

# An Erythropoietin Autocrine/Paracrine Axis Modulates the Growth and Survival of Human Prostate Cancer Cells

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## Abstract

Erythropoietin receptors have been identified on a variety of cancer-derived cell lines and primary cancer cells, including those of prostate cancer. The functional status of these extrahematopoietic erythropoietin receptors remains a matter of some dispute. The publication of several important clinical trials suggesting a direct effect of erythropoietin on the growth and survival of primary tumors adds further importance to the question of whether erythropoietin receptors on cancer cells are functional. We have reported previously that human prostate cancer cell lines and primary prostate cancer cells express functional erythropoietin receptors that respond to exogenous erythropoietin by increased cell proliferation and STAT5 phosphorylation. We now show that prostate cancer cell lines express both the *EPO* gene and the biologically active erythropoietin. The coexpression of functional receptor and biologically active ligand in the cells has led us to hypothesize an autocrine/paracrine mechanism, driven by endogenous erythropoietin, which may modulate the growth and progression of prostate cancer. To test our hypothesis, we have knocked down, independently, erythropoietin receptor and erythropoietin on prostate cancer cells by transfection with short hairpin RNAs. Erythropoietin receptor knockdown cells grow significantly more slowly than their erythropoietin receptor-bearing counterparts in monolayer culture, produce fewer, smaller colonies in soft agar, and do not exhibit erythropoietin-induced signaling. Erythropoietin knockdown cells exhibit dramatically slower rates of growth, which could be restored by transfecting the cells with a murine erythropoietin gene. Taken together, our data suggest that the coordinated regulation of a functional erythropoietin/erythropoietin receptor axis in prostate cancer cells may be integral to the growth and progression of prostate cancer. (*Mol Cancer Res* 2009;7(7):1150–7)

## Introduction

Erythropoietin is a glycoprotein produced mainly by the kidneys and is the principal regulator of RBC formation from bone marrow-derived progenitor cells. Additionally, a substantial body of evidence now points to erythropoietin as a multi-functional, pleiotropic hormone (reviewed in refs. 1-5), and erythropoietin receptors have been identified on a wide variety of nonhematopoietic cells. Among the nonhematopoietic cells expressing erythropoietin receptors are tumor cells, including (but not limited to) those of the breast (6-13), ovaries (14-18), and prostate (19, 20).

Previously, we showed the expression of erythropoietin receptor, at both mRNA and protein levels, by prostate cancer cell lines and by primary prostate cancer cells (20). Furthermore, we showed that the erythropoietin receptors on prostate cancer cell lines are functional in that erythropoietin treatment of the receptor-bearing cells resulted in increased cell proliferation and increased STAT5 phosphorylation (20).

We now show that erythropoietin treatment of prostate cancer cells increases dramatically the number of anchorage-independent colonies formed in soft agar, further proof of erythropoietin receptor function. Additionally, we show that prostate cancer cells also express the erythropoietin gene and produce biologically active erythropoietin. This coexpression of biologically active erythropoietin and functional erythropoietin receptor led us to hypothesize that an erythropoietin-responsive autocrine/paracrine regulatory mechanism may be integral to the growth and survival of prostate cancer cells. To test our hypothesis, we interrupted the functional erythropoietin/erythropoietin receptor axis by independently knocking down the expression of either the erythropoietin receptor gene (*EPOR*) or the erythropoietin gene (*EPO*) by short hairpin RNA (shRNA) and showing that disruption of the erythropoietin/erythropoietin receptor autocrine/paracrine axis slows the growth of the cells in liquid culture and decreases dramatically the number of colonies that form in soft agar. Taken together, the data support our hypothesis that a functional erythropoietin/erythropoietin receptor autocrine/paracrine axis is integral to prostate cancer cell growth. We propose this erythropoietin/erythropoietin receptor axis as a novel, druggable target for the treatment of prostate cancer.

## Results

### *Erythropoietin Increases Anchorage-Independent Growth of Prostate Cancer Cells in Soft-Agar Culture*

We showed previously that prostate cancer cell lines express functional erythropoietin receptors (20) and that concentrations of exogenous erythropoietin that are physiologically and/or

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therapeutically relevant stimulate the proliferation of the cells under serum-free and reduced serum conditions. Therefore, we asked whether recombinant human erythropoietin (rhEpo) has a stimulatory effect on the ability of prostate cancer cells to form anchorage-independent colonies in semisolid medium, a model of predicted *in vivo* tumorigenicity (21, 22).

We plated PC-3 cells in two-layer soft-agar cultures in the presence of specified doses of rhEpo (0.1-10 units/mL) that correspond to therapeutically attainable *in vivo* concentrations of the drug (23). Figure 1A shows a soft-agar culture experiment in which an increase in colony number is observed, after 6 and 12 days, in the presence of 1 unit/mL rhEpo. Figure 1B quantifies results from similar soft-agar plating experiments, in which lower cell numbers were plated to make colony counting more manageable. An erythropoietin concentration-dependent increase in the number of colonies is observed ( $19 \pm 2$  colonies per well in the absence of rhEpo,  $41 \pm 5$  in the presence of 0.1 unit/mL rhEpo,  $60 \pm 6$  in the presence of 1 unit/mL rhEpo, and  $95 \pm 6$  in the presence of 10 units/mL rhEpo).

#### Knockdown of Erythropoietin Receptor Expression in Prostate Cancer Cells Using shRNA (*EpoR<sup>KD</sup>* Cells) Markedly Reduces Cell Proliferation

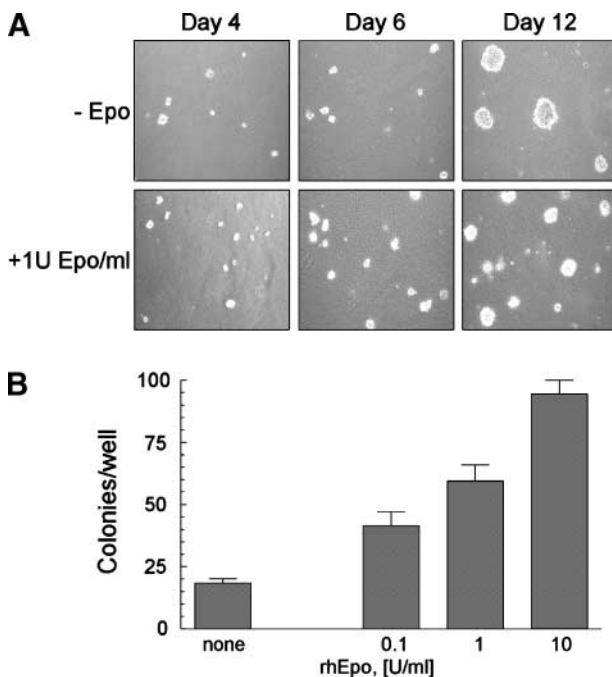
To test our hypothesis that erythropoietin receptor expression itself has an important role for the growth and maintenance of prostate cancer cells, we elected to stably knock down endogenous *EPOR* in PC-3 cells using a vector-based shRNA

approach. PC-3 cells were transfected either with shRNA constructs targeting the human *EPOR* mRNA sequence, with a nontargeting shRNA construct (pLKO-NTC), or with empty vector (V) as controls. Puromycin-resistant stable clones were expanded from single-cell colonies and their *EPOR* gene expression levels were analyzed by semiquantitative real-time PCR (sqRT-PCR; Fig. 2A). We identified two clones, designated PC-3/*EpoR<sup>KD</sup>*-2C10 and PC-3/*EpoR<sup>KD</sup>*-4B6, in which *EPOR* expression levels were reduced most dramatically. qRT-PCR (Fig. 2B) was employed to confirm the comparative *EPOR* expression levels of these two clones relative to that of untransfected PC-3 cells or PC-3/NTC cells. qRT-PCR results indicated that PC-3/*EpoR<sup>KD</sup>*-2C10 expressed ~45% as much *EPOR* as did PC-3 cells, whereas PC-3/*EpoR<sup>KD</sup>*-4B6 expressed ~20% as much *EPOR* as did the untransfected cells.

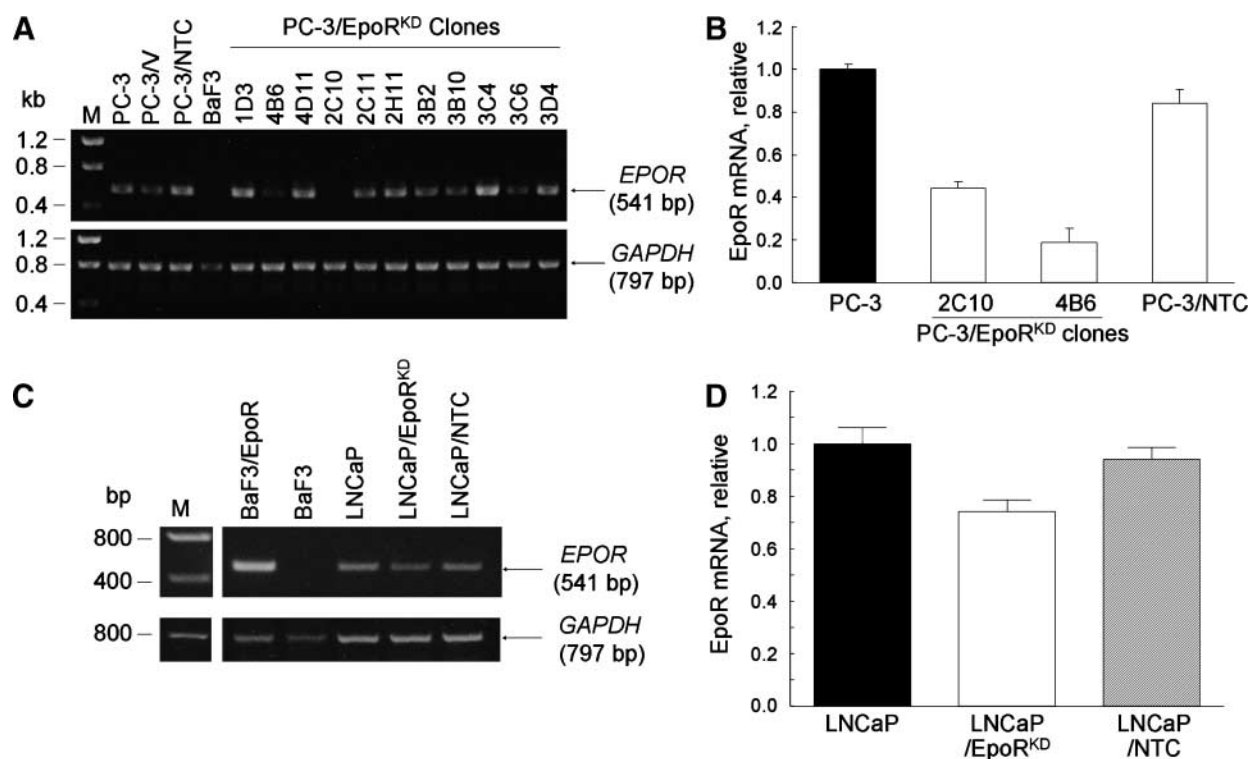
We also performed knockdown of *EPOR* in LNCaP cells. LNCaP cells were transiently transfected with a mixture of three shRNA constructs targeting *EPOR* or with pLKO-NTC by electroporation as described in Materials and Methods. The *EPOR* gene expression levels at 5 days post-transfection, corresponding to day 3 of a cell proliferation experiment (see Fig. 3B), were analyzed by sqRT-PCR (Fig. 2C) and qRT-PCR (Fig. 2D). qRT-PCR results indicated that LNCaP cells transiently transfected with *EPOR* targeting shRNA constructs (LNCaP/*EpoR<sup>KD(transient)</sup>*) expressed ~70% as much *EPOR* as did untransfected LNCaP cells, whereas LNCaP transfected with pLKO-NTC (LNCaP/NTC<sup>(transient)</sup>) maintained ~90% *EPOR* expression compared with LNCaP cells.

To determine the effect of erythropoietin receptor knockdown on PC-3 cell growth/proliferation, equal numbers of PC-3, PC-3/NTC, and clones PC-3/*EpoR<sup>KD</sup>*-2C10 or PC-3/*EpoR<sup>KD</sup>*-4B6 were established in standard serum-containing liquid culture. Cells from replicate plates were enumerated by manual cell counting, in the presence of trypan blue, daily for 6 days. Figure 3A shows the results of manual cell counting of PC-3/*EpoR<sup>KD</sup>* clones as fold increase in cell growth for each cell line compared with the number of cells plated on "day 0." A clear decrease in cell growth for the *EpoR<sup>KD</sup>* clones is seen and is proportional to the degree of *EPOR* down-regulation. The growth rate, as measured by change in cell number, of PC-3/*EpoR<sup>KD</sup>*-2C10 cells is ~57% that of PC-3, and the growth rate of PC-3/*EpoR<sup>KD</sup>*-4B6 is ~38% that of PC-3, after 6 days in culture. The number of cells positively stained with Annexin V did not differ among the four cell lines (data not shown), indicating that decreased proliferation rather than increased apoptosis may be responsible for the reduced growth rate, at least during steady-state cell culture conditions. Figure 3B includes corresponding data generated from transient *EPOR* knockdown in LNCaP cells. Again, the rate of growth of LNCaP/*EpoR<sup>KD(transient)</sup>* is reduced significantly in comparison with LNCaP/NTC<sup>(transient)</sup>. The growth rate of LNCaP/*EpoR<sup>KD(transient)</sup>* is ~36% that of control (LNCaP/NTC<sup>(transient)</sup>) after 3 days in culture and 61% after 6 days in culture.

Knockdown of the erythropoietin receptor also affected the ability of prostate cancer cells to form anchorage-independent colonies. Equal numbers of PC-3, PC-3/NTC (control), PC-3/*EpoR<sup>KD</sup>*-2C10, or PC-3/*EpoR<sup>KD</sup>*-4B6 cells were plated in semisolid medium and incubated for 4 weeks. Cultures were stained with crystal violet, and colonies from replicate wells



**FIGURE 1.** Erythropoietin stimulates the anchorage-independent growth of PC-3 prostate cancer cells in soft-agar culture. PC-3 prostate cancer cells were plated in two-layer soft-agar cultures in the absence or presence of designated doses of rhEpo. **A.** Series of photographs from cultures in which 5,000 cells were plated per well in the absence or presence of 1 unit/mL rhEpo. **B.** Numbers of colonies derived from cultures in which 1,000 cells per well were plated in the absence or presence of 0.1, 1, or 10 unit/mL rhEpo. Mean  $\pm$  SD from two replicate wells for each condition.



**FIGURE 2.** Knockdown of *EPOR* gene expression by transfection with shRNA constructs. **A.** sqRT-PCR for PC-3 derivatives. cDNA (10 ng each) from PC-3, PC-3/V, PC-3/NTC, or stable clones of PC-3/EpoR<sup>KD</sup> was used as template to amplify a specific 541 bp fragment of the human *EPOR* gene and a 797 bp fragment of the *GAPDH* gene using RT-PCR. BaF3 cell cDNA was used as negative control. Note significant reductions of the *EPOR* expression in PC-3/EpoR<sup>KD</sup> clones 4B6 and 2C10. **B.** qRT-PCR for PC-3 derivatives. *EPOR* expression levels in PC-3, PC-3/NTC, or PC-3/EpoR<sup>KD</sup> clones 2C10 and 4B6 were determined by qRT-PCR using *GAPDH* as endogenous control. Results are presented as relative *EPOR* level to that of PC-3 cells. Mean  $\pm$  SD of triplicates. **C.** sqRT-PCR for LNCaP derivatives. cDNA (10 ng each) from LNCaP cells, transiently transfected LNCaP/EpoR<sup>KD</sup>, or LNCaP/NTC was used as template to amplify a specific 541 bp fragment of the human *EPOR* gene using RT-PCR. BaF3/EpoR cell cDNA (10 pg) and BaF3 cell cDNA (10 ng) were used as positive and negative controls, respectively. For *GAPDH* amplification, 10 ng cDNA each was used. Note a modest but noticeable reduction of the *EPOR* amplification in transient LNCaP/EpoR<sup>KD</sup>. **D.** qRT-PCR for LNCaP derivatives. *EPOR* expression levels in LNCaP, transiently transfected LNCaP/EpoR<sup>KD</sup>, and LNCaP/NTC were determined by qRT-PCR.

for each culture condition were counted microscopically. Figure 3C illustrates that PC-3 cells formed 38 to 42 colonies per well and PC-3/NTC had 44 colonies per well in soft-agar culture, whereas PC-3/EpoR<sup>KD</sup>-2C10 had 28 to 30 colonies per well, a reduction of  $\sim$ 25% from control cells. Soft-agar cultures of PC-3/EpoR<sup>KD</sup>-4B6 cells, which express the least erythropoietin receptor transcript, yielded an average of  $<1$  colony per well. This effect on anchorage-independent colony formation was far more dramatic than the decrease in cell growth observed in liquid cultures compared with PC-3 or PC-3/NTC cells.

#### Down-Regulation of the Erythropoietin Receptor in PC-3 Cells Abolishes Erythropoietin-Dependent Signaling

PC-3, PC-3/EpoR<sup>KD</sup>-2C10, PC-3/EpoR<sup>KD</sup>-4B6, and PC-3/NTC cells were used to examine erythropoietin-dependent phosphorylation of Jak2 and Akt, important intermediates for cellular survival pathways in both normal and malignant cells (24). As shown in Fig. 4, PC-3 and PC-3/NTC cells responded to erythropoietin stimulation in a time-dependent manner, exhibiting a maximum phosphorylation of Jak2 (*top*) at 10 min and maintaining up for 60 min or a maximum phosphorylation of Akt (*bottom*) at 10 min and then decreasing. The maximum relative phospho-Jak2 stimulation was 2.7-fold for PC-3 and 2.5-fold for PC-3/NTC. Neither PC-3/EpoR<sup>KD</sup>-2C10 nor PC-3/

EpoR<sup>KD</sup>-4B6 showed any increase in phospho-Jak2 following erythropoietin stimulation. The maximum relative phospho-Akt stimulation was 2.1-fold for PC-3 cells and 3.2-fold for PC-3/NTC. In marked contrast, both PC-3/EpoR<sup>KD</sup>-2C10 and PC-3/EpoR<sup>KD</sup>-4B6 cells failed to exhibit increased phospho-Akt on erythropoietin stimulation. The results show that the erythropoietin-dependent increase in either phospho-Jak2 or phospho-Akt is mediated specifically by the erythropoietin receptor in PC-3 cells and that the  $\sim$ 55% knockdown of the *EPOR* mRNA expression (as seen in Fig. 3B for PC-3/EpoR<sup>KD</sup>-2C10 cells) is sufficient to abolish erythropoietin-dependent signaling through these intermediates.

#### Prostate Cancer Cells Express Biologically Active Erythropoietin and Erythropoietin Expression Contributes to PC-3 Cell Growth

We showed previously that prostate cancer cells exhibit a moderately enhanced proliferation in response to physiologically and/or therapeutically relevant doses of rhEpo (20). We questioned whether the magnitude of this response might have been dampened by the presence of endogenous erythropoietin already produced by these cells and that an erythropoietin/erythropoietin receptor autocrine/paracrine axis may be integral to regulation of their growth and survival. Firstly, to determine

if the cells expressed erythropoietin, we performed sqRT-PCR using specific primers derived from the human *EPO* gene sequence. As shown in Fig. 5A, we used decreasing amounts of a plasmid containing the full-length *EPO* cDNA (marked as control) to show that the PCR was done within a linear range of amplification. The positive control BHK/Epo cells yielded a robust PCR fragment of 324 bp and the negative control BHK cells did not, as expected. Both LNCaP and PC-3 prostate cancer cell lines, as well as the nontumorigenic transformed prostate epithelial cell line 267B1, express *EPO* at the mRNA level.

We collected serum-free conditioned medium from each of these cell lines and quantified the amount of biologically active erythropoietin in a cell-based bioassay (25). Under the conditions we examined, LNCaP cells expressed between 10 and 15 mU erythropoietin/mL medium, PC-3 cells expressed between 3 and 13 mU erythropoietin/mL medium, and 267B1 cells expressed ~8 mU erythropoietin/ml.

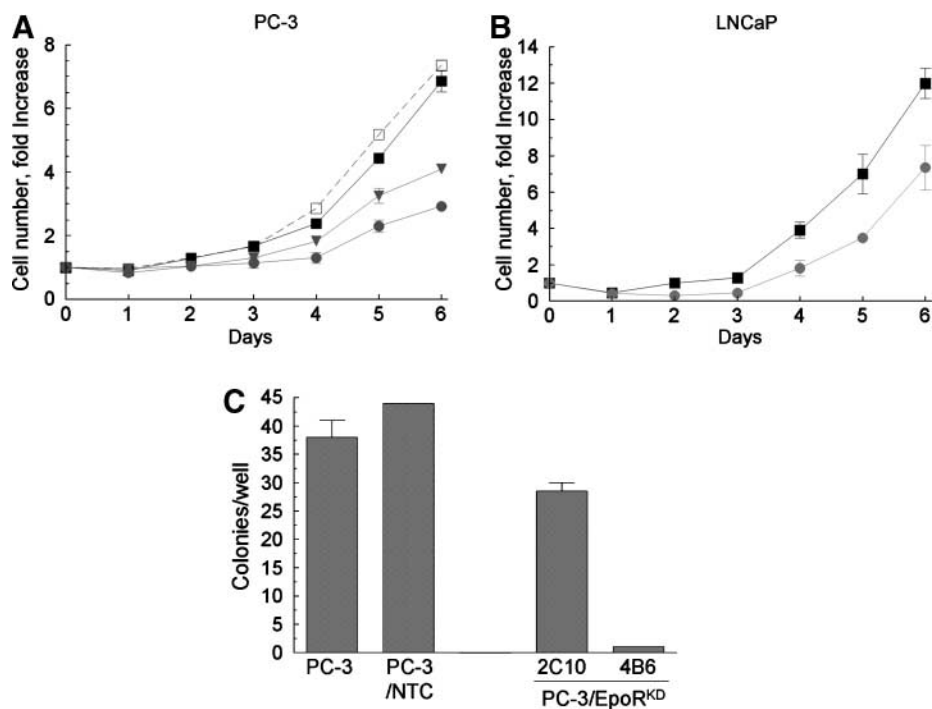
The coexpression of erythropoietin receptor and erythropoietin by prostate cancer cells led us to hypothesize that an endogenous erythropoietin/erythropoietin receptor autocrine/paracrine mechanism may exist in the cells and may contribute to their growth and survival. To begin to test this hypothesis, we plated PC-3 cells in the absence or presence of a neutralizing anti-erythropoietin antibody, or in the presence of normal (control) IgG, and determined their growth by manual cell counting. As shown in Fig. 5B, the neutralizing anti-erythropoietin antibody decreased cell growth ~20%, whereas an equivalent (protein) amount of normal IgG was without effect. To confirm that the decreased cell growth was specifically due to inhibition of endogenous erythropoietin produced, we added an excess of erythropoietin (10 units/mL) to some of the samples at the same time that the antibody was added and showed that the growth inhibition was reversed.

To test further our hypothesis of an autocrine/paracrine mechanism integral to the growth and maintenance of prostate cancer cells, we knocked down endogenous *EPO* in PC-3 cells using gene-specific shRNA (PC-3/Epo<sup>KD</sup>) in a manner similar to erythropoietin receptor knockdown, as described (Materials and Methods). Additional aliquots of cells were subjected to transfection with nontargeting control shRNA (PC-3/NTC). Both stably selected PC-3/NTC and PC-3/Epo<sup>KD</sup> cells then were subjected to a second transfection with the murine erythropoietin gene (mEpo) or empty vector (V) as control. Sequence differences in mEpo from that of the human erythropoietin sequence make it resistant to the shRNAs that were used for the initial erythropoietin knockdown. As shown in Fig. 5C, PC-3/Epo<sup>KD</sup> cells (column 3) exhibit markedly reduced growth (65% of control cell growth) in comparison with PC-3/NTC cells (column 1). Reversal of the PC-3/Epo<sup>KD</sup> knockdown phenotype was achieved by transfection of the mEpo gene into these cells (column 4). Transfection of the mEpo gene into PC-3/NTC cells did not result in significant change in growth rate of the cells (column 2).

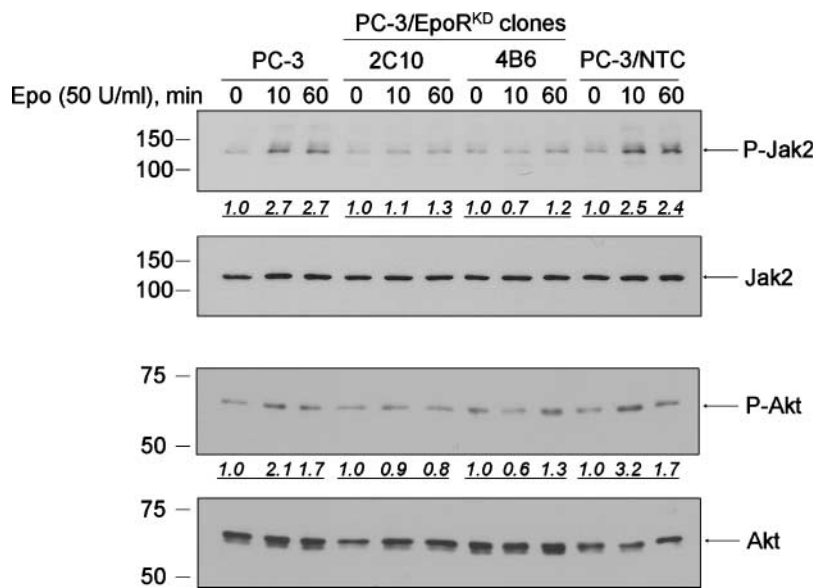
## Discussion

The potential physiologic significance of extrahematopoietic erythropoietin receptors is an area of great interest and even greater debate. Although there now may be a consensus that nonhematopoietic cells, including many tumor cells, express the *EPOR* gene (as identified by RT-PCR), much disagreement remains as to whether the cells express erythropoietin receptor protein and, more specifically, plasma membrane-associated protein.

In hematopoietic cells, only a small percentage of the total erythropoietin receptor protein produced actually makes its way



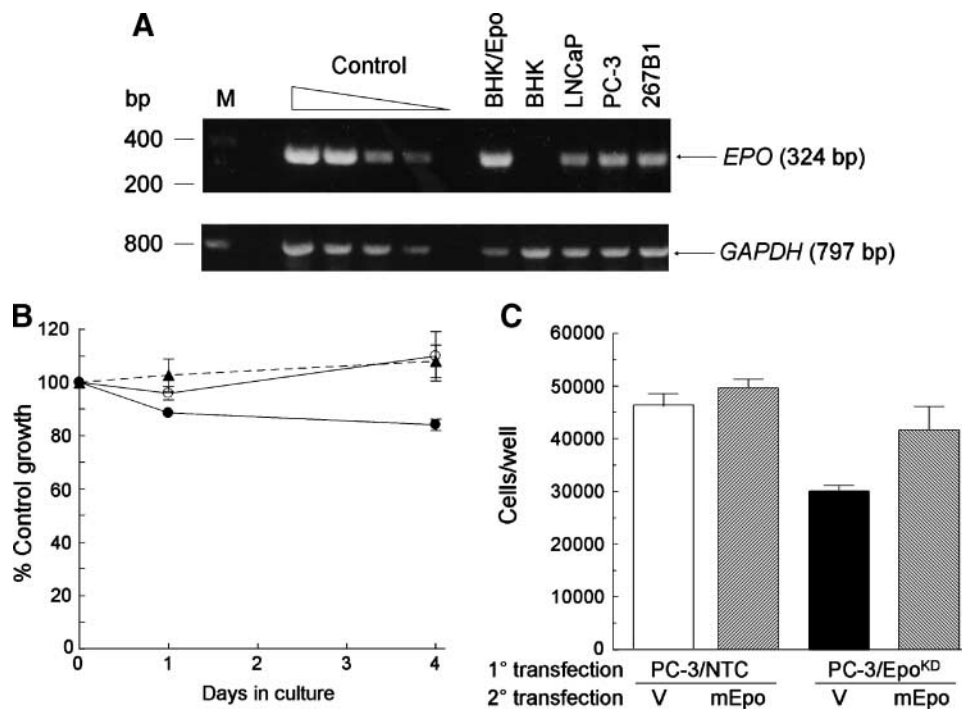
**FIGURE 3.** Knockdown of *EPOR* slows both anchorage-dependent and anchorage-independent growth of prostate cancer cells in culture. **A.** Equal numbers (5,000 per well) of PC-3 (■), PC-3/NTC (□), PC-3/EpoR<sup>KD</sup>-2C10 (▼), and PC-3/EpoR<sup>KD</sup>-4B6 (●) cells were plated in triplicate in replicate plates and counted manually for each of 6 d. Cell growth is represented in fold increase in cell number compared with day 0. **B.** Equal numbers (10,000 per well) of LNCaP/NTC (■) and LNCaP/EpoR<sup>KD(transient)</sup> (●) cells were plated in triplicate and counted as in **A.** **C.** Equal numbers (1,000 per well) of PC-3, PC-3/NTC, PC-3/EpoR<sup>KD</sup>-2C10, and PC-3/EpoR<sup>KD</sup>-4B6 were plated in soft-agar cultures and incubated for 4 wk. The number of crystal violet-stained colonies from duplicate wells for each cell population was counted.



**FIGURE 4.** Knockdown of *EPO*R abolishes erythropoietin-dependent signaling. PC-3 (*left*), PC-3/NTC (*right*), and two PC-3/*EpoR*<sup>KD</sup> clones (2C10 and 4B6; *middle*) were incubated in the absence or presence of erythropoietin (50 units/ml) for the times indicated. Cell lysates were electrophoresed, and the resultant Western blots were developed with phospho-Jak2 and Jak2 (*top*) or phospho-Akt and Akt (*bottom*) specific antibodies. Left, positions of molecular weight standards (kDa). Relative changes in phospho-Jak2/Jak2 or phospho-Akt/Akt were quantified using the ImageJ software.

to the cell surface (26–28), where it can interact with erythropoietin and function in transducing the signal of erythropoietin. Furthermore, up to 95% of the surface receptor may be internalized and down-regulated within 48 h following ligand bind-

ing (29), and the continuous presence of ligand may result in depletion of surface erythropoietin receptor. Identification of erythropoietin receptor protein expressed by tumor cells has most frequently been made immunohistochemically or



**FIGURE 5.** Prostate cells express the *EPO* gene and down-regulation of erythropoietin expression slows PC-3 cell growth. **A.** sqRT-PCR with gene-specific primers to amplify a 324 bp fragment of the *EPO* gene from LNCaP, PC-3, and 267B1 cells. Control concentration curves to determine the linear range of PCR product detection were established using 50, 10, 2, 0.4, and 0 fg of a plasmid containing the entire erythropoietin coding sequence for *EPO* and 100, 20, 4, 0.8, and 0 ng cDNA from BHK cells for *GAPDH*. cDNA from LNCaP, PC-3, and 267B1 cells was used as template to amplify a specific 324 bp fragment of the human *EPO* gene and a 797 bp fragment of the *GAPDH* gene using RT-PCR. The lane labeled BHK/Epo used 0.1 ng BHK/Epo cell cDNA. All other cell lanes used 100 ng cDNA. For *GAPDH* amplification, 10 ng cDNA each was used. **B.** Growth of PC-3 cells in the presence of AB286, a neutralizing anti-erythropoietin antibody (●), in the presence of AB286 + 10 units/ml erythropoietin (○), or in the presence of normal IgG (▲) was compared with the growth of untreated PC-3 cells (100% control growth). **C.** PC-3 cells transfected with pLKO-NTC (PC-3/NTC) or *EPO* targeting shRNA constructs (PC-3/*Epo*<sup>KD</sup>) were further transfected with empty vector (V) or murine erythropoietin expression plasmid (mEpo). Equal numbers (10,000 per well) of each cell were plated in 12-well plates in triplicate and cell numbers were counted after 4 d.

immunochemically (6, 11, 19, 30-34) and the specificity of some of the most commonly used commercial anti-erythropoietin receptor antibodies has been questioned (35-39). The controversy as to whether erythropoietin receptors expressed by tumor cells are functional is fueled not only by suboptimal detection methods and/or reagents but also by the estimates of extremely low receptor numbers on nonhematopoietic cells. These low receptor numbers make classic receptor binding studies inadequate for measuring erythropoietin receptors on (most) nonhematopoietic cells; however, novel methods have been applied not only to measure extrahematopoietic receptors (40) but also to show their function (41) in signal transduction.

The evidence that tumor cells express not only erythropoietin receptor but also erythropoietin, shown by us in the present study and also suggested by others, implies that these cells possess an autocrine/paracrine mechanism of erythropoietin receptor activation. Under these conditions, erythropoietin receptor may be fractionally/maximally stimulated and/or constitutively activated by endogenously produced erythropoietin. This could explain why, under some conditions and in some cells, there may be a failure to exhibit a robust proliferative response to exogenous rhEpo. The rapid turnover of constitutively or maximally activated erythropoietin receptor also could explain the difficulty in showing cell surface receptors by standard (Western blotting) methods and might clarify why tumor cell-derived erythropoietin receptors sometimes appear to be of different sizes than the "classic" hematopoietic cell-derived erythropoietin receptor. In addition, a recent study concluded that erythropoietin receptor signaling is strongly affected by both ligand (erythropoietin) and receptor (erythropoietin receptor) variants (42), which can affect receptor conformation. The concept of splice variants in nonhematopoietic (specifically tumor cell) erythropoietin receptors has been introduced previously (43), although no study yet has been made of potential tumor cell-derived erythropoietin.

We now have shown that such an autocrine/paracrine mechanism is operative in prostate cancer cells. Should this type of mechanism be operative in primary or metastatic tumors in humans, it would have profound implications. Firstly, increasing circulating erythropoietin levels by administration of rhEpo (epoetin) or other erythropoiesis stimulating agents (darbepoetin, etc.) might increase cancer growth/survival (inhibit apoptosis), resulting in increased progression. This possibility continues to be debated widely (44). Additionally, compounds are under development that are designed to increase endogenous erythropoietin in patients by stabilizing hypoxia-inducible factor-1 $\alpha$  globally (45), thus mimicking the effect of hypoxia. The results of continuous, long-term treatment with such agents remain to be determined.

On the other hand, we propose that a functional erythropoietin/erythropoietin receptor autocrine/paracrine axis could provide several novel, druggable targets, the erythropoietin receptor being one of them, as we have shown in this study. Targeted delivery of appropriate shRNA-based therapeutic agents could be a very powerful approach to inhibit this process and thereby inhibit the growth of prostate cancer and other cancers exhibiting an erythropoietin/erythropoietin receptor axis.

## Materials and Methods

### *Growth and Maintenance of Cell Lines*

Human prostate cancer cell lines PC-3 and LNCaP cells were purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Mediatech) containing 5% to 10% heat-inactivated fetal bovine serum (FBS; Hyclone). In some instances, cells were grown in DMEM/F-12 (Mediatech) instead of RPMI 1640, containing the same amounts of serum. No obvious differences were noted between cells grown in either of the media. 267B1, a human transformed nontumorigenic prostate epithelial cell line (20), was a generous gift of Dr. John S. Rhim (Center for Prostate Disease Research, Uniformed Services University of the Health Sciences). 267B1 cells were maintained in RPMI 1640-5% heat-inactivated FBS. All cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>.

Serum-free prostate cell conditioned medium was prepared by plating equal numbers of cells ( $2.5 \times 10^6$  per well in a 6-well plate) in their normal growth medium and allowing the cells to adhere overnight. The medium was removed, adherent cells were washed two times with serum-free medium, and fresh serum-free medium (RPMI 1640 or DMEM/F-12 depending on the experiment) was added. Conditioned medium was harvested after 72 h incubation, filter sterilized, and stored at 4°C until bioassay.

BaF3 and BaF3/EpoR cells (ref. 46; gift of Dr. Mark Showers, Brigham & Women's Hospital) were maintained in RPMI 1640-5% heat-inactivated FBS containing 5% WEHI-3B cell conditioned medium (as a source of interleukin-3) or 1 unit/mL rhEpo, respectively, as described previously (14). BHK and BHK/Epo cells were maintained in RPMI 1640-10% heat-inactivated FBS as described previously (14).

### *RNA Isolation and RT-PCR*

Total RNA was isolated from cells grown to 70% to 80% confluency using the RNAqueous-4PCR kit (Ambion) according to the manufacturer's instructions, including DNase I treatment. First-strand cDNA was generated by using the RETROscript kit (Ambion) with random decamer as primer. Semi-quantitative PCR was done using the predetermined number of cycles so that the band intensity of amplified DNA would be within a linear range of amplification as described previously (14). Primer sequences were 5'-gcgatatcacctgtcatccacat (sense) and 5'-attctgtacagccacagctggaa (antisense) for *EPOR*, 5'-ctgtctgaacactgcagcttgaa (sense) and 5'-tcagcagtgattgtcg-gagtggga (antisense) for *EPO*, and 5'-aaggctgagaacgggaagctt (sense) and 5'-tccaccacctgttctgtga (antisense) for *GAPDH*. We have confirmed specific amplification of the *EPOR* and *EPO* DNA fragments under these conditions by restriction enzyme pattern analysis as well as by DNA sequencing (14). qRT-PCR was done using the ABI 7300 RT-PCR system (Applied Biosystems) with iTaq SYBR Green Supermix (Bio-Rad). The same primer pair described above for semiquantitative PCR was used for *EPOR*. Gene expression levels were normalized by qRT-PCR validated *GAPDH* primers, 5'-gagtcacg-gattgtctgt (sense) and 5'-ttgatttggaggatctcg (antisense; real-time primers). All the primers were designed to include or encompass one or more introns, such that amplified DNA fragments from genomic contamination, if any, could

be distinguished by size. No significant PCR amplification was observed with control cDNA samples lacking reverse transcriptase under the conditions used.

### Bioassays

**Erythropoietin Bioassay.** BaF3/EpoR cells were washed two times in RPMI 1640-2% heat-inactivated FBS and plated at 3,000 per well in 96-well plates. An erythropoietin standard curve was generated by diluting rhEpo (Elanex Pharmaceuticals) in serum-free RPMI 1640 and incubating the cells with specified concentrations of rhEpo from 0 to 1 units/mL. In some wells, serum-free prostate cell conditioned medium was added in place of rhEpo. Plates were incubated 48 h at 37°C, 5% CO<sub>2</sub> in humidified air and then developed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described previously (20, 47).

**Cell Proliferation Assay.** Cell proliferation was quantified by manual cell counting. Equal numbers of cells (5,000 or 10,000) in normal growth medium were plated in replicate 12-well plates (with triplicate wells for each cell type) and incubated under standard growth conditions. Each day, the cells from a separate plate were disadhered by trypsinization and counted in the presence of trypan blue using a hemacytometer. Alternatively, cell numbers were determined using an automated cell counter, ADAM (NanoEnTek), according to the manufacturer's instructions. Cell counts from each day were compared with the "day 0" number for that cell type to determine fold increase in cell number.

**Soft-agar Colony Formation Assay.** To monitor the effect(s) of rhEpo supplementation or erythropoietin receptor knockdown on the ability of prostate cancer cells to form anchorage-independent colonies, cells were plated in two-layer soft-agar cultures. Soft-agar cultures were established in 24-well plates, with duplicate wells for each cell population and/or growth condition. The bottom layers comprised 0.5 mL RPMI 1640-5% heat-inactivated FBS-0.5% agar with or without the addition of 0.1, 1, or 10 units/mL rhEpo. After the bottom layers were incubated for ≥30 min at room temperature to solidify, the top layers (containing cells) were added. Top layers contained, in different experiments, 1,000 to 5,000 cells per well in 0.5 mL RPMI 1640-5% heat-inactivated FBS-0.35% agarose in the presence of the same concentration of rhEpo as in the bottom layer of that well. Cultures were incubated for up to 4 weeks at 37°C, 5% CO<sub>2</sub> and examined microscopically each week. Cultures were not re-fed during the total incubation time. At the end of the experiment, the cells were stained with 0.05% crystal violet to aid in visualization of the colonies, and the colonies from replicate wells were enumerated microscopically.

**Anti-Erythropoietin Antibody Growth Inhibition Assay.** To determine the effect of neutralizing anti-erythropoietin antibody on prostate cancer cell growth, cells were plated (5,000 per well in replicate 12-well plates) in the presence of specified concentrations of a neutralizing anti-erythropoietin antibody (AB286; R&D Systems) or normal IgG (Jackson ImmunoResearch Laboratories) as control. To show antibody specificity, in some wells, an amount of rhEpo in excess of the neutralizing capacity of the antibody was added, thereby reversing antibody-mediated growth inhibition. Cells from one plate were counted on day

1 (24 h following antibody addition) and its replicate plate was counted on day 4. Growth in the presence of antibody (or of antibody + erythropoietin) was compared with growth of control cells in the absence of any addition.

### RNAi Inhibition of Erythropoietin Receptor and Erythropoietin Expression (Erythropoietin Receptor and Erythropoietin "Knockdown")

PC-3 cells were transfected with the RNAi Consortium (TRC) shRNA constructs targeting the human *EPOR* (Open Biosystems) using FuGENE 6 transfection reagent (Roche). Puromycin-resistant clones grown from single-cell colonies were subjected to sqRT-PCR and qRT-PCR to determine the *EPOR* mRNA expression levels. The targeting sequences for the shRNA constructs used in the present study were as follows: 5'-ccctatgagaacagccttat for clone 2C10 and 5'-tgccagctttgagtacactat for clone 4B6. Empty vector (pLKO) and nontargeting control vector (pLKO-NTC; containing a target sequence of 5'-caacaagatgaagaccacaa) were purchased from Sigma and used as controls. pLKO expresses no shRNA, whereas pLKO-NTC expresses shRNA, which contains 5 bp mismatches to known human or mouse genes.

For transient knockdown of *EPOR*, LNCaP cells were transfected with a mixture of three shRNA constructs targeting *EPOR* (the two described above plus one additional construct, targeting 5'-cgtgtcatccatcaatgaa) or pLKO-NTC by electroporation at 0.23 kV and 950 μF using a Gene Pulser II electroporation system (Bio-Rad). Two days after transfection, cells were plated in 12-well plates (10,000 live cells per well determined by trypan blue exclusion) and their cell growth was determined by manual cell counting every day for 6 days. RNA from the same batches of cells was prepared on day 3 of cell counting (corresponding to 5 days after transfection) to examine the *EPOR* mRNA levels.

Erythropoietin expression was down-regulated in PC-3 cells by transfecting a mixture of five shRNA constructs targeting *EPO* (Open Biosystems; targeting sequences: 5'-cccagaccacaaagttaatt, 5'-ccgcaaacctctccgagctca, 5'-gctgaacactcagcttgaat, 5'-ccgagtactccaattct, and 5'-gaacaataactgtgactact). Puromycin-resistant cells grown from pooled colonies were then transfected with either pcDNA3.1(+) (Invitrogen) or pcDNA3.1(+)-mEpo containing the entire coding sequence of murine erythropoietin (48, 49), which has mismatches against all five shRNA sequences used to down-regulate human *EPO* expression. Cells were plated at 10,000 per well in replicate 12-well plates and counted after incubation for 4 days. PC-3 cells transfected with pLKO-NTC were used as control.

### Erythropoietin-Dependent Cell Signaling

Cells were plated in 6-well plates (10<sup>5</sup> per well) and incubated in growth medium for 24 h. Cells were washed twice with PBS and serum starved overnight by incubation in phenol red-free DMEM/F-12 (Mediatech) containing 0.5% FBS. Cells were treated with 50 units/mL rhEpo or with vehicle alone (0.1% bovine serum albumin in PBS) for 0 to 60 min. After washing cells with ice-cold PBS, lysates were prepared by scraping the cells into 2× SDS-PAGE sample buffer containing Protease Inhibitor Cocktail Set III (Calbiochem) and Phosphatase Inhibitor Cocktail Set III (Calbiochem) and by boiling for 5 min. Equal protein

amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting using antibodies specific for phospho-Jak2 (Santa Cruz Biotechnology) and total Jak2 (Cell Signaling Technology) or phospho-Akt (Cell Signaling Technology) and total Akt (Cell Signaling Technology). Band intensities were quantified using the ImageJ software (NIH).

### Disclosure of Potential Conflicts of Interest

A.J. Sytkowski received a commercial research grant from DNAPrint Genomics and was a consultant for DNAPrint Genomics.

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