Published in final edited form as:
J Cell Biochem. 2013 November ; 114(11): 2471–2478. doi:10.1002/jcb.24592.

Erythropoietin Supports the Survival of Prostate Cancer, But Not Growth and Bone Metastasis

Yusuke Shiozawa[1], **Samantha McGee**[1], **Michael J. Pienta**[1,2], **Natalie McGregor**[2], **Younghun Jung**[1], **Kenji Yumoto**[1], **Jingcheng Wang**[1], **Janice E. Berry**[1], **Kenneth J. Pienta**[2], and **Russell S. Taichman**[1,*]

[1]Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, Michigan, 48109

[2]Departments of Urology and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, 48109

Abstract

Erythropoietin (Epo) is used in clinical settings to enhance hematopoietic function and to improve the quality of life for patients undergoing chemotherapy by reducing fatigue and the need for transfusions. However, several meta-analyses have revealed that Epo treatments are associated with an increased risk of mortality in cancer patients. In this study, we examined the role of Epo in prostate cancer (PCa) progression, using in vitro cell culture systems and in vivo bone metastatic assays. We found that Epo did not stimulate the proliferation of PCa cell lines, but did protect PCa cells from apoptosis. In animal models of PCa metastasis, no evidence was found to support the hypothesis that Epo enhances metastasis. Together, these findings suggest that Epo may be useful for treating severe anemia in PCa patients without increasing metastatic risk.

Keywords

PROSTATE; CANCER; ERYTHROPOIETIN; TUMOR; GROWTH; METASTASIS

Although high-dose chemotherapy and radiotherapy prolong survival in cancer patients, the side effects of these therapies, including pancytopenia, remain serious concerns. Pancytopenia, a condition in which the bone marrow is not able to supply sufficient numbers of blood cells to the body, occurs due to the cytotoxic effects of dose-intensive treatments on rapidly dividing hematopoietic progenitor cells and slowly dividing hematopoietic stem cells (HSCs). Under these conditions, external compensation for blood loss is often required, including red blood cell and platelet transfusions. White blood cell transfusions, while feasible, are rarely performed, in large measure due to allergic reactions.

Although a blood transfusion is a simple medical treatment, which can immediately replace blood loss, it is also a potential source of infection [Finucane et al., 2000]. Therefore, to minimize the risks to patients, alternative treatments are often considered. Among the well-accepted treatment options for pancytopenia is supportive care with growth factors and/or

*Correspondence to: Russell S. Taichman, D.M.D., D.M.Sc., Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Room 3307, 1011 North University Avenue, Ann Arbor, MI 48109-1078. rtaich@umich.edu.

Conflict of interest: None.

The present address of Kenneth J. Pienta is Departments of Urology, Oncology, and Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Brady Urological Institute, 600 N Wolfe Street, Marburg 121, Baltimore, MD, 21287-2101.

cytokines that stimulate blood production. Granulocyte colony-stimulating factor (G-CSF) has been widely used to stimulate white blood cell production [Dempke et al., 2000]. Thrombopoietin is involved in platelet production, but is not used for the treatment of thrombocytopenia despite numerous trials, due to the frequency of side effects [Dempke et al., 2000]. Erythropoietin (Epo) is the primary growth factor known to regulate the production of erythrocytes [Dempke et al., 2000]. Although Epo improves quality of life (QoL) in anemic patients, there remains considerable controversy over the use of Epo for cancer patients as an alternative to blood transfusion.

In adults, the major source of Epo is the kidney, from which it travels through the blood stream to the bone marrow to control red blood cell production. In addition to its enhancement of red blood cell production, Epo has recently been demonstrated to have protective effects on non-erythropoietic tissues (e.g., neuroprotection and cardioprotection) through its receptor (EpoR) [Arcasoy, 2008]. This suggests that Epo not only improves hematopoietic conditions, but also provides other health benefits. Yet several meta-analyses have revealed an association between Epo treatment and increased mortality in cancer patients [Dicato and Plawny, 2010; Hedley et al., 2011]. Moreover, it has been demonstrated that various cancer cells express EpoR [Acs et al., 2001; Arcasoy et al., 2003, 2005; Jeong et al., 2008], and that Epo may promote tumor progression by stimulating tumor growth [Acs et al., 2001], or angiogenesis [Ribatti, 2010]. To date, however, little is known regarding the mechanisms whereby Epo may stimulate tumor progression, the roles of EpoR in tumor progression, and the relationship between Epo and metastasis.

Recently, we reported that high dose Epo enhances bone formation by directly stimulating HSC production of BMPs, and by activating bone formation by mesenchymal stem cells [Shiozawa et al., 2010]. We also recently demonstrated that prostate cancer (PCa) cells that disseminate to the bone marrow compete directly with HSCs for occupancy of the HSC niche [Shiozawa et al., 2011]. We therefore hypothesized that one mechanism whereby Epo could increase mortality in cancer patients is by expanding the number of HSC niches, and therefore increasing the opportunity for metastasis. In this study we found that Epo did not stimulate the growth of PCa cells in vitro, nor did Epo enhance PCa bone metastasis in vivo. However, we did observe that Epo protected PCa cell lines from apoptosis. Although our findings reveal that Epo may not directly support PCa tumor growth and metastasis to bone, there remains a significant need to further understand the biological effects of Epo treatment in cancer.

MATERIALS AND METHODS

CELL CULTURE

The human PCa cell line PC3 and normal human prostate epithelial cell line RWPE1 were obtained from the American Type Culture Collection (Rockville, MD). The human metastatic subline LNCaP C4-2B was originally isolated from a lymph node of a patient with disseminated bony and lymph node involvement [Wu et al., 1998]. PCa cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% (v/v) fetal bovine serum (FBS; Invitrogen), and 1% (v/v) penicillin–streptomycin (Invitrogen), and maintained at 37°C, 5% CO_2, and 100% humidity.

RNA EXTRACTION AND REAL-TIME RT-PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized in a 20 μl reaction volume using 0.4 μg of total RNA. RT products were analyzed by real-time PCR in TaqMan® Gene Expression Assays for human EpoR and β-actin (Applied Biosystems, Foster City, CA). Reactions without template and/or enzyme

were used as negative controls. The PCR reactions were run for 50 cycles (95°C for 15 s and 60°C for 1 min) after an initial single cycle of 50°C for 2 min and 95°C for 10 min. The PCR product was detected as an increase in fluorescence using an ABI PRISM 7,700 instrument (Applied Biosystems). RNA quantity (C_R) was normalized to the housekeeping gene β-actin by using the formula $\mathbf{C_R = 2(40 - C_t \text{ of sample}) (40 - C_t \text{ of control}).}$ The threshold cycle (C_t) is the cycle at which a significant increase in fluorescence occurs.

FLOW CYTOMETRY

PCa cell lines and RWPE1 cells were stained with APC anti-human monoclonal EpoR antibody (R&D systems, Minneapolis, MN). The levels of EpoR were analyzed by a FACSAria dual laser flow cytometer (Becton Dickinson, Franklin Lakes, NJ). In some cases, PCa cells and RWPE1 cells (80–90% confluent) were cultured in 6-well plates in RPMI medium without FBS for 5 h. After serum starvation, the cells were treated with 20 U/ml of Epo (EPOGEN; Amgen, Thousand oaks, CA) for 30 min. The phosphorylation of p44/42 MAP kinase (Thr202/Tyr204), or Erk1/2, and Akt were analyzed with flow cytometry, using total Erk1/2 and Akt as internal controls. Antibodies for phosphorylated Erk1/2, phosphorylated Akt, total Erk1/2, and total Akt were purchased from Cell Signaling Technology.

WESTERN BLOT ANALYSES

PCa cells and RWPE1 cells were extracted for protein and analyzed for EpoR (R&D systems) by Western blot analysis. β-actin (Cell Signaling Technology) was used as an internal control for loading.

SDS–PAGE was performed in 10% polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were then incubated with the primary antibody overnight, followed by horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Danvers, MA). Proteins were visualized by autoradiography using an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ).

PROLIFERATION ASSAYS

PCa cells were plated into triplicate 96-well plates at a concentration of 5,000 cells per well in growth medium with 0.1% FBS. After 24 h, cells were treated with serial doses of Epo. Thereafter, the cultures were incubated in an atmosphere of 5% CO_2 and 95% O_2 at 37°C for 2 days. Proliferation was quantified using sodium 3′-[1-[(phenyl-amino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) colorimetric assay (Sigma–Aldrich, St. Louis, MO). Optical intensities were read on a multiwall scanning spectrophotometer at OD_{492} (Molecular Devices, Sterling Heights, MI).

APOPTOSIS ASSAYS

PCa cells (80–90% confluent) were cultured in 6-well plates in RPMI medium without FBS for 5 h. After serum starvation, the cells were treated with 5 U/ml of Epo for 24 h. The cells were stained for apoptosis using annexin V PE (BD Biosciences, San Jose, CA) and 7AAD (BD Biosciences), according to the manufacturer's protocol, and apoptotic cells were quantified by flow cytometry.

IMMUNOHISTOCHEMISTRY

Murine bones were harvested, fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin, and 5–7 μm sections were prepared. Masson's trichrome staining (Sigma–Aldrich) was done, and measurements were made of numbers of osteoblasts per

millimeter of bone surface using NIS elements (Nikon Instruments Inc., Melville, NY) imaging software. For each condition, slides were counted in eight fields. In some case, murine bones were stained with antibodies to SDF-1 (Abcam, Cambridge, MA: pre-stained with Zenon® Alexa Fluor® 488 rabbit IgG_1 labeling kit; Invitrogen). Confocal laser scanning was performed with a FV500 microscope (Olympus, Center Valley, PA).

IN VIVO METASTASIS ASSAYS

Luciferase-expressing PC3 ($PC3^{Luc}$) cells were introduced into male SCID mice by intracardiac injections. For 4 weeks prior to or post tumor inoculation, the recipient mice were treated by intraperitoneal injections with Epo (6,000 U/kg/day, three times per week) or vehicle. Bone metastatic regions were measured by bioluminescence imaging. Total tumor burden of each animal was calculated using regions of interest (ROI) that encompassed the entire animal.

COL2.3Δ-TK ANIMALS AND TREATMENTS

Transgenic mice bearing a fusion gene composed of the 2.3-kb fragment of the rat type I collagen α1 (Col1α1) promoter and HSV-TK (Col2.3Δ-TK) [Visnjic et al., 2001] were graciously provided by Dr. David W. Rowe (University of Connecticut, Farmington, CT). Four- to 6-week-old mice bearing the Col2.3ΔTK transgene and age-matched control CD-1 mice were intraperitoneally injected with ganciclovir (Cytovene-IV; Roche, Nutley, NJ; 3–8 mg/kg per day), or vehicle (0.9% saline) for 3 weeks with/without PTH ($hPTH_{1–34}$; Bachem, Torrence, CA; 50–80 μg/kg per day), or Epo (6,000 U/kg/day, three times per week) injections.

BONE MARROW TRANSPLANTATION ASSAYS

Prior to bone marrow transplantation, mice were treated with PTH or Epo to expand the bone marrow niche. Recipient mice were exposed twice to 570 cGy given 3 h apart in a gamma cell 40 cesium source. One hour later the mice were injected intravenously with a mixture of a radioprotective dose of CD45.2 cells (2×10^5 cells) and CD45.1 donor cells in 100 μl. Starting at 4 weeks after transplantation and continuing for at least 16 weeks, the CD45.1 phenotypes were measured in peripheral blood.

STATISTICAL ANALYSES

All in vitro experiments were performed at least three times with similar results. Results from representative assays are shown. Numerical data are expressed as mean ± SEM. Statistical analysis was performed by ANOVA or unpaired two-tailed Student's *t*-test using GraphPad Instat (GraphPad). For QPCR assays, Kruskal–Wallis test and Dunn multiple-comparisons test was used. For all analyses, a P value <0.05 was considered significant.

RESULTS

EPO DOES NOT STIMULATE PROLIFERATION OF PCA CELLS

In many systems, the binding of Epo to its receptor is believed to regulate a variety of cellular responses, including cell proliferation and cell survival. Therefore, we evaluated the effects of Epo on PCa cells in these fundamental cellular functions. To explore the interaction of exogenous Epo treatment and EpoR expression in PCa cells, we first examined the basal mRNA expression levels of the EpoR in PCa cells by QRT-PCR. A higher level expression of EpoR was observed in PCa cells, compared to the normal prostate epithelial cell line RWPE1 cells (Fig. 1A). To further evaluate if EpoR mRNA expression is correlated with protein levels, cell surface EpoR expression was analyzed by flow cytometry using commercially available anti-EpoR antibodies. Although the expression of EpoR is

extremely low in all cell types, the level of expression of EpoR protein by PCa cells was detected and was higher than that seen in RWPE1 cells (Fig. 1B). These observations were further confirmed by Western blotting (Fig. 1C).

To determine if EpoR expressed on PCa cells is activated in response to Epo treatment, phosphorylation of Erk1/2 and Akt in treated PCa and RWPE1 cells were evaluated by flow cytometry. The data demonstrated that Epo induces phosphorylation of Erk1/2 in both PC3 and C4-2B cells, while it had no effect on RWPE1 cells (Fig. 2A). A similar trend was observed in the phosphorylation of Akt (Fig. 2B). These data suggest that activation of PCa cells by Epo through EpoR is possible.

To determine whether Epo regulates proliferation of PCa cells through EpoR, in vitro XTT assays were performed. After 2 days of culture with and without varying concentrations of Epo, we observed no effect on proliferation of the PCa cells (Fig. 2C,D).

EPO PROTECTS PCA CELLS FROM APOPTOSIS

To determine if Epo can regulate PCa survival, apoptosis initiated by serum starvation in the presence or absence of Epo was evaluated by flow cytometry using annexin V staining methods. The data demonstrate that significantly fewer apoptotic cells were detected in Epo-treated cells than controls (Fig. 3A).

The maintenance and protection from apoptosis and injury of HSCs in the marrow is predominantly a function of the HSC endosteal niche, a region largely populated by osteoblasts [Shiozawa and Taichman, 2012]. Recently, we demonstrated that PCa cells target this region in marrow to establish metastatic footholds, and that the niche protects PCa cells from apoptosis [Shiozawa et al., 2011]. To address whether the protective role of Epo is dependent on the osteoblastic niche, we used a genetically engineered animal model, in which thymidine kinase is placed under control of the collagen I promoter (hereinafter referred to as Col2.3Δ-TK animals). In this animal model ganciclovir treatment conditionally ablates osteoblasts in the marrow [Visnjic et al., 2004]. In this experiment, parathyroid hormone (PTH) was used as a control, since it has also been shown to expand osteoblastic niches [Calvi et al., 2003]. Without ganciclovir, there were no differences in the number of hematopoietic cells in the marrow between vehicle, PTH, and Epo treatment in this animal model (Fig. 3B). In contrast, the number of osteoblasts in PTH and Epo treated animals were increased (Fig. 3C). Ganciclovir alone had no effect on the number of hematopoietic cells in the marrow of control animals (Fig. 3B), but dramatically decreased the number of osteoblasts in Col2.3Δ-TK animals regardless of treatment (Fig. 3C). Interestingly, Epo treatment prevented the cells from undergoing apoptosis initiated by the ablation of osteoblastic niche, whereas neither vehicle nor PTH treatment protected the marrow cells from apoptosis (Fig. 3B). Therefore, although this experiment was performed in the hematopoietic system, the data suggest that Epo protects cells from apoptosis, but in a niche-independent manner.

EPO DOES NOT DIRECTLY REGULATE BONE METASTASIS OF PCA CELLS

To explore if Epo is critical for the development of bone metastases in vivo, luciferase-expressing PC3 cells ($PC3^{Luc}$ cells) were inoculated into SCID mice by intracardiac injection. The animals were treated either with vehicle, Epo prior to tumor inoculation, or Epo post tumor inoculation. Development of bone metastases was followed over time using bioluminescence imaging. Surprisingly, neither pre-treatment with Epo nor treatment after tumor inoculation altered the development of bone metastases (Fig. 4A).

Recently, we demonstrated that pre-treatment of animals with anabolic dosing of PTH increased bone metastasis from PCa cells by expanding the bone marrow niche size

[Shiozawa et al., 2011]. Based upon the fact that the Epo has a protective effect on the cells in the marrow (Fig. 3B), we hypothesized that the occupancy of the marrow space by the cells protected by Epo would prevent the cell trafficking process to the marrow, even if Epo also expands the size of niche. To test this hypothesis, two sets of studies were performed. First, bone marrow transplantation assays with/without Epo treatment were performed, since the homing process of HSCs well represents the cell trafficking process to the marrow. As a control for the niche expansion, mice were also treated with PTH. There were no significant differences between treatments when analyzing % engraftment of transfused cells to the marrow of recipient mice (Fig. 4B). As before, although this experiment was performed in the hematopoietic system, the data suggest that the cell trafficking to the marrow may not be directly correlated to niche numbers.

At the molecular level, it has been well accepted that SDF-1 secreted by the bone marrow microenvironment plays an important role in bone metastasis, including PCa [Muller et al., 2001; Taichman et al., 2002]. Therefore, we hypothesized that SDF-1 expression in marrow is responsible for the differences in the metastatic potential between PTH- and Epo-treated mice, and thus SDF-1 levels in the marrow were evaluated by immunohistochemistry. As expected, PTH-treated mice express more SDF-1 in the marrow, compared to vehicle-treated mice (Fig. 4C). Surprisingly, lower levels of SDF-1 expression were observed in the bone marrow of Epo-treated mice, compared to PTH-treatments (Fig. 4C).

DISCUSSION

In this study, we examined the role of Epo in PCa tumor progression using in vitro cell culture models and in vivo metastatic assays. We found that Epo does not enhance the proliferation of PCa cell lines in vitro, however, it does prevent cells from undergoing apoptosis when stressed by serum starvation. More importantly, we observed that Epo does not affect the metastatic cascade of PCa cells in an in vivo model of metastasis. Together, our data suggest that promotion of PCa metastasis or induction of proliferation by PCa cells are unlikely to be the cause of enhanced mortality following Epo therapy in PCa.

While the biological roles of Epo in erythropoiesis and tissue protection has been revealed [Arcasoy, 2008], the use of Epo for treatment of anemia in cancer patients is still controversial. Recent clinical studies have suggested that Epo adversely affects the prognosis in cancer patients. It has been suggested that Epo promotes tumor growth through EpoR, and that Epo activates several signaling pathways (Jak2/Stat3, PI3K/AKT, MAPK) [Hedley et al., 2011]. Indeed, some in vitro studies have demonstrated that Epo enhances tumor proliferation [Westenfelder and Baranowski, 2000; Acs et al., 2001; Yasuda et al., 2003; Feldman et al., 2006]. However, others have demonstrated that no effects of Epo on tumor growth were observed in vitro [Dunlop et al., 2006; Jeong et al., 2008], and in vivo [Kataoka et al., 2010]. Our current study failed to reveal any stimulatory effects of Epo on tumor growth, however, we did find that Epo inhibits PCa cell apoptosis. As most assays to determine cell proliferation measure the number of viable cells, data obtained from these assays do not always correlate with cell proliferation. Therefore, an alternative explanation for the differences seen in various studies may be in the nature of the assays themselves. Consistent with these findings, it has been demonstrated that Epo protects tumor cells from cytotoxic insults through EpoR [Brines et al., 2004; Arcasoy, 2008]. Previous in vitro studies may have interpreted the cell protective role of Epo as cell proliferation rather than a decrease in apoptosis.

Epo is believed to activate a signal transduction cascade in cancer cells through its receptor on their surface. However, the roles of Epo in non-hematopoietic tissues, including cancer cells, remain conflicting. EpoR mRNA expression has been detected in several tumors and

cancer cell lines [Acs et al., 2001; Arcasoy et al., 2003; Arcasoy et al., 2005; Jeong et al., 2008]. Although EpoR protein was detected by flow cytometry and Western blotting in this study, there remains debate as to whether commercially available antibodies for EpoR are adequately specific and EpoR, a membrane receptor, is often detected in cytoplasm [Jelkmann and Laugsch, 2007]. Moreover, Epo may bind to diverse receptors to activate cancer cells, since EpoR exist as a multi-receptor complex with a β common receptor [Jubinsky et al., 1997; Brines et al., 2004]. As a result, the direct interaction between Epo and EpoR in cancer cells is not yet fully understood. Conversely, Epo may affect tumor progression indirectly by stimulating angiogenesis [Ribatti, 2010]. For example, studies have demonstrated that anti-angiogenic therapy reduces tumor sizes [Ingber et al., 1990; O'Reilly et al., 1994, 1997; Folkman, 1995], suggesting that vasculature surrounding tumor cells supports tumor growth. It has also been reported that Epo enhances angiogenesis by mobilizing endothelial progenitor cells (EPCs) from bone marrow [Heeschen et al., 2003; Bahlmann et al., 2004]. However, the contribution of either Epo itself or to EPC-induced tumor vascularization has not yet been observed in an in vivo setting [Jelkmann and Elliott, 2012]. In addition to these observations, endothelial cells in other non-hematopoietic tissue may also express Epo mRNA and functional EpoR, yet these results remain contentious [Sinclair et al., 2010]. Collectively, while the subject of Epo production outside of the kidney and EpoR expression remains in debate, the effects of Epo on tumor progression may be substantially limited. Clearly, further studies are needed.

Epo has been thought to improve QoL in the patients undergoing chemotherapy by reducing fatigue and the need for blood transfusions. Recent clinical trials testing the prophylactic effects of Epo on chemotherapy-induced anemia were terminated early, since Epo increased mortality in cancer patients [Leyland-Jones, 2003; Wright et al., 2007]. It was initially speculated this is due mainly to tumor growth initiated by Epo. However, growing evidence suggests that Epo may initiate thrombosis, resulting in a higher mortality [Fandrey and Dicato, 2009], although detailed mechanisms are still unknown. One potential means behind this may be due to hyperviscosity caused by increased red blood cell production and reduction of plasma volume [Spivak et al., 2009]. Epo is also believed to enhance platelet levels by stimulating the production or release of other inflammatory cytokines [Spivak et al., 2009]. Often in cancer patients, hypercoagulation and enhancedplateletaggregationareobserved.Importantly,recentstudies suggest that platelets play a crucial role in tumor progression and metastasis [Gay and Felding-Habermann, 2011]. Platelets are believed to promote the migration/invasion of tumor cells, maintain their survival in the blood stream, protect them from immunosurveillance, andsupporttheestablishmentofmetastasesinsecondarysites[Gay and Felding-Habermann, 2011]. Nevertheless, many of these postulated events in tumor progression were not mimicked in our model of bone metastasis. Therefore, a direct effect of Epo on tumor progression may be possible, or Epo may influence the metastatic cascade in different ways than were examined in our study.

Little is known regarding the impact of Epo on the metastatic process, although this drug may have profound effects on local tumor progression. It has been demonstrated that primary solid tumors spread to the marrow utilizing similar mechanisms as HSC homing [Muller et al., 2001; Taichman et al., 2002]. We have recently demonstrated that disseminated PCa cells travel to the HSC niche and then take up the residence in the niche to eventually form a metastasis [Shiozawa et al., 2011]. These findings suggest that the HSC niche plays an essential role in the development of metastasis. As seen with PTH (12), we recently reported that Epo expands the HSC niche in the marrow by either directly driving the differentiation of mesenchymal stem cells or indirectly through BMP signaling by HSCs [Shiozawa et al., 2010]. In our current study, Epo did not increase bone metastasis of PCa, despite a resultant increase in the niche with treatment in this transgenic animal model. A

potential reason for this discrepancy may be the SDF-1 response in the bone marrow to Epo and PTH. SDF-1 is a ligand for the chemokine receptors CXCR4 and CXCR7 [Sun et al., 2010]. The one of major roles of SDF-1 is to serve as a chemoattractant for CXCR4/7 expressing tumor cells and HSCs [Sun et al., 2010]. We found that PTH treatment stimulates the secretion of SDF-1 in the marrow, however, we observed here that when animals were treated with Epo, SDF-1 levels in the marrow were suppressed. These data suggest that Epo affects the HSC niche differently from PTH, and as a result these two hormones may have opposite effects on bone metastasis, even though they both expand the niche size.

Excluding venous thrombosis, adverse effects of Epo treatment for cancer patients are still largely controversial. Despite concerns about the mortality risk, Epo is, however, still a viable treatment option for cancer patients with anemia, as it could potentially improve their QoL and reduce the need for transfusions. Therefore, further well- designed, controlled clinical trials in this area are clearly warranted in order to ensure the safe use of Epo for treatment of anemia in cancer patients. Ultimately, whether prophylactic Epo treatment is appropriate should be decided on a case-by-case basis until the existence of functional EpoR, the impacts of EpoR on the prognosis, and the roles of Epo/EpoR in tumor progression are biologically determined.

Acknowledgments

This work is directly supported by the National Cancer Institute (CA093900 to K.J. Pienta and R.S. Taichman and CA163124 to Y. Shiozawa, K.J. Pienta, and R.S. Taichman), the Department of Defense (Y. Shiozawa, K.J. Pienta, and R.S. Taichman), and the Prostate Cancer Foundation (Y. Shiozawa, K.J. Pienta, and R.S. Taichman). K.J. Pienta receives support as an American Cancer Society Clinical Research Professor, NIH SPORE in prostate cancer grant P50 CA69568, and the Cancer Center support grant P30 CA46592.

Grant sponsor: National Institutes of Health; Grant numbers: P50 CA69568, P30 CA46592; Grant sponsor: National Cancer Institute; Grant numbers: CA093900, CA163124; Grant sponsor: Department of Defense; Grant sponsor: Prostate Cancer Foundation.

REFERENCES

Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A. Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res. 2001; 61:3561–3565. [PubMed: 11325818]

Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. Br J Haematol. 2008; 141:14–31. [PubMed: 18324962]

Arcasoy MO, Jiang X, Haroon ZA. Expression of erythropoietin receptor splice variants in human cancer. Biochem Biophys Res Commun. 2003; 307:999–1007. [PubMed: 12878211]

Arcasoy MO, Amin K, Vollmer RT, Jiang X, Demark-Wahnefried W, Haroon ZA. Erythropoietin and erythropoietin receptor expression in human prostate cancer. Mod Pathol. 2005; 18:421–430. [PubMed: 15467711]

Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. Blood. 2004; 103:921–926. [PubMed: 14525788]

Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick CJ, et al. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci USA. 2004; 101:14907–14912. [PubMed: 15456912]

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003; 425:841–846. [PubMed: 14574413]

Dempke W, Von Poblozki A, Grothey A, Schmoll HJ. Human hematopoietic growth factors: Old lessons and new perspectives. Anticancer Res. 2000; 20:5155–5164. [PubMed: 11326688]

Dicato M, Plawny L. Erythropoietin in cancer patients: Pros and cons. Curr Opin Oncol. 2010; 22:307–311. [PubMed: 20498598]

Dunlop EA, Percy MJ, Boland MP, Maxwell AP, Lappin TR. Induction of signalling in non-erythroid cells by pharmacological levels of erythropoietin. Neurodegener Dis. 2006; 3:94–100. [PubMed: 16909043]

Fandrey J, Dicato M. Examining the involvement of erythropoiesis-stimulating agents in tumor proliferation (erythropoietin receptors, receptor binding, signal transduction), angiogenesis, and venous thromboembolic events. Oncologist. 2009; 14(Suppl1):34–42. [PubMed: 19762515]

Feldman L, Wang Y, Rhim JS, Bhattacharya N, Loda M, Sytkowski AJ. Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells. Prostate. 2006; 66:135–145. [PubMed: 16161153]

Finucane ML, Slovic P, Mertz CK. Public perception of the risk of blood transfusion. Transfusion. 2000; 40:1017–1022. [PubMed: 10960532]

Folkman J. Seminars in medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med. 1995; 333:1757–1763. [PubMed: 7491141]

Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011; 11:123–134. [PubMed: 21258396]

Hedley BD, Allan AL, Xenocostas A. The role of erythropoietin and erythropoiesis-stimulating agents in tumor progression. Clin Cancer Res. 2011; 17:6373–6380. [PubMed: 21750199]

Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood. 2003; 102:1340–1346. [PubMed: 12702503]

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature. 1990; 348:555–557. [PubMed: 1701033]

Jelkmann W, Elliott S. Erythropoietin and the vascular wall: The controversy continues. Nutr Metab Cardiovasc Dis. 2012 In Press.

Jelkmann W, Laugsch M. Problems in identifying functional erythropoietin receptors in cancer tissue. J Clin Oncol. 2007; 25:1627–1628. author reply 1628. [PubMed: 17443006]

Jeong JY, Feldman L, Solar P, Szenajch J, Sytkowski AJ. Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells. Int J Cancer. 2008; 122:274–280. [PubMed: 17893874]

Jubinsky PT, Krijanovski OI, Nathan DG, Tavernier J, Sieff CA. The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor. Blood. 1997; 90:1867–1873. [PubMed: 9292519]

Kataoka M, Moriya Y, Moriguchi Y, Iwai T, Fujimoto-Ouchi K, Shirane M, Kondoh K, Mori K. Effect of erythropoietin on human tumor growth in xenograft models. Mol Med Rep. 2010; 3:95–101. [PubMed: 21472206]

Leyland-Jones B. Breast cancer trial with erythropoietin terminated unexpectedly. Lancet Oncol. 2003; 4:459–460. [PubMed: 12901958]

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001; 410:50–56. [PubMed: 11242036]

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell. 1994; 79:315–328. [PubMed: 7525077]

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. Cell. 1997; 88:277–285. [PubMed: 9008168]

Ribatti D. Erythropoietin and tumor angiogenesis. Stem Cells Dev. 2010; 19:1–4. [PubMed: 19886790]

Shiozawa Y, Taichman RS. Getting blood from bone: An emerging understanding of the role that osteoblasts play in regulating hematopoietic stem cells within their niche. Exp Hematol. 2012; 40:685–694. [PubMed: 22640993]

Shiozawa Y, Jung Y, Ziegler AM, Pedersen EA, Wang J, Wang Z, Song J, Wang J, Lee CH, Sud S, et al. Erythropoietin couples hematopoiesis with bone formation. PLoS ONE. 2010; 5:e10853. [PubMed: 20523730]

Shiozawa Y, Pedersen EA, Havens AM, Jung Y, Mishra A, Joseph J, Kim JK, Patel LR, Ying C, Ziegler AM, et al. Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow. J Clin Invest. 2011; 121:1298–1312. [PubMed: 21436587]

Sinclair AM, Coxon A, McCaffery I, Kaufman S, Paweletz K, Liu L, Busse L, Swift S, Elliott S, Begley CG. Functional erythropoietin receptor is undetectable in endothelial, cardiac, neuronal, and renal cells. Blood. 2010; 115:4264–4272. [PubMed: 20124513]

Spivak JL, Gascon P, Ludwig H. Anemia management in oncology and hematology. Oncologist. 2009; 14(Suppl1):43–56. [PubMed: 19762516]

Sun X, Cheng G, Hao M, Zheng J, Zhou X, Zhang J, Taichman RS, Pienta KJ, Wang J. CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. Cancer Metastasis Rev. 2010; 29:709–722. [PubMed: 20839032]

Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res. 2002; 62:1832–1837. [PubMed: 11912162]

Visnjic D, Kalajzic I, Gronowicz G, Aguila HL, Clark SH, Lichtler AC, Rowe DW. Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice. J Bone Miner Res. 2001; 16:2222–2231. [PubMed: 11760835]

Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J, Aguila HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood. 2004; 103:3258–3264. [PubMed: 14726388]

Westenfelder C, Baranowski RL. Erythropoietin stimulates proliferation of human renal carcinoma cells. Kidney Int. 2000; 58:647–657. [PubMed: 10916088]

Wright JR, Ung YC, Julian JA, Pritchard KI, Whelan TJ, Smith C, Szechtman B, Roa W, Mulroy L, Rudinskas L, et al. Randomized, double-blind, placebo-controlled trial of erythropoietin in non-small-cell lung cancer with disease-related anemia. J Clin Oncol. 2007; 25:1027–1032. [PubMed: 17312332]

Wu TT, Sikes RA, Cui Q, Thalmann GN, Kao C, Murphy CF, Yang H, Zhau HE, Balian G, Chung LW. Establishing human prostate cancer cell xenografts in bone: Induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. Int J Cancer. 1998; 77:887–894. [PubMed: 9714059]

Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, Onozaki M, Hashimoto M, Musha T, Ogawa K, et al. Erythropoietin regulates tumour growth of human malignancies. Carcinogenesis. 2003; 24:1021–1029. [PubMed: 12807756]

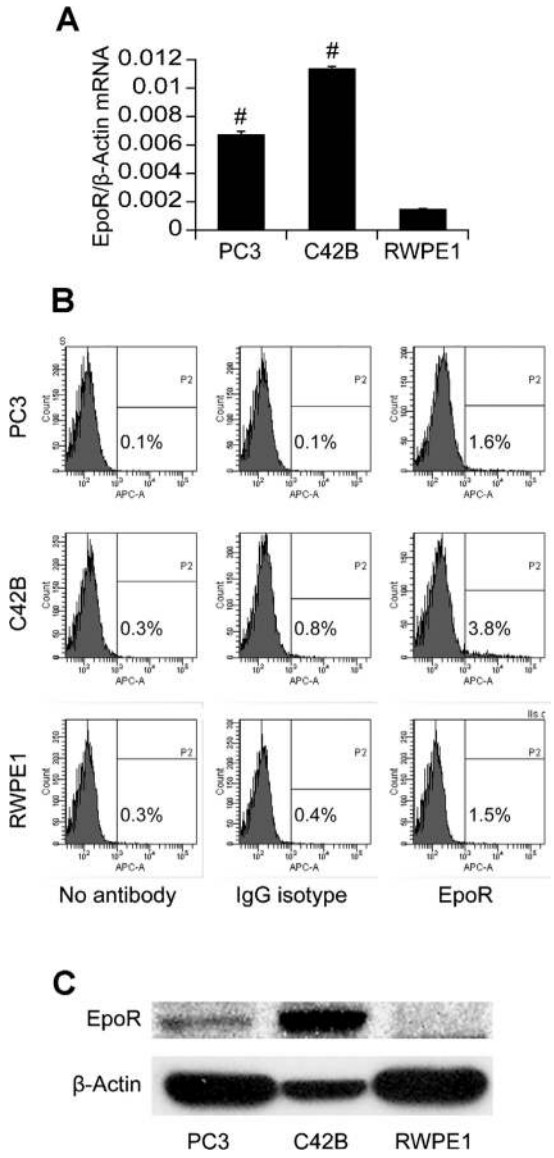

Fig. 1.

PCa cells express EpoR. (A) EpoR mRNA levels of PCa cell lines (PC3 and C4-2B) and RWPE1 cells were determined by real time RT-PCR. Data were normalized to β-actin and are presented as mean ± SEM from three independent PCRs. Representative (B) flow cytometric analyses and (C) Western blotting of EpoR on PCa cells.

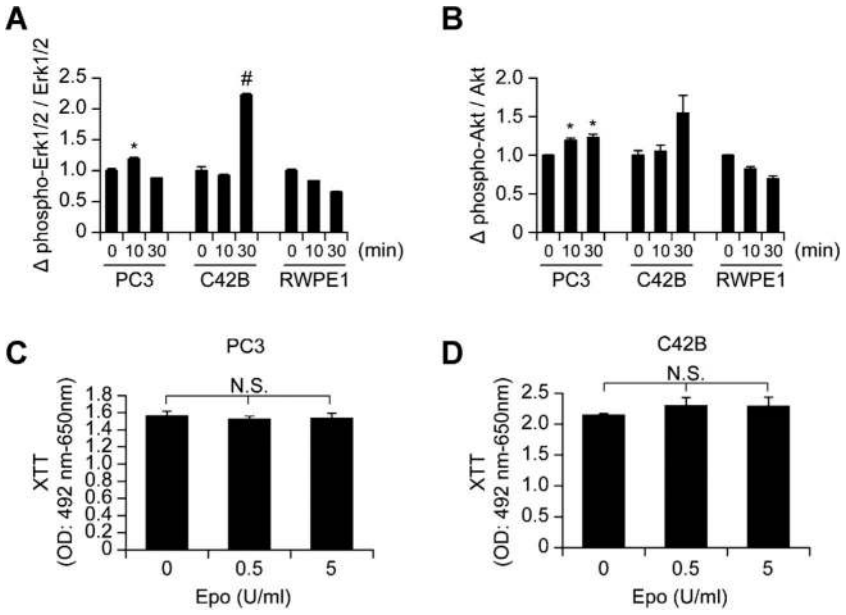

Fig. 2.

Epo activate MAPK pathway in PCa cells, but does not stimulate proliferation. (A, B) PCa cell lines (PC3 and C4-2B) and RWPE1 cells were cultured in medium without FBS for 5 h. After serum starvation, the cells were treated with 20 U/ml of Epo for 30 min, and phosphorylation of (A) Erk1/2 and (B) Akt were analyzed by flow cytometry at 0, 10, and 30 min. Total Erk1/2 and Akt were used as internal controls, respectively. Data are presented as mean ± SEM from triplicate determinations. * $P < 0.05$ and $^{\#}P < 0.01$ versus 0 min in the titration period. (C) PC3 cells and (D) C4-2B cells were seeded at 5,000 cells per well in 96-well plates and cultured with 0.1% FBS in the presence or absence of Epo (0–5 U/ml). After 48 h, cell proliferation was assessed using the XTT assay. Data are presented as mean ± SEM from triplicate determinations. N.S. indicates not significant.

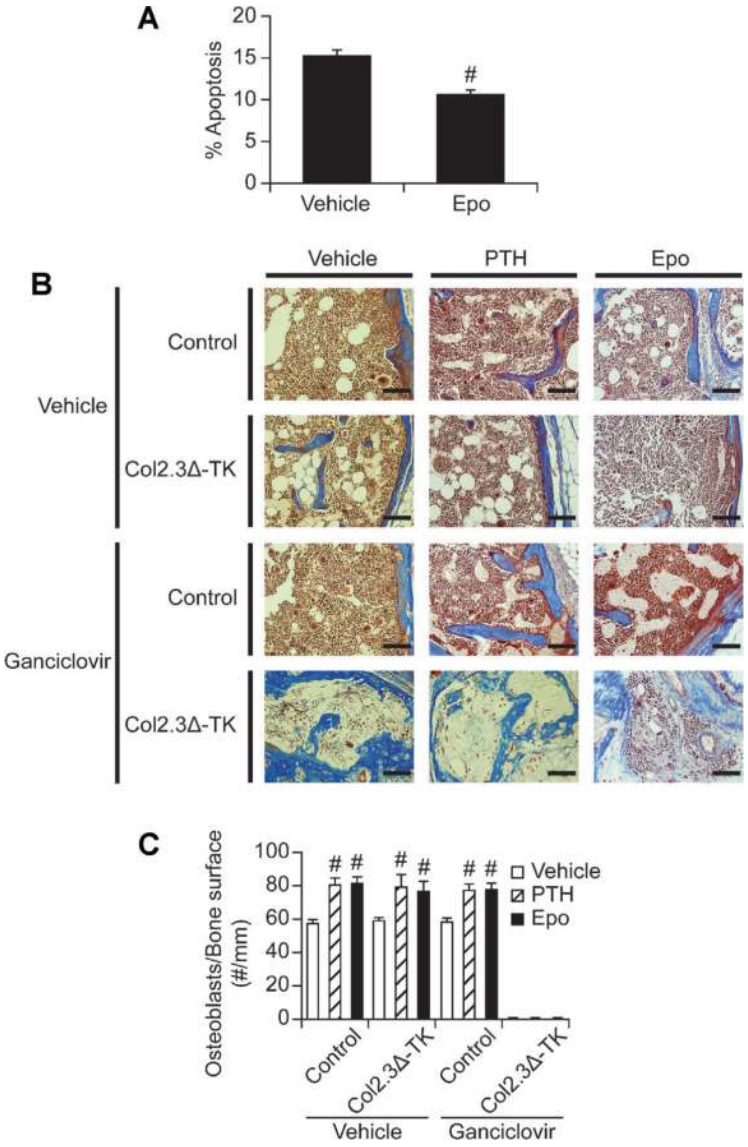

Fig. 3.

Epo protects PCa cells from apoptosis. (A) PC3 cells were treated with Epo (5 U/ml) in serum-free medium for 24 h. The effect of Epo on apoptosis of PCa cells was measured by flow cytometry using annexin V staining. Data are presented as mean SEM from triplicate determinations. $^{\#}P < 0.01$ versus vehicle treatment. B, C: Col2.3Δ-TK mice were treated with either ganciclovir to ablate the osteoblast niche or vehicle, with/without PTH or Epo for 3 weeks. (B) Representative Masson's trichrome staining of long bones. Original magnification, 40×. Scale bars: 50 μm. (C) Osteoblast number was quantified with Masson's trichrome staining. Data are presented as mean ± SEM. $^{\#}P < 0.01$ versus vehicle treatment.

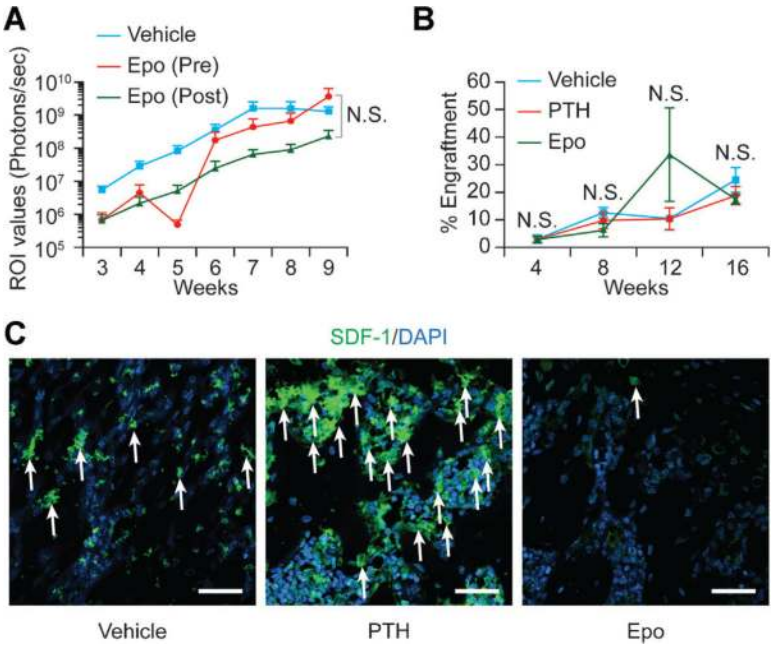

Fig. 4.

Epo does not alter bone metastasis of PCa cells. (A) Luciferase-expressing PCa cells were injected systemically by intracardiac injection into SCID mice. Either prior to or post tumor inoculation, mice were treated with Epo. Metastatic regions were measured by a bioluminescence imaging. Bioluminescence images are presented as the relative photon counts of each individual (n = 10). Data are presented as the mean SEM. N.S. indicates not significant. (B) Competitive long-term bone marrow transplantations were used to determine the effect of the occupancy of the marrow space on cell trafficking to the bone marrow. Bone marrow cells derived from CD45.1 mice (2×10^5 cells) were transplanted into CD45.2 congenic C57BL6 mice along with a mixture of a radioprotective dose of CD45.2 cells (2×10^5 cells). Epo or PTH was used to expand the osteoblastic niche prior to irradiation. The percentages of engraftment of CD45.1 cells were determined in peripheral blood analyzed by flow cytometry over time (n = 10). Data are presented as the mean ± SEM. N.S. indicates not significant. (C) Representative SDF-1 immunostaining of long bones. Arrows indicate the region of SDF-1-positive. Original magnification, 60×. Scale bars: 25 μm.