed structure of podosomes (actin ring) is a characteristic of polarized OCLs. Cell viability was expressed as morphologically intact $TRAP^+$ multinucleated cells. Other cultures were further incubated for the indicated times, and then the number of living OCLs was counted. The number of viable cells remaining at the different time points is shown as a percentage of total cells at time zero.

Pit formation assay. The pit formation assay was performed according to a protocol previously described [4] except that osteologic slides were used. Briefly, mature OCLs were seeded onto 16-well BD BioCoat Osteologic® calcium phosphate-coated quartz slides. Forty-eight hours after seeding mature OCLs on osteologic slides (Bedford, MA, USA), slides were treated with NH_4OH for 10 min. The resorbed area was measured using an image analysis system Image J linked to a Nikon Eclipse TE2000-U microscope (Nikon, Australia) and photographed with SPOT Advanced software (SciTech, USA).

In vivo inflammatory bone destruction assay. Balb/c mice, 1 week old (n = 6), were administered a local calvarial injection of lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at 25 mg/kg body weight. At the same site of LPS injection, the mice were subcutaneously administered with recombinant human PEDF at 100 ng per kg weight (n = 6) or control normal saline (n = 8) for 4 successive days. Five days after LPS injection, the number of OCLs per millimetre of trabecular bone surface was measured.

Histology of murine bone. The harvested limbs were fixed in 4% *p*-formaldehyde for 24 h. All tissues were embedded in paraffin for histological analysis according to standard conditions without decalcification. Immunohistological procedures were performed according to a protocol previously described [12]. For von Kossa staining, deparaffinized specimens were subjected to dehydration using a series of ethanol gradients. The slides were stained with 5% silver nitrate solution under halogen light for 10 min, incubated in 5% sodium thiosulfate for 5 min, rinsed in distilled water and allowed to dry [6]. For ALP staining, deparaffinized slides were stained with Alkaline/Acid Phosphatase Assay Kit (Millipore, North Ryde, NSW, Australia). Tartrate-resistant acid phosphatase (TRAP) staining was performed according to the manufacturer's protocol (Acid Phosphatase, Leukocyte Kit from Sigma–Aldrich, Sydney, NSW, Australia).

Statistical analyses. In vitro and *in vivo* data were analyzed for statistical significance using the Student's *t*-test. Means, standard deviations and degrees of significance are shown on individual data graphs in the 'Results' section. A probability (p) value of <0.05 was considered significant unless otherwise indicated.

Results

PEDF is expressed mainly in calcifying skeletal tissue

To determine the expression of PEDF in skeletal tissues, we performed immunohistochemical analysis using antibodies for PEDF and several prevalent bone markers. Strong expression of PEDF was observed mainly in periosteum and calcified cartilage of E18.5 embryo mice radius, which was co-localized with osteopontin (OPN) (indicating maturing osteoblasts), Von Kossa staining (indicating calcified lesion) and tartrate-resistant acid phosphatase (TRAP) enzymatic staining (indicating OCLs) (Supplementary Fig. 1). On the other hand, PEDF was hardly detectable in immature osteoblasts, resting and proliferative chondrocytes. Type-I collagen and alkaline phosphatase were expressed in all osteoblasts and mature chondrocytes (Supplementary Fig. 1).

PEDF stimulated OPG expression in OCL precursor cells and osteoblasts

RT-PCR revealed that mRNA of PEDF expressed in OCL (Fig. 1A), osteoblast (Fig. 1B) and chondrocyte (Fig. 1C). In all types of cells evaluated, the expression level was not affected by cell differentiation status. Thus, it seems that the *in vivo* expression pattern is controlled by protein stability or protein–protein affinity. PEDF prevented OCL precursor cells from differentiating to mature OCLs, and instead channeled them to differentiate into macrophages (Fig. 1A). PEDF administration increased osteoprotegerin (OPG) expression level in both bone marrow-derived cells (Fig. 1D) and primary osteoblasts (Fig. 1E). OPG is an intrinsic inhibitor of RANKL, by acting as a decoy receptor for RANKL [11]. The RANKL expression level of primary osteoblasts was completely suppressed by PEDF (Fig. 1E). The osteoblast supports OCL differentiation *via* RANKL presentation on its cell surface *in vivo*. Thus, PEDF suppressed RANKL activity directly and indirectly.

PEDF inhibited OCL differentiation in vitro

RANKL is an OCL differentiation factor. Hence, in light of the findings above, PEDF can be considered an inhibitory factor for OCL differentiation. PEDF administration inhibited OCL formation in not only the RANKL/M-CSF system (Fig. 2A) but also co-culture system where OCLs were cultured with osteoblasts (Fig. 2B). Further proof of this phenomenon was attained when a PEDF neutralizing antibody supported OCL formation in a dose-dependent manner (Fig. 2C).

PEDF abrogated RANKL-dependent OCL survival and bone resorption activity

RANKL is not only an OCL differentiation factor but also a factor promoting OCL survival and bone resorption activity. PEDF inhibited RANKL-dependent OCL survival, but not survival reliant on M-CSF (Fig. 3A). OCL bone resorption activity was suppressed by PEDF dose-dependently (Fig. 3B). These data suggest that PEDF effects on OCLs depend on RANKL inhibition by OPG expression.

PEDF suppressed OCL induction in vivo

These observations prompted us to examine the *in vivo* efficacy of PEDF for the treatment of OCL-mediated pathological conditions, using a model of endotoxin-induced inflammatory bone destruction. Injection of LPS into the calvaria bone induced sixfold more OCLs as control (Fig. 4A, B and E). Administration of PEDF resulted in marked inhibition of OCL formation at the site of inflammation (Fig. 4D and E), and PEDF administration without LPS stimulation failed to show an alteration in bone biology (Fig. 4C). Thus, exogenous application of PEDF indeed has a beneficial effect against bone destruction in this disease model.

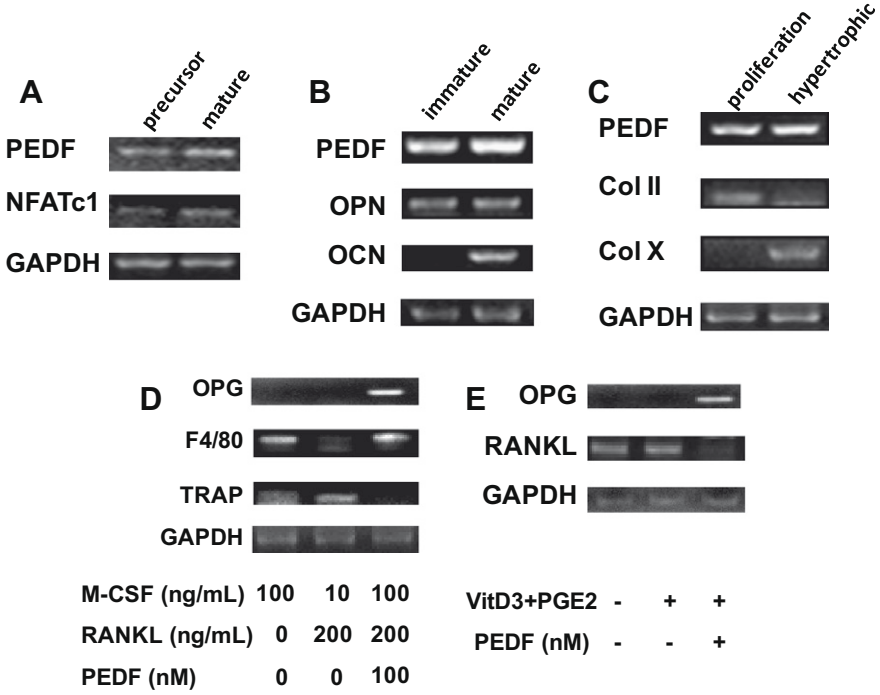

Fig. 1. Expression of PEDF in OCLs, osteoblasts and chondrocytes. PEDF expression was detected in murine OCLs (A), osteoblasts (B) and chondrocytes (C). NFATc1 is a master gene of OCL differentiation. Osteopontin (OPN) is a marker of osteoblast differentiation. Osteocalcin (OCN) is a marker for mature osteoblast. Type-II collagen (Col II) is a marker for proliferative chondrocyte, Type X collagen (Col X) is for hypertrophic chondrocyte. PEDF expression levels in each cell is not altered with the differentiation status. PEDF administration stimulated OPG expression in bone marrow-derived cells (OCL precursor) (D) and primary osteoblasts (E). PEDF upregulates F4/80 macrophage (immature OCL marker) in bone marrow-derived cells and suppressed the secretion of OCL differentiation factor RANKL in primary osteoblasts.

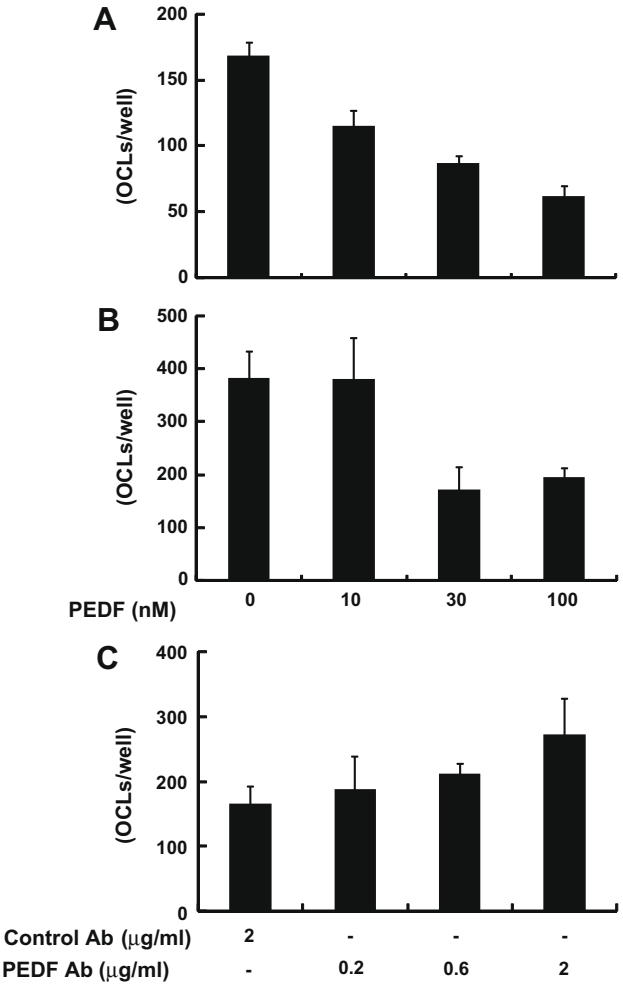

Fig. 2. PEDF suppresses OCL formation. PEDF suppressed OCL formation in the presence of RANKL and M-CSF (A) and when co-cultured with osteoblasts (B). PEDF neutralizing antibody dose-dependently supported OCL formation in the presence of RANKL and M-CSF (C).

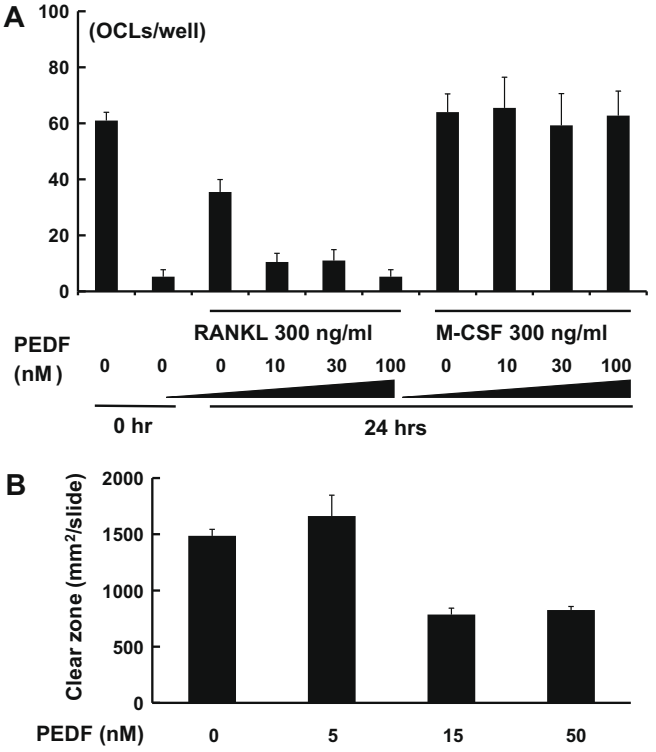

Fig. 3. PEDF inhibits RANKL-mediated OCL survival. Although RANKL-mediated OCL survival was inhibited by PEDF, M-CSF-mediated OCL survival was not (A). PEDF suppressed OCL bone resorption activity (B) at a threshold between 5 and 15 nM.

Discussion

PEDF is a 50 kDa secreted glycoprotein, identified and isolated from conditioned medium of cultured primary human fetal retinal pigment epithelial cells [9]. PEDF is widely expressed throughout fetal and adult tissues, including the adult brain, spinal cord, plasma, liver, lung, eye, heart, and bone [10]. PEDF was identified as a neurotrophic factor at first, but it also possesses neuro-protective, pro-differentiation, anti-proliferative and anti-angiogenic functions [13]. PEDF has been shown to be the most potent endogenous inhibitor of angiogenesis, being more than twice as potent as angiostatin, and more than seven times as potent as endostatin.

PEDF is a rather interesting candidate for an anti-cancer biological drug. Tumor growth and progression requires unchecked proliferation, undifferentiated cellular status, invasive capacity, and abundant angiogenesis. In the case of one type of tumor, osteosarcoma, all these biological phenomena yield to regulation by PEDF [4,5]. Interestingly, the anti-tumor effects are limited to bone milieu, not observed in soft tissue tumor. Hence, PEDF probably disturbs tumor development mechanisms that are idiosyncratic to bone.

OCLs are the primary cells for physiological and pathological bone resorption. Recent studies have shown that osteolysis at the interface between bone and metastatic bone tumor is caused by OCL stimulation – not by the direct effects of cancer cells on bone [11]. The importance of the relationship between bone metabolism and angiogenesis has been reported repeatedly, and the effects of VEGF are closely related to the activation of OCL function [2]. OCLs express two distinct VEGF receptors, KDR/Flk-1 and Flt-1. VEGF stimulates the bone-resorbing activity of OCLs and partially enhances the survival of OCLs [2]. VEGF can substitute for M-CSF, and Flt-1 signaling is essential in OCL development [14,15]. A single injection of M-CSF or VEGF can induce OCL recruitment in op/op mice, which are osteopetrotic because of M-CSF deficiency [16]. Flt-1 tyrosine kinase-deficient homozygous mice (flt-1($TK^{-/-}$)) develop normal vessels and survive. However, VEGF-induced macrophage migration is strongly suppressed and mild OCL reduction without bone marrow suppression is observed in flt-1($TK^{-/-}$) mice [17].

However, the effects of anti-angiogenic factors on bone metabolism have not been reported. Previously, we reported that PEDF was expressed by osteoblasts lining the bone spicules in the ossification zone of metaphyseal bone, as well as by osteoblasts lining cortical periosteum [12]. Another group reported that both osteoblasts and OCLs expressed PEDF as well [15]. PEDF possesses a type-I collagen binding motif [18]. Type-I collagen is not only an angiogenic scaffold but also an important matrix for bone formation. Thus, these reports suggest that PEDF is linked to bone homeostasis.

Our current data demonstrates that PEDF inhibits OCL formation, survival and bone resorption activity *via* OPG induction in OCL precursor cells and osteoblasts. To date, the contribution of an anti-angiogenic factor to bone homeostasis has not been reported. The retinas of $PEDF^{-/-}$ mice have malpositioned vessels, irregular pigmentation and a reduced number of ganglion cells [19]. Their pancreases appear enlarged and have an excessive number of blood vessels. Their prostates showed hyperplasia. PEDF was

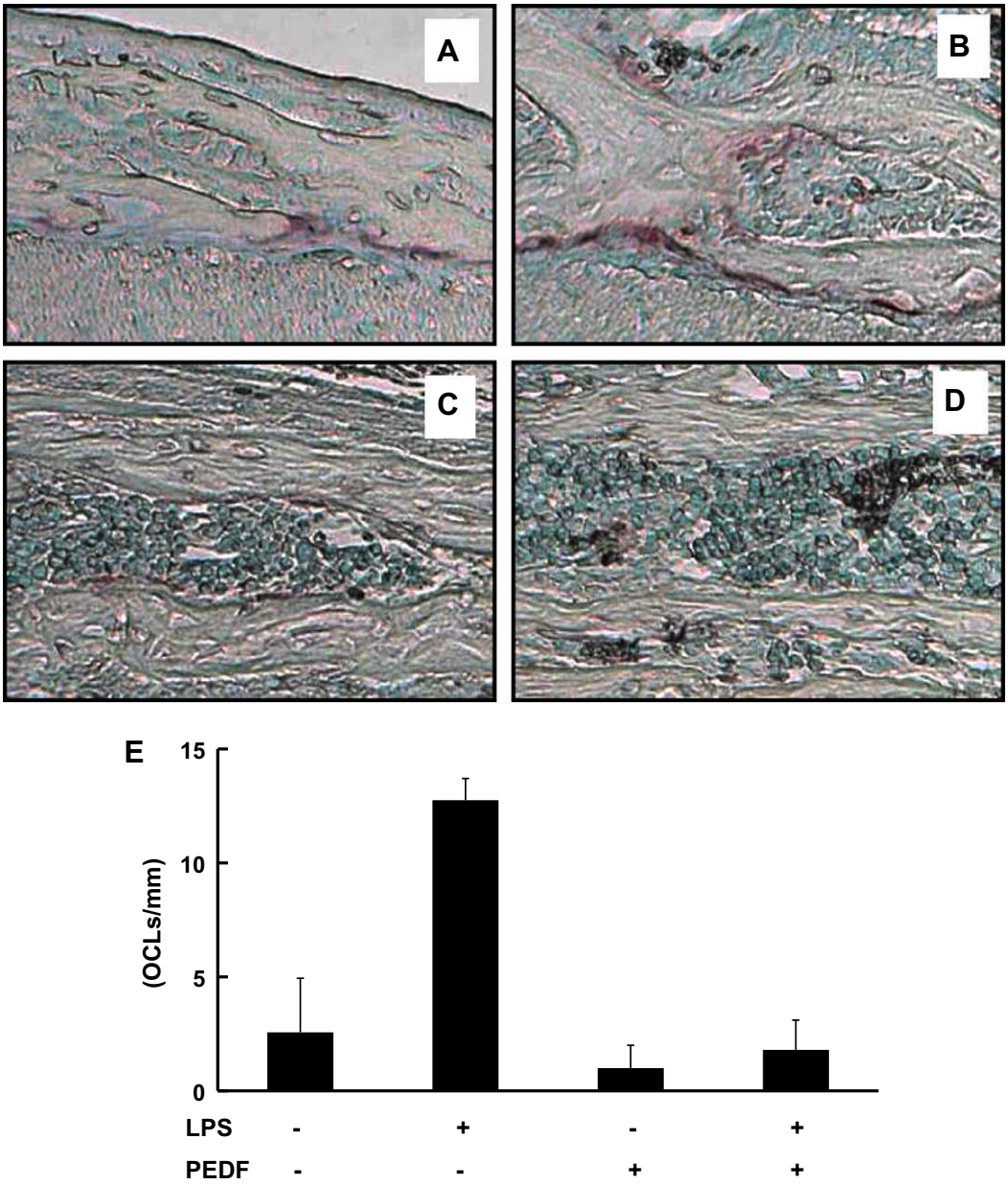

Fig. 4. PEDF inhibits OCL numbers *in vivo*. PEDF inhibited OCL induction in a lipopolysaccharide (LPS)-induced inflammation model. Control (A), LPS (B), PEDF (C) and LPS + PEDF (D). LPS-induced OCLs were suppressed to baseline levels with PEDF treatment (E).

a functional inhibitor of prostate angiogenesis. However, skeletal abnormalities in PEDF knockout mice have not been reported. Prostate and bone are sex hormone-responsive organs. Interestingly, androgens can stimulate VEGF secretion and increase prostate vascularity [16]. PEDF had opposite effects on PEDF. Furthermore, prostate cancer shows a decreased expression level of PEDF [17].

Hormone therapy is a common therapeutic strategy for pathologies involving hormone-sensitive organs including prostate, breast, ovary and bone [3,18]. Thus, PEDF is a potential mediator of hormone therapy for some tumors. Actually, PEDF inhibits prostate and ovarian cancers *in vitro*. PEDF suppresses angiogenesis and induces differentiation in prostate cancer [19]. PEDF is estrogen-sensitive in ovarian surface epithelium cells and inhibits the development of human ovarian cancer *in vitro* [20]. Decreased expression level of PEDF in breast cancer is associated with increased tumor progression [21].

The prognosis of osteosarcoma in females is better than in males, and males are more frequently affected than females [22]. Our current data and previous reports suggest the existence of the correlation between skeletal system and PEDF in various fields including sex hormone regulation and pathologies involving the bone. Moreover, osteolytic skeletal disorders including not only osteoporosis but also rheumatoid arthritis, tumor-induced bone diseases, periodontal disorders, periprosthetic implant loosening and Paget's disease are mediated by OCLs [11]. Thus, we propose that PEDF may have potential therapeutic value against skeletal disorders, though further studies are required to test these *in vivo*, and to delineate the exact molecular signaling pathways involved.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.139.

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