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Author manuscript

Oncogene. Author manuscript; available in PMC 2012 September 29.

Published in final edited form as:

Oncogene. 2012 March 29; 31(13): 1649–1660. doi:10.1038/onc.2011.366.

Erythropoietin Receptor Contributes to Melanoma Cell Survival *in vivo*

Suresh M. Kumar[1], **Gao Zhang**[2], **Boris C. Bastian**[3], **Murat O. Arcasoy**[4], **Pankaj Karande**[5], **Anitha Pushparajan**[1], **Geza Acs**[6], and **Xiaowei Xu**[1]

[1]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

[2]The Wistar Institute, 3601 Spruce Street, Philadelphia, PA, USA

[3]Departments of Dermatology and Pathology, University of California, San Francisco, CA, USA

[4]Department of Medicine, Duke University School of Medicine, Durham, NC, USA

[5]Departments of Chemical and Biological Engineering, Rensselaer Polytechnology Institute, Troy, NY, USA

[6]Departments of Anatomic Pathology and Women's Oncology, Moffitt Cancer Center, Tampa, FL, USA

Abstract

Erythropoietin (Epo) is widely used clinically to treat anemia associated with various clinical conditions including cancer. Data from several clinical trials suggest significant adverse effect of Epo treatment on cancer patient survival. However, controversy exists whether erythropoietin receptor (EpoR) is functional in cancer cells. In this study, we demonstrated that EpoR mRNA expression was detectable in 90.1% of 65 melanoma cell lines, and increased copy number of the Epo and EpoR loci occurred in 30% and 24.6% of 130 primary melanomas, respectively. EpoR knockdown in melanoma cells resulted in diminished ERK phosphorylation in response to Epo stimulation, decreased cell proliferation, and increased response to the inhibitory effect of hypoxia and cisplatin *in vitro*. EpoR knockdown significantly decreased melanoma xenograft size and tumor invasion *in vivo*. On the contrary, constitutive activation of EpoR activated cell proliferation pathways in melanoma cells and resulted in increased cell proliferation and resistance to hypoxia and cisplatin treatment *in vitro*. EpoR activation resulted in significantly larger xenografts with increased tumor invasion of surrounding tissue *in vivo*. Daily administration of recombinant Epo fails to stimulate melanoma growth *in vivo*, but the treatment increased vascular size in the xenografts. Increased local recurrence after excision of the primary tumors was observed after Epo treatment. Epo induced angiogenesis in Matrigel plug assays, and neutralization of Epo secreted by melanoma cells results in decreased angiogenesis. These data support that EpoR is functional in

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Correspondence should be addressed to: Xiaowei Xu, MD, PhD, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, 19104, xug@mail.med.upenn.edu.

Conflict of interest

The authors declare no conflict of interest.

melanoma and EpoR activation may promote melanoma progression, and suggest that Epo may stimulate angiogenesis and increase survival of melanoma cells under hypoxic condition *in vivo*.

Keywords

Erythropoietin; Erythropoietin receptor; melanoma; hypoxia

Introduction

Erythropoietin (Epo) regulates erythropoiesis by binding to its transmembrane receptor (EpoR) (Wu et al. 1995). EpoR activation in hematopoietic cells results in the phosphorylation of the tyrosine kinase JAK2 and activation of ERK and PI3K/AKT pathways (Constantinescu et al. 1999). The recombinant forms of Epo are widely used to treat anemia associated with various clinical conditions including cancer (Arcasoy 2008). Recent randomized clinical trials in patients with breast (Leyland-Jones et al. 2005) and head and neck cancer (Henke et al. 2003) unexpectedly reported that Epo treatment decreased patients survival and accelerated tumor progression. Following the reports of the results of three additional phase III studies and a phase II trial, all suggesting a statistically significant adverse effect of Epo treatment on survival (Blau 2007), a “black box warning” was issued for Epo products by the United States Food and Drug Administration (FDA). While some of these studies have been criticized based on inadequate study design and lack of rigorous controls (Jelkmann et al. 2008). A meta-analysis of 57 studies did not find a survival benefit of patients receiving Epo or darbepoetin, but raised the possibility of a negative impact on survival (Bohlius et al. 2009). A more recent meta-analysis of more than 10,000 cancer patients confirmed decreased overall survival in patients treated with Epo (Bennett et al. 2008).

The effects of Epo on non-hematopoietic normal and cancerous tissues are still poorly understood (Noguchi et al. 2008). Previous studies have shown that EpoR expression is not restricted to hematopoietic cells but rather, it is also expressed in a variety of other cell types such as embryonic neural crest progenitor cells and their derivatives (Knabe et al. 2005; Tsai et al. 2006), neurons, glial cells, endothelial cells and cardiomyocytes (Arcasoy 2008; Okazaki et al. 2008; Tovari et al. 2008; Wright et al. 2004). We and others have previously shown that a variety of cancer cells, including carcinomas of ovary, breast, lung, thyroid, prostate, endometrium, cervix, head and neck (squamous), renal cell carcinoma, glioma, and melanoma, express EpoR mRNA transcripts and protein(Acs et al. 2001; Acs et al. 2004b; Arcasoy et al. 2002; Arcasoy et al. 2003; Arcasoy et al. 2005; Jeong et al. 2009; Lai et al. 2005; Yates et al. 2006). A number of studies suggested that Epo/EpoR may play a role in tumor progression (Acs et al. 2004a; Hardee et al. 2007; Kumar et al. 2005; Mohyeldin et al. 2005) whereas other reports contradicted these findings (Belda-Iniesta et al. 2007; LaMontagne et al. 2006) and thus, the role of Epo/EpoR during tumor progression is still controversial.

The current study shows for the first time that increased copy number of the Epo and EpoR loci occurs in surprisingly high percentage of primary melanomas; EpoR is functional in a

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series of *in vitro* and *in vivo* assays. These data suggest that EpoR activation may contribute to melanoma progression.

Results

Genomic alterations in Epo and EpoR in melanoma

We have previous shown that melanoma express Epo and EpoR (Kumar et al. 2005; Paragh et al. 2009). To study whether genomic changes could drive increased Epo and/or EpoR expression, we first determined the expression and DNA copy number status of Epo and EpoR in early passage melanoma cell lines and human foreskin melanocytes using gene expression profiling and high-density single nucleotide polymorphism (SNP) arrays. EpoR gene expression was detectable in 59/65 melanoma cell lines (90.1%). While no copy number increases of EpoR were found in any of 47 cell lines analyzed by SNP, gain of Epo was identified in 5/47 melanoma cells lines.

To correlate the results from cell lines with primary melanomas, we analyzed array CGH (aCGH) data obtained from 130 formalin fixed paraffin embedded (FFPE) tissue samples of primary invasive melanomas. A detail breakdown of these cases is shown in Supplemental Figure 1. 39 samples (30%) showed gain of the Epo region on chromosome 7p22 and 32 samples (24.6%) had copy number increases at the EpoR region on chromosome 19p13.3-p13.2. 59 cases (45.4%) showed either Epo or EpoR gain and 12 cases (9.2%) showed simultaneous gain of both regions (Table 1). Genomic amplification of the Epo gene was seen in 1 case. The majority of melanomas with Epo or EpoR copy number increases were from acral or mucosal sites (Supplemental Table 1). These data indicate that Epo and EpoR region genomic gain is common in primary melanomas.

Effects of EpoR knockdown in vitro

Three different human melanoma cells lines from different stages, including WM35 (radial growth phase, RGP), WM793 (vertical growth phase, VGP) and 1205Lu (metastatic melanoma), were transfected with EpoR shRNA and selected for stable expression clones ($WM35^{EpoRkd}$, $WM793^{EpoRkd}$, $1205Lu^{EpoRkd}$). Respective melanoma cell lines transfected with empty plasmids were used as controls. EpoR knockdown was confirmed by quantitative RT-PCR (Figure 1A) and western blotting (Figure 1B) using a goat anti-EpoR (N-terminal portion of the protein) antibody. This antibody has been shown to be specific for human EpoR (LaMontagne et al. 2006; Paragh et al. 2009). To confirm the specificity of EpoR knockdown, we also used commercially available EpoR siRNA (Qiagen Hs EpoR 5 Cat No: S102780351) for transient EpoR knockdown. EpoR knockdown was verified by western blot (supplementary Figure 1A) and quantitative PCR (supplementary Figure 1B). We previously showed that Epo stimulates ERK phosphorylation in melanoma cells (Kumar et al. 2005). To confirm the biological effect of EpoR knockdown on ERK phosphorylation, we treated control and $WM793^{EpoRkd}$ with Epo and showed that Epo induced phosphorylation of ERK was significantly decreased in EpoR knockdown cells (Figure 1C). To avoid the influence of mutant $BRAF^{V600E}$ in the ERK phosphorylation assay, we used EpoR siRNA for transient transfection in WM3211 melanoma cells which have wild type BRAF and NRAS proteins. EpoR knockdown by siRNA also resulted in significantly

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diminished phosphorylation of ERK in response to Epo (Figure 1D). The basal phospho-ERK level was also decreased in EpoR knockdown cells suggesting that endogenous EpoR may regulate basal ERK activation in the absence of Epo stimulation (Figure 1C and Figure 1D).

Using a Matrigel migration assay, we found that the migration capacity of melanoma cells increased from WM35 to 1205Lu, whereas EpoR knockdown significantly decreased the migration capacity of the most aggressive cell line 1205Lu (Figure 1E), similar to the effect of EpoR in other cancers (Fu et al. 2009; Mohyeldin et al. 2005). We found that $WM35^{EpoRkd}$ and $WM793^{EpoRkd}$ cell exhibited significantly decreased growth rate compared to control cells under normoxic conditions, while no significant change was observed in $1205Lu^{EpoRkd}$ during the 4-day culture period (Figure 1F). Similar results were seen in melanoma cells with transfected EpoR siRNA (Supplementary Figure 1C).

To assess effect of chemotherapeutic agents on EpoR knockdown melanoma cells, control WM35 and $WM35^{EpoRkd}$ cells were treated with Epo (5U/ml), cisplatin 100 μM, or combination of Epo/cisplatin for 24 hours. Epo significantly inhibited cisplatin induced cell death in the control cells but not in EpoR knockdown melanoma cells (Figure 1G). In addition, EpoR knockdown cells were more sensitive to cisplatin induced cell death (Figure 1G). We then examined the effect of EpoR knockdown on hypoxia tolerance of melanoma cells by culturing these cells under 1% O2. EpoR knockdown resulted in a 60% decrease of 1205Lu cell survival compared to control cells under hypoxic conditions (Figure 1H).

EpoR knockdown in vivo

We injected $WM35^{EpoRkd}$, $WM793^{EpoRkd}$, $1205Lu^{EpoRkd}$ cells or respective control cells subcutaneously into the flank of nude mice. Following EpoR knockdown, WM35 cells were not able to form any xenografts in mice (Figure 2A and 2B); tumor volume of WM793 and 1205Lu EpoR knockdown cells was significantly reduced compared to control groups (Figure 2A and 2B). During necropsy, we noticed that half of the mice receiving control 1205Lu cells developed ascites (Figure 2A), while this was not seen in any mice injected with $1205Lu^{EpoRkd}$ cells. Histological examination showed that despite the xenografts formed by EpoR knockdown cells were significantly smaller compared to control tumor cells, there was significant amount of tumor necrosis (Figure 2C). We quantified the area of tumor necrosis on representative H&E stained sections and found that 50.0±11.6% of the area in $1205Lu^{EpoRkd}$ xenografts were necrotic whereas 32.5±13.2% of the area in control xenografts were necrotic. However, the difference was not statistically significantly. Metastasis of control 1205Lu cells were identified in the lymphatic vessels and internal organs such as liver in the mice that developed ascites (Supplementary Figure 2), while metastasis were not observed in any of the mice injected with $1205Lu^{EpoRkd}$ cells. We counted the number of mitotic figures in the xenografts and found that EpoR knockdown significantly reduced the mitotic rate in the xenografts (Figure 2D). We performed cleaved caspase-3 staining for apoptosis by immunohistochemistry in the xenografts, and no significant changes were identified (Supplemental Figure 2).

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Constitutive EpoR activation in vitro

To further evaluate the role of EpoR in melanoma progression, we transfected 451Lu, WM793 and 1361C melanoma cells with expression plasmids containing EpoR-R129C, a constitutively active EpoR mutant (Pharr et al. 1993). Transfected cells were selected and well-formed clones were isolated, expanded and screened for EpoR expression. Random clones with increased expression of EpoR were used for further experiments (Figure 3A for 451Lu; supplemental Figure 3A and 3B for WM793 and 1361C cells). Melanoma cells transfected with empty expression vectors were used as controls. EpoR-R129C transfected melanoma cells exhibited increased phosphorylation of JAK2, ERK and AKT, supporting constitutive activation of the EpoR pathway in these cells (Figure 3A). We found that constitutively active EpoR significantly increased melanoma cell proliferation under normoxic (Figure 3B) or hypoxic conditions (Figure 3C) *in vitro*. To examine the effect of cisplatin on melanoma cells with EpoR activation, 451Lu with EpoR-R129C or control cells were treated with cisplatin or cisplatin/Epo(5U/ml). The presence of EpoR activation or Epo significantly inhibited cisplatin-mediated cell death compared to control cells (Figure 3D).

Constitutive EpoR activation in vivo

To study the effect of constitutive activation of EpoR *in vivo*, we injected melanoma cells with EpoR-R129C or control vectors into the flanks of immunocompromised mice. As shown in Figure 4A, expression of EpoR-R129C in melanoma cells was associated with significantly faster growth of tumors, and the average tumor size was 3 times that of controls. The mice were sacrificed after 4 weeks, and the xenografts were excised, weighed and processed for histology. As shown in Figure 4B, the tumors formed by EpoR-R129C melanoma cells were significantly larger than tumors consisting of control cells. The xenografts formed by control cells were confined to the dermis at the inoculation site and showed focal tumor necrosis (Figure 4C) after 4 weeks, whereas EpoR-R129C expressing melanoma cells invaded into the surrounding structures, such as the femoral bone (Figure 4C). To dissect the underlying mechanism, we studies the degree of hypoxia within the xengrafts by staining the tumor sections with an antibody to CAIX, a well established tissue hypoxia marker (Carlin et al. 2010) and quantified the staining using a modify H score method as we previously described (Kumar et al. 2005). The CAIX expression was significantly increased in tumors formed by EpoR-R129C cells. Nevertheless, we found that the area with tumor necrosis was significantly smaller in the xenografts formed by EpoR-R129C cells compared to control cells (Figure 4E), further supporting that constitutive EpoR activation may enhance melanoma cell survival under hypoxia *in vivo*.

Effect of recombinant Epo treatment on melanoma growth in vivo

Since constitutive activation of EpoR increases melanoma growth, we then tested whether exogenous Epo treatment may influence melanoma growth *in vivo*. To better monitor tumor growth, we used GFP-labeled 1232Lu melanoma cells which express EpoR by RT-PCR and western blot (data not shown). These cells were injected subcutaneously into the flanks of nude mice. On the following day, animals randomized to the Epo treatment group started to receive daily subcutaneous Epo injections and the control group received saline for 8 weeks. Tumor growth was measured twice a week using a caliper. As expected, there was a

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significant increase in hemotocrits in Epo treated group (Figure 5A), but exogenous Epo had no significant effect on tumor growth in these mice (Figure 5B). After 8 weeks, the xenografts were surgically removed, the surgical area was inspected to ensure that no visible tumor was left, and the wound was closed. We stained the sections with antibody against CD34, an endothelial cell marker, and found that blood vessels in the Epo-treated group were more dilated compared to these in the control group (Figure 5C). We quantified the vessels with open lumen and confirmed that Epo treatment significantly increased the vessel size in the xenografts (Figure 5D). We also stained the primary xenografts with antibodies to Ki67 and cleaved caspase-3 by immunohistochemistry and we did not found significant difference between Epo treated and control tumors (data not shown).

After removing the primary tumor, these mice were observed for an additional 6 weeks for local recurrence or metastasis. Three of four mice in the control group and all four mice in the Epo-treated group developed recurrence near the previous inoculation site. *In vivo* imaging was used to visualize the GFP expressing tumor cells and showed that recurrent/ metastatic tumors in the Epo-treated group were larger compared to controls (Figure 5E). The tumor volume was measured at the end of the sixth week. Recurrent tumors were significantly larger in the Epo treated group (Figure 5F).

Effect of Epo in angiogenesis assays in vivo

Since Epo had an effect on blood vessels in the xenografs, we then directly assessed whether Epo induces angiogenesis. The growth factor deleted Matrigel plugs were supplemented with VEGF or Epo. There was little new vessel formation in control Matrigel plugs macroscopically (Figure 6A control) and histology showed little growth of vessels in these Matrigel plugs (Figure 6B control). Angiogenesis was increased in the plugs supplemented with VEGF (Figure 6A VEGF) and histology showed blood filled vessels in the Matrigel plugs (Figure 6B VEGF). Angiogenesis was also increased in plugs supplemented with Epo (Figure 6A Epo) and histology showed blood vessels in the Matrigel plugs (Figure 6B Epo).

We have previously shown that WM35 melanoma cells can produce and secrete Epo *in vitro* (Kumar et al. 2005). We performed a modified Matrigel plug assay by mixing Matrigel with WM35 melanoma cells (2×10^6 in 500μl Matrigel) in the presence of a blocking anti-Epo antibody (25 μg/plug). The Matrigel mixtures were injected into the flank region of nude mice and left in place for 9 days. The Matrigel plugs were harvested and processed for histology and immunohistochemical staining with an antibody to CD34. We found that the plugs mixed with melanoma cells showed significant growth of blood vessels (Figure 6C control). In contrast, the Matrigel plugs with melanoma cells and anti-Epo antibody showed significantly less well formed vessels (Figure 6C, Anti-Epo antibody). The vessel density was significantly higher in the control plugs (Figure 6D).

Discussion

The demonstration of adverse clinical outcomes of poor survival and potential for increased tumor progression reported in a series of trials associated with rHuEpo use in cancer patients has raised serious safety concerns. The mechanisms of the observed adverse clinical effects have remained elusive and whether expression of EpoR mRNA transcripts and protein found

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in many different types of primary human tumors modulates tumor cell behavior has been controversial (Sytkowski 2007). In this study, we showed that EpoR gene is frequently expressed with copy number increase in melanoma and EpoR activation supports melanoma cell survival *in vivo*.

Our gene expression profiling studies showed that a majority of early passage melanoma cell lines express EpoR. This result is supported by a recent study showing that expression of EpoR protein can be detected by immunohistochemistry in about 50% of the analyzed melanoma metastasis specimens (Mirmohammadsadegh et al. 2010). The aCGH study using primary melanoma tissues with a broader representation of different melanoma subtypes showed that DNA copy number increases at the Epo and EpoR loci are common (30% and 24.6% respectively) and that these aberrations are particularly frequent in acral and mucosal melanomas.

It has been shown that embryonic neural crest cells express functional EpoR (Knabe et al. 2005; Tsai et al. 2006). Since melanocytes are neural crest derivatives and it may be not surprising that EpoR is functional in melanoma. Lodish and colleagues have demonstrated that small numbers of high-affinity Epo surface binding sites are present on tumor cells of neural origin, and the high affinity binding of Epo to these tumor cells is mediated by EpoR (Um et al. 2007). The antibody we used has been shown to recognize the human EpoR protein (Knabe et al. 2005; LaMontagne et al. 2006; Paragh et al. 2009). In this study, EpoR protein expression as detected by this antibody decreased significantly after EpoR shRNA or siRNA transfection, with a concomitant decrease in EpoR mRNA expression determined by quantitative RT-PCR. In addition, Epo induced activation of ERK pathway was significantly down-regulated in melanoma cells with EpoR knockdown. Taken together, our studies suggest that melanoma cells express biologically active and functional EpoR.

We found that EpoR expression supports tumorigenesis in the xenograft model. $WM35^{EpoRkd}$ cells were incapable of forming xenografts, whereas the tumor mass of $WM793^{EpoRkd}$ and $1205Lu^{EpoRkd}$ cells were significantly smaller compared to controls. Although EpoR knockdown did not change the growth rate of 1205Lu cells under normoxia, hypoxia significantly reduced $1205Lu^{EpoRkd}$ cell growth *in vitro*, suggesting that EpoR function in tumors is oxygen tension dependent. Since hypoxia is very common in solid tumors (Koritzinsky and Wouters 2007; Wouters and Koritzinsky 2008), this may explain the profound effect of EpoR knockdown in the xenograft model whereas EpoR knockdown only has modest effect on cell proliferation *in vitro* under normoxia. In addition, unlike the parent 1205Lu cells which are highly aggressive and are able to metastasize, $1205Lu^{EpoRkd}$ cells were confined to the primary inoculation sites, suggesting that EpoR expression may also contribute to tumor invasion and metastasis. This conclusion is further supported by the experiments showing that melanoma cells with EpoR-R129C formed larger xenografts with deeper invasion into surrounding tissues. We showed that larger xenografts formed by melanoma cells with EpoR-R129C have higher degree of hypoxia demonstrated by stronger CAIX staining, however, necrotic area in these xenografts was significantly smaller comparing to control cells, further supporting that activation of EpoR increases tumor cell hypoxia tolerance.

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Given our data suggesting that EpoR activation support melanoma growth *in vivo*, it was surprising to see that exogenous Epo treatment failed to enhance melanoma growth *in vivo*. However, these results are consistent with the reported findings of prior studies showing that recombinant Epo failed to increase human squamous cell, colorectal and breast carcinoma growth in animal models (LaMontagne et al. 2006; Tovari et al. 2005). These result led us to devise a novel animal model to study the role of exogenous Epo administration on *in vivo* tumor growth. One of the limitations of previous studies has been the relatively short duration of Epo treatment. Another limitation has been the difficulty presented by continuing tumor growth follow-up in animals bearing large tumors after 8 weeks (Arcasoy 2008; Sinclair et al. 2007). To address these limitations, we surgically removed the primary tumors and continued the Epo treatment, an approach mimicking excision of primary human tumor. Importantly, we found that that the local recurrence of melanoma was increased by this regimen, suggesting that despite the lack of effect of Epo on tumor cell proliferation, it may enhance the survival of melanoma cells under less favorable, hypoxic growth conditions in the wounded areas (Daniel and Groves 2002; Kumar et al. 2005). Since our study also showed that Epo increases angiogenesis in melanoma, these data indicate that although melanoma cells express biologically functional EpoR, short term Epo treatment (8 weeks) does not induce proliferation of melanoma cells *in vivo*. However, it may increase the survival of EpoR expressing melanoma cells under less favorable growth conditions through both its direct effect on tumor cells and/or its indirect effects on tumor neoangiogenesis. The effect of Epo on tumor growth is likely dosage and duration dependent. Nevertheless, more studies are needed to fully elucidate the effect of long term Epo treatment on tumor growth *in vivo*.

In summary, our data strongly suggest that EpoR activation enhances melanoma cell survival *in vivo*. However, the effects of Epo on tumor cells appear to be complex, and the potential effect of exogenous Epo may depend not only on the presence of EpoR on tumor cells, but also treatment duration and dosage.

Materials and Methods

Cell Culture, EpoR knockdown and constitutively active EpoR transfection

Melanoma cells were cultured under normoxic and hypoxic conditions as previously described (Kumar et al. 2007). We used EpoR shRNA oligonucleotides (top strand, 5'-GAT**CCCTACAGCTTCTCCTACCAG**TTCAAGAGA**CTGGTAGGAGAAG CTGTAGTT**A-3'; bottom strand, 5’-AGCTT**AACTACAGCTTCTCCTACCAG**TCTCT TGAA**CTGGTAGGAGAAGCTGTAG**G-3’) contained a region specific to bases 362 to 382 of EpoR mRNA (bold), a hairpin loop region, and 5’ and 3’ linker sequences in p*Silencer*™ 4.1-CMV hygro vector. EpoR siRNA oligo (Hs EpoR 5 Cat No: S102780351) was purchased from Qiagen. EpoR-129C vectors were used for constitutive EpoR activation (Pharr et al. 1993).

Cell viability and proliferation assays

The cell 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega Corp.,

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Madison, WI) according to the manufacturer's instructions. Absorbance was recorded at 570 nm using a 96-well plate reader. Experiments were carried out in triplicate. Cell proliferation was also measured using a WST-1 Cell Proliferation Assay (Roche Diagnostics, Indianapolis, IN). Absorbance was measured at 450 nm using a 96-well plate reader. Experiments were carried out in triplicate. Trypan blue dye exclusion assay was done as we described previously (Kumar *et al.* 2007).

Matrigel™-GFR invasion assay—The membrane invasion culture system was used to measure cell invasion. Plates pre-coated with growth factor reduced matrigel (Matrigel-GFR) chambers (BD Biosciences, San Jose, CA) were prepared and assay performed according to the manufacturer's instructions.

Quantitative RT-PCR—Total RNA was prepared using the RNeasy kit (Qiagen Inc, Valencia, CA) and cDNAs were prepared using SuperScript™ First –Strand Synthesis system for RT-PCR according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Real Time PCR was performed with specific primer for EpoR (forward 5'CCT GAC GCT CTC CCT CAT CC 3'; reverse 5'GCC TTC AAA CTC GCT CTC TGG 3') and β-actin (forward 5'CTA CCTCAT GAA GAT CCT CAC CGA 3'; reverse 5'ACG TAG CAC AGC TTC TCC TTA ATG 3'). cDNA corresponding to 1μg of RNA was added to the iQ™ SYBER green super mix (Bio-Rad Laboratories, Hercules, CA). PCR reactions were carried out in an iCycler PCR machine (Bio-Rad Laboratories, Hercules, CA).

Western Blotting and immunohistochemistry

Western blot was performed using antibodies to EpoR (goat polyclonal anti-human EpoR antibody, 1:200 dilution, R&D systems, Minneapolis, MN), phospho-ERK p42/44 (1: 1000 dilution), phospho-AKT (1: 1000 dilution), AKT (1: 1000 dilution) (Cell signaling INC, Danvers, MA), p42/44(1: 500 dilution) JAK2 (1: 200 dilution) (Santa Cruz Biotechnology, Inc.), phospho-JAK2 (1:1000 dilution, Upstate, Billerica, MA) and β-actin (1:1000 dilution, Sigma-Aldrich, St. Louis, MO). Immunohistochemistry was performed using antibodies to CAIX (1:200 dilution, Abcam, Cambridge, MA); CD34 (1:50 dilution, Abcam, Cambridge, MA); Cleaved Caspase-3 using Cleaved Caspase-3 (Asp175) (Asp175) IHC Detection Kit (Cell Signaling Technology) as per manufacturer's recommendations. A detailed method for quantification of staining and vascular density was described previously (Kumar et al. 2005; Seaman et al. 2011).

Animal studies

All animal studies were performed under a protocol approved by the IACUC at the University of Pennsylvania. For EpoR knockdown studies *in vivo*, 4 million EpoR knockdown cells (WM35^{EpoRkd}, WM793^{EpoRkd}, 1205Lu^{EpoRkd}) or respective control cells transfected with scramble shRNA were injected into the flank region of nude mice. The mice were followed for 5 weeks and tumor size was measured weekly (Reynolds et al. 2005). For EpoR constitutive activation, 2 million 451Lu stably transfected with EpoR-R129C or empty expression vector were used.

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To study the effect of recombinant Epo in melanoma xenograft model, we used 1232Lu cells expressing green fluorescent protein (GFP). Mice randomized to Epo treatment (2000 U/kg) and control groups and they received daily injection of saline (100μl). Mice were treated with Epo for 8 weeks, the primary tumors were then removed, the surgical wounds were inspected to ensure that no visible tumor remained, and the wounds were closed. Mice were observed for an additional 6 weeks for signs of local recurrence or metastasis. The Epo group continued to receive twice a week injections of Epo during this period of time. Tumor recurrence was visualized using the IVIS imaging system at the Bioluminescence Molecular Imaging Core facility at the University of Pennsylvania. To assess angiogenic effects of Epo *in vivo*, we used a well established Matrigel plug assay (Ley et al. 2004). 6–8 week old athymic nude mice received 500 μl subcutaneous injection of growth factor reduced Matrigel (BD Biosciences) alone, Matrigel with rHuEpo (500 U/ml of Matrigel), or Matrigel with rhVEGF (60 ng/ml of Matrigel) (RD system, Minneapolis, MN). In addition, we also used a modified Matrigel plug assay by mixing Matrigel with WM35 melanoma cells (2×10^6 in 500μl Matrigel) or WM35 cells and anti-Epo antibody (25 μg, R&D systems). Six Matrigel plugs in each group were analyzed.

Genomic Data Analysis in early passage melanoma cell lines

Gene expression profiles of 65 cultured melanoma and 5 human neonatal melanocyte cell lines were described elsewhere (Lin et al. 2008) and are used in this study and analyzed as previously described (Yu et al. 2008).

Array CGH in primary melanoma

Existing array CGH data from 130 primary invasive melanomas from University of California, San Francisco were included in the analysis (Curtin et al. 2005). A detailed breakdown of the cases is shown in supplemental Table 1. All statistical analyses were performed using the freely available R/Bioconductor software.

Statistical analyses

The data represent mean ± s.e.m values. The effect of treatments and differences among experimental groups were assessed using analysis of variance (ANOVA) and appropriate post-hoc test. The differences between two experimental groups were determined using Student's t-tests. A two-tailed value of $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We are grateful to M. Herlyn from the Wistar Institute for providing melanoma cell lines. Financial support was provided by CA-093372, CA-116103, AR-054593 and Melanoma Research Foundation (to X.Xu) and CA-100844 (to M.O. Arcasoy).

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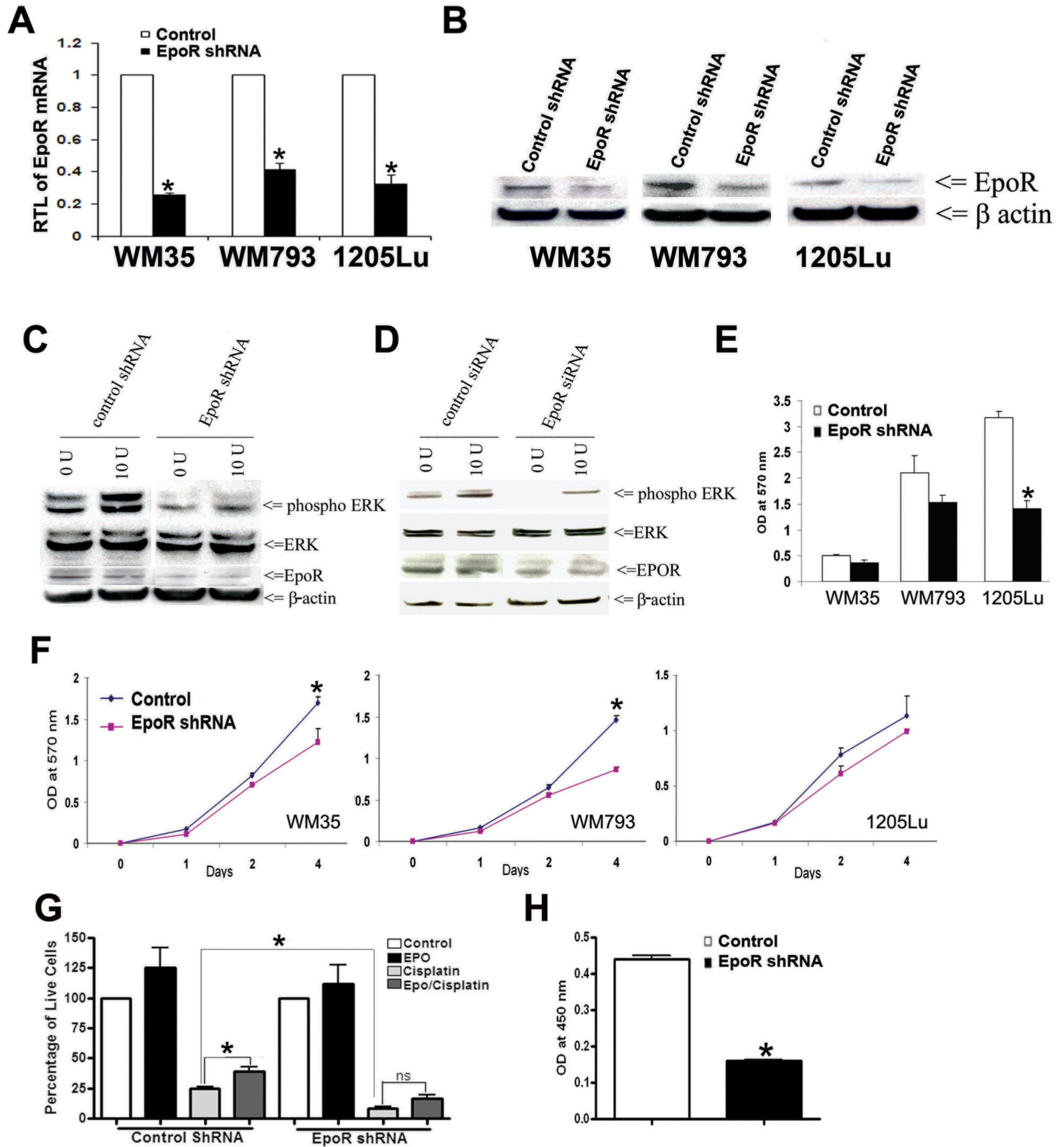

Figure 1. Effects of EpoR knockdown *in vitro*

A. EpoR mRNA expression. Quantitative RT-PCR of EpoR in melanoma cells with control scrambled or EpoR shRNA. * indicates p<0.05 comparing to control shRNA. Bars show mean ± SEM from three separate experiments. **B.** EpoR protein expression. Western blot analysis of WM35, WM793 and 1205Lu cells transfected with control or EpoR shRNA. Representative image from three independent experiments. **C.** ERK phosphorylation in response to Epo. Melanoma cells with control or EpoR shRNA were stimulated with Epo (10U/ml). **D.** ERK phosphorylation in response to Epo. Melanoma cells with control or

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EpoR siRNA were stimulated with Epo (10U/ml). **E.** EpoR knockdown and cell migration. WM35, WM793 and 1205Lu melanoma cells were placed in growth factor reduced Matrigel Boyden chambers. Cells that migrated through the chamber were quantified. Bars show mean ± SEM from three separate experiments. * indicates $p<0.05$ comparing to 1205Lu control cells. **F.** Cell proliferation under nomoxia *in vitro*. WM35, WM793 and 1205Lu cells with control or EpoR shRNA were cultured under nomoxia and quantified. * indicates $p<0.05$ comparing to corresponding controls. **G.** Effect of Epo and cisplatin on cell proliferation. WM35 melanoma cells with control or EpoR shRNA were treated with cisplatin (100 μM), Epo (5U/ml) or both for 24 hours. Live cells were counted using trypan blue dye exclusion assay. Bars show mean ± SEM from three separate experiments. * indicates $p<0.05$ comparing to corresponding controls. NS indicates not statistically significant. **H.** Cell proliferation under hypoxia *in vitro*. 1205Lu cells with control or EpoR shRNA were cultured under 1% O2 for 48 hours, and cell proliferation was quantified. Bars show mean ± SEM from three separate experiments. * indicates $p<0.05$ compared with 1205Lu cells with control shRNA.

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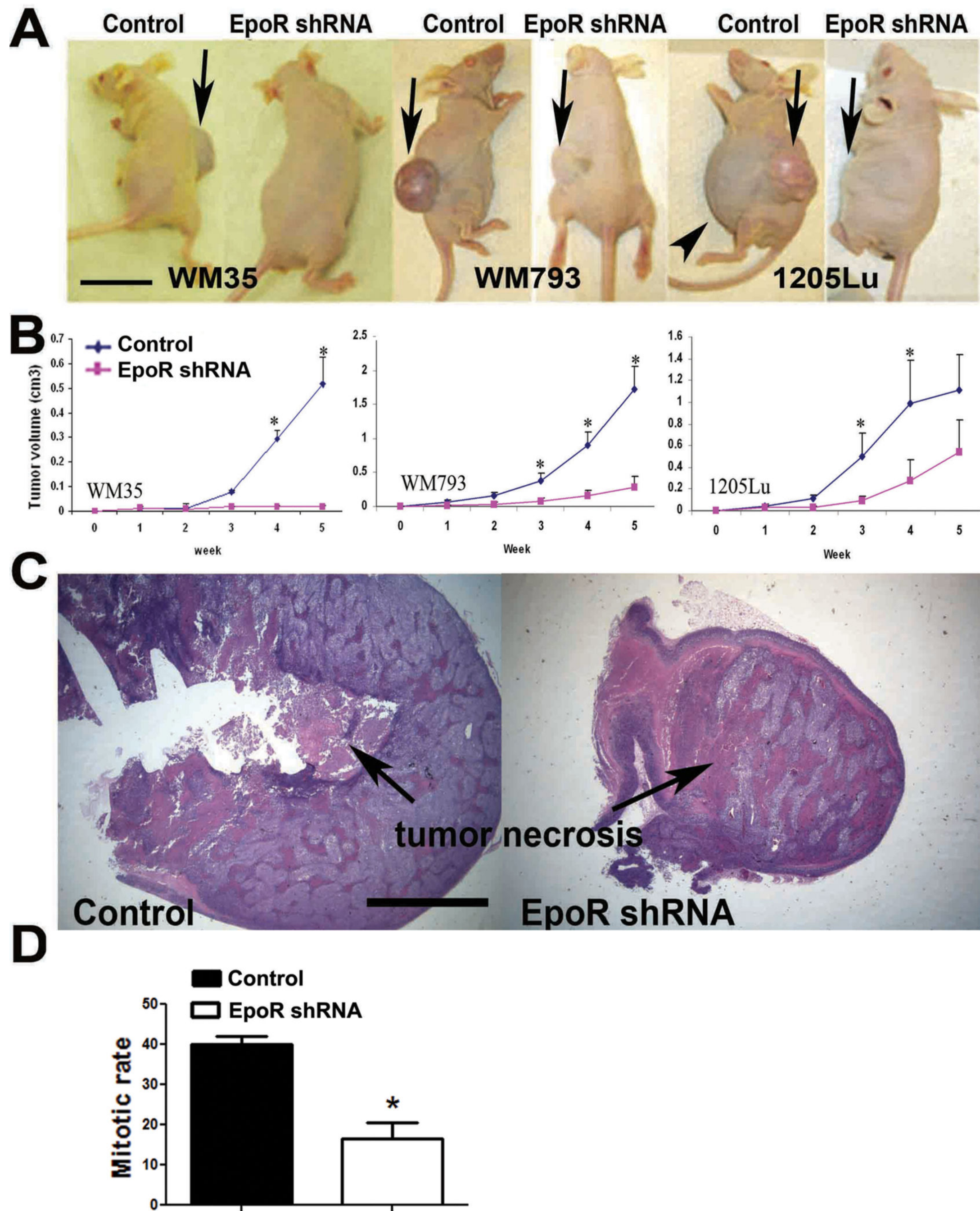

Figure 2. Effects of EpoR knockdown *in vivo*

WM35^{EpoRkd}, WM793^{EpoRkd}, 1205Lu^{EpoRkd} or respective control cells were injected into the flanks of nude mice. All mice were sacrificed after 5 weeks. **A.** Photographs of xenografts. Arrows point to the tumors and arrow head points to bulging abdomen with ascites in the mouse injected with 1205Lu cells. Bar indicates 4 cm. **B.** Tumor growth rate. After subcutaneous injection, tumor size was measured weekly. * indicates $p<0.05$ comparing to respective control cells. Bars show mean ± SEM from 4 xenografts. **C.** Histology of xenografts formed by WM793 or WM793^{EpoRkd} cells. Arrows points to the

area with tumor necrosis. Bar indicates 3 mm. **D.** Mitotic rate in the xenografts. Mitotic rate was quantified per mm^2. * indicates $p<0.05$ compared with the control.

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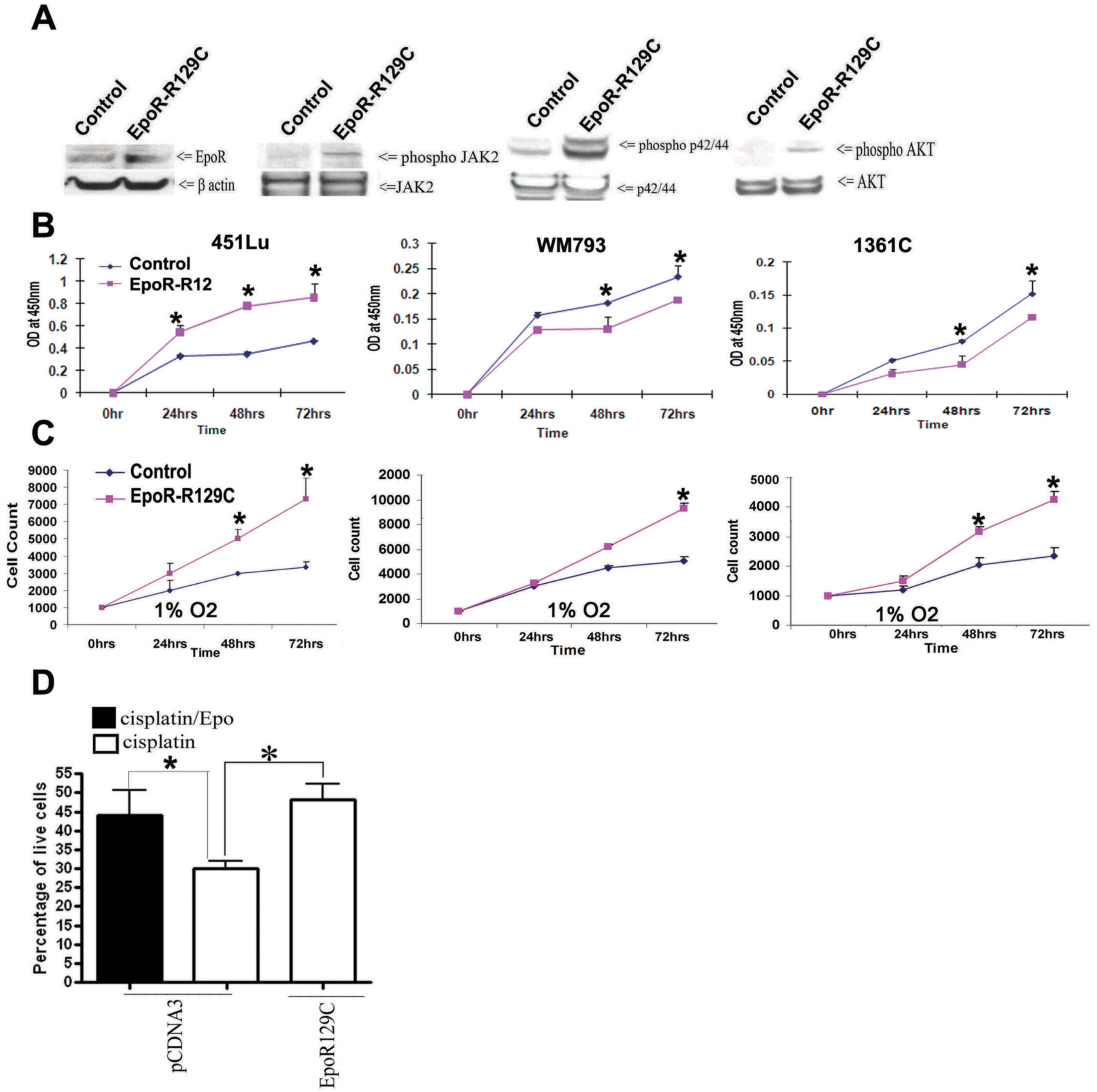

Figure 3. Effects of constitutive EpoR activation *in vitro*

We transfected the 451Lu melanoma cells with EpoR-R129C or control empty vectors. **A.** Activation of EpoR signaling pathways. Western blot analysis phosphorylated and total JAK2, ERK and AKT proteins in melanoma cells with control or EpoR-129C vectors. **B.** Cell proliferation under nomoxia. 451Lu, WM793, and 1361C cells with control or EpoR-R129C plasmids were cultured under normoxic condition and cell proliferation was quantified. * indicates $p<0.05$ comparing to respective controls. The data represent mean ± SEM from three separate experiments. **C.** Cell proliferation under hypoxia. 451Lu, WM793,

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and 1361C cells with control or EpoR-R129C plasmids were cultured under hypoxic condition and cell proliferation was quantified. * indicates $p<0.05$ comparing to respective controls. The data represent mean ± SEM from three separate experiments. **D.** Cell proliferation after Epo and cisplatin treatment. 451Lu melanoma cells with control or EpoR-R129C plasmids were treated with Epo (5U/ml), cisplatin (100μM), or Epo/cisplatin for 24 hours, and surviving cells were quantified. Bars show mean ± SEM from three separate experiments. * indicates $p<0.05$ compared to cells with control shRNA.

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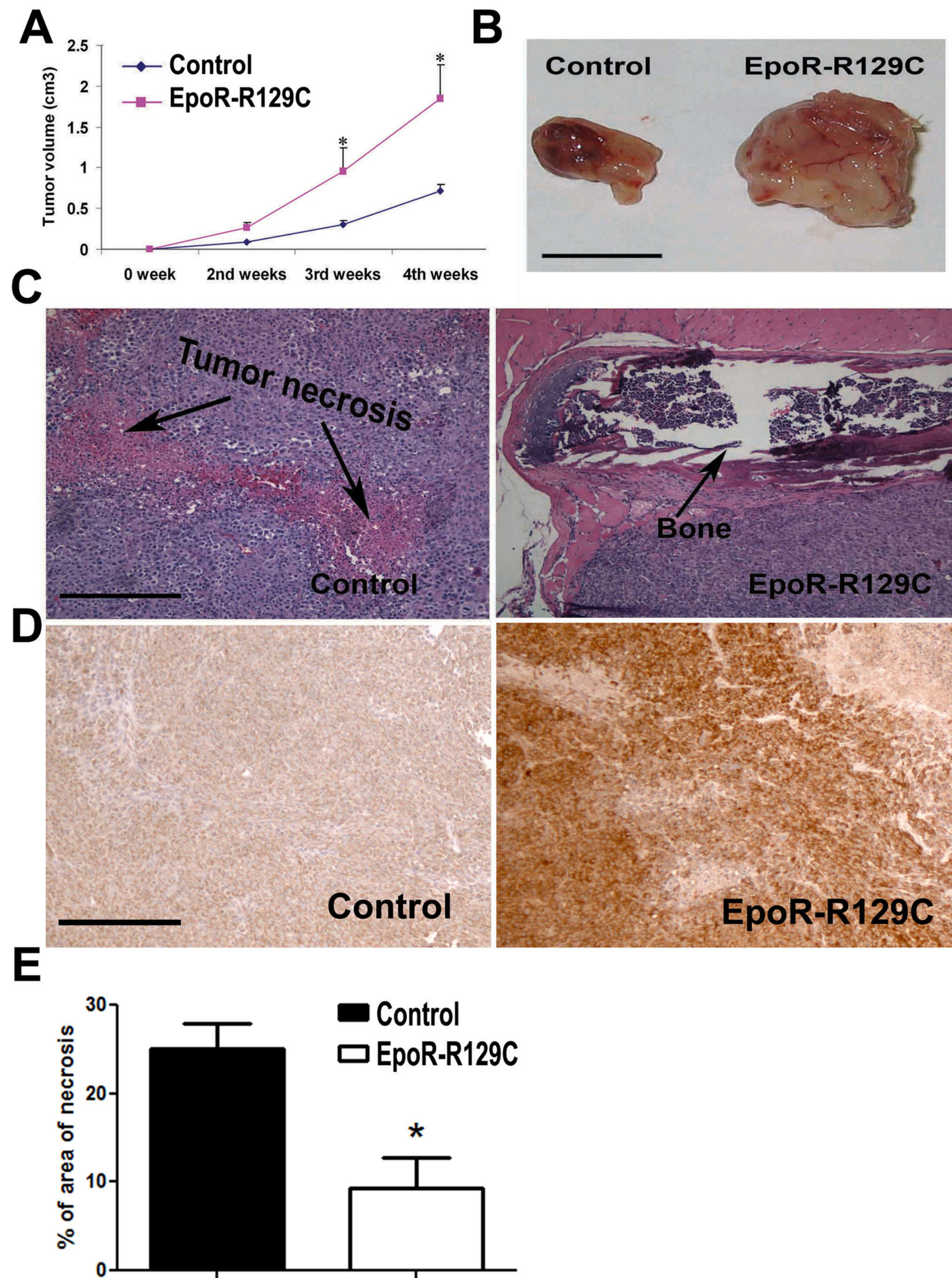

Figure 4. Effects of constitutive EpoR activation *in vivo*

A. Tumor growth rate *in vivo*. Melanoma cells with control or EpoR-R129C were injected into the flank area of NOD/SCID mice and tumor size was measured weekly. * indicates $p<0.05$ comparing to control. Bars show mean ± SEM from 5 xenografts. **B.** Morphology of xenografts. Bar indicates 1 cm. **C.** Tumor histology. Arrows points to tumor necrosis in xenografts formed by melanoma cells with control shRNA (left panel). EpoR-R129C expressing tumor cells invaded into surrounding femoral bone. Arrow points to bone (right panel). Bar indicates 200 μm. **D.** CAIX expression. Tumor sections were stained with an

antibody to CAIX. Bar indicates 200 μm. **E.** Quantification of tumor necrosis. The area with tumor necrosis was measured using a micrometer. * indicates p<0.05 comparing to control.

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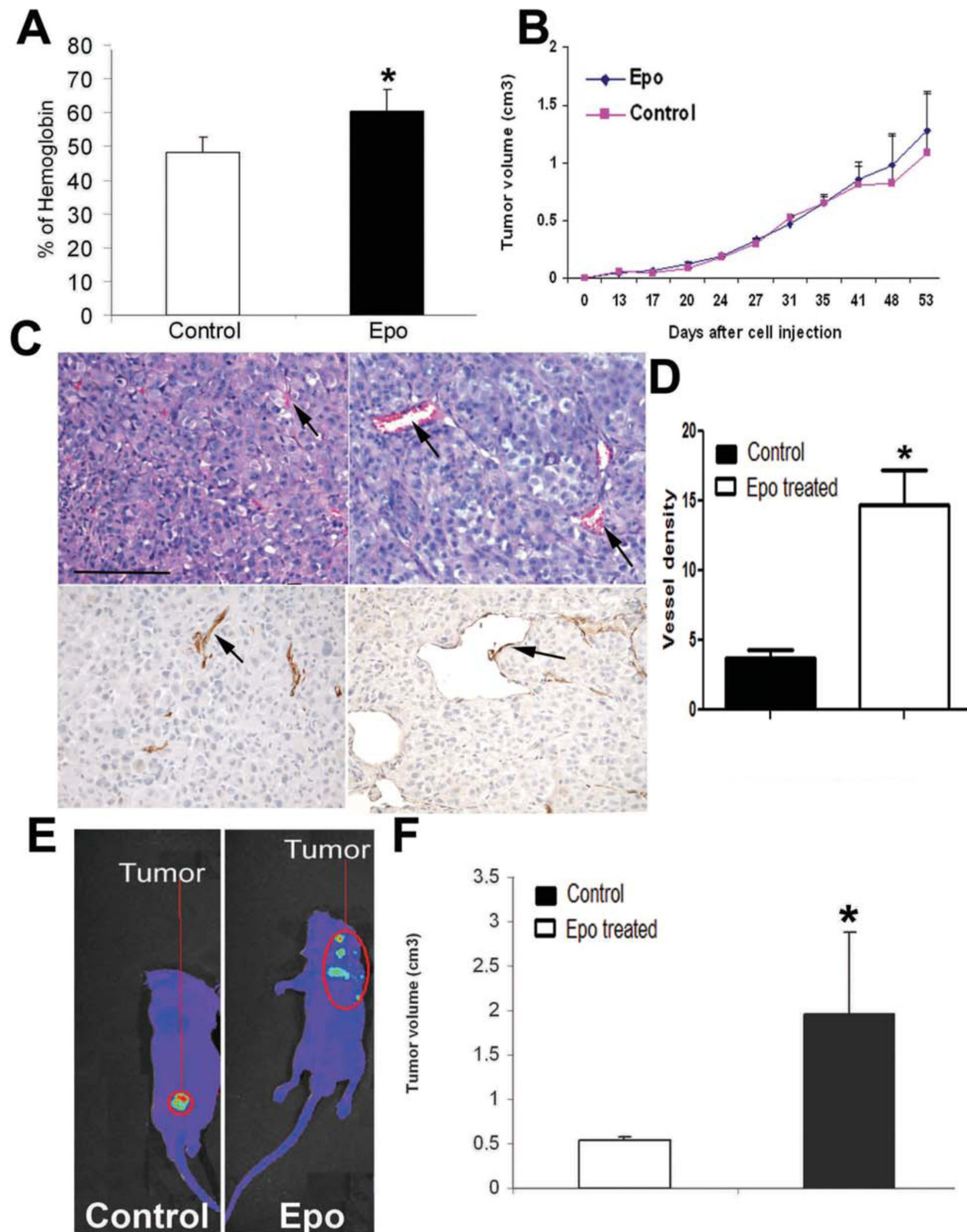

Figure 5. Effects of recombinant Epo treatment *in vivo*

GFP-1232Lu melanoma cells were injected subcutaneously into athymic nude mice. Epo group received daily subcutaneous Epo injection (2000 U/kg) in 100ul saline and the control group receiving daily injection of saline (100 ul) for 8 weeks. **A.** Hematocrit. Hematocrit was measure after 8 weeks of Epo treatment. * indicates $p<0.05$ comparing to control. Bars show mean ± SEM from 4 mice. **B.** Tumor growth rate *in vivo*. Tumor size was measured twice weekly. **C.** Histology and CD34 stain of primary xenografts. The tumor vasculature in the Epo-treated group appeared more dilated than the control group. The sections were

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stained with anti-CD34 antibody. **D.** Open vessel density. Vessels with lumen width more than 0.03 mm were quantified per mm^2. * indicates $p<0.05$ comparing to control. **E.** The primary tumors were removed, and the wounds were closed. These mice were observed for another 6 weeks. The Epo treated group continued to receive twice a week injection of Epo (2000U/kg) during that period of time. *In vivo* imaging showed that the recurrent tumors were larger in Epo treated group. **F.** Tumor volume was measured at the end of the sixth week. The secondary tumors at the site of local recurrence were significantly larger in the Epo treated group. * indicates $p<0.05$ comparing to control. Bars show mean ± SEM from 4 mice.

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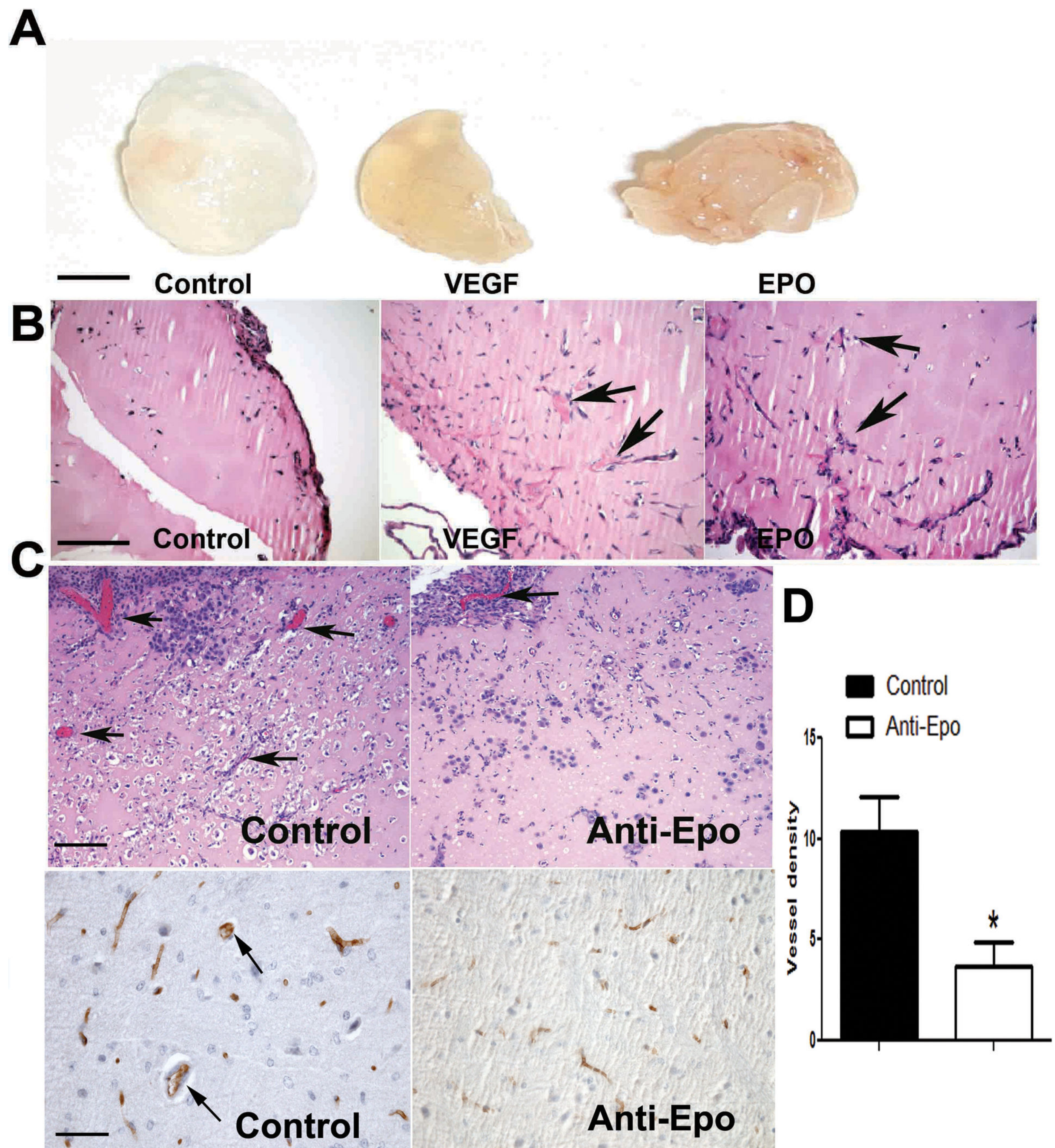

Figure 6. Epo and angiogenesis *in vivo*

A and B. Matrigel plug assay (A: gross; B: Histology). Athymic nude mice received subcutaneous injection of growth factor reduced Matrigel plugs supplemented with saline, Epo or VEGF. Angiogenesis was observed in plugs supplemented with VEGF or Epo. Arrows point to the blood vessels. **C.** Modified Matrigel plug assay. Athymic nude mice received subcutaneous injection of growth factor reduced Matrigel plugs supplemented with WM35 melanoma cells or WM35 cells plus anti-Epo antibody. Arrows points to blood filled vessels (upper panels). The sections were stained with an antibody to CD34. Arrows points

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to the stained open blood vessels (lower panel). **D.** Quantitative analysis of vessel density. The vessel density in the plugs was counted per mm^2. * indicates $p<0.05$ comparing to control. Bars show mean ± SEM from 6 Matrigel plugs.

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Table 1

Genomic changes in primary melanomas at Epo and EpoR loci.

Type of aberration	Epo locus (%)	EpoR locus (%)
Gain	39 (30.0)	32 (24.6)
Amplification	1 (0.8)	0 (0)
Loss	7 (5.4)	4 (3.1)

130 primary melanomas were included in the analysis. Gain at both Epo and EpoR loci was present in 12 cases (9.2%); and 59 cases (45.4%) of melanomas showed either Epo or EpoR gain.