Angiogenesis (2011) 14:467–479
DOI 10.1007/s10456-011-9230-4

ORIGINAL PAPER

Heme oxygenase 1 (HO-1) challenges the angiogenic switch in prostate cancer

M. Ferrando · G. Gueron · B. Elguero · J. Giudice · A. Salles · F. Coluccio Leskow · E. A. Jares-Erijman · L. Colombo · R. Meiss · N. Navone · A. De Siervi · E. Vazquez

Received: 26 May 2011 / Accepted: 30 July 2011 / Published online: 11 August 2011

Abstract Prostate cancer (PCa) is the second leading cause of cancer-associated death in men. Once a tumor is established it may attain further characteristics via mutations or hypoxia, which stimulate new blood vessels. Angiogenesis is a hallmark in the pathogenesis of cancer and inflammatory diseases that may predispose to cancer. Heme oxygenase-1 (HO-1) counteracts oxidative and inflammatory damage and was previously reported to play a key role in prostate carcinogenesis. To gain insight into the anti-tumoral properties of HO-1, we investigated its capability to modulate PCa associated-angiogenesis. In the present study, we identified in PC3 cells a set of inflammatory and pro-angiogenic genes down-regulated in response to HO-1 overexpression, in particular VEGFA, VEGFC, HIF1α and $\alpha5\beta1$ integrin. Our results indicated that HO-1 counteracts oxidative imbalance reducing ROS levels. An in vivo angiogenic assay showed that intradermal inoculation of PC3 cells stable transfected with HO-1 (PC3HO-1) generated tumours less vascularised than controls, with decreased microvessel density and reduced CD34 and MMP9 positive staining. Interestingly, longer term grown PC3HO-1 xenografts displayed reduced neovascularization with the subsequent down-regulation of VEGFR2 expression. Additionally, HO-1 repressed nuclear factor κB (NF-κB)-mediated transcription from an NF-κB responsive luciferase reporter construct, which strongly suggests that HO-1 may regulate angiogenesis through this pathway. Taken together, these data supports a key role of HO-1 as a modulator of the angiogenic switch in prostate carcinogenesis ascertaining it as a logical target for intervention therapy.

Keywords Heme oxygenase 1 (HO-1) · Angiogenesis · NF-κB · VEGF · Prostate cancer

M. Ferrando and G. Gueron contributed equally to this work and are co-first authors.

M. Ferrando · G. Gueron · B. Elguero · A. Salles · F. C. Leskow · A. De Siervi · E. Vazquez (✉)
Department of Biological Chemistry, School of Sciences, CONICET, University of Buenos Aires, Intendente Guiraldes 2160, Ciudad Universitaria, Pabellón II, 2do Piso, 1428 Buenos Aires, Argentina
e-mail: elba@qb.fcen.uba.ar

J. Giudice · E. A. Jares-Erijman
Department of Organic Chemistry, School of Sciences, CIHIDECAR CONICET, University of Buenos Aires, Ciudad Universitaria, Pabellón II, 2do Piso, 1428 Buenos Aires, Argentina

L. Colombo
Research Area, Inst of Oncology "A.H.Roffo", University of Buenos Aires, Buenos Aires, Argentina

R. Meiss
Department of Pathology, Institute of Oncological Studies, National Academy of Medicine, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina

N. Navone
Department of Genitourinary Medical Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

Background

Angiogenesis is a rare phenomenon in healthy adults, occurring only locally and transiently under distinctive physiological conditions such as wound healing and inflammation. It is required for an efficient blood supply and also plays a critical role in tumor growth and metastasis. Many stimuli including hypoxia, growth factors,

cytokines and oxidative stress can increase the expression of pro-angiogenic factors in tumor cells, which correlates with increased microvessel formation and poor prognosis in many human cancers [1]. VEGF, one of the most important angiogenic factors, promotes angiogenesis by signaling through the interaction with specific transmembrane receptors as VEGFR1, VEGFR2 and VEGFR3 [2]. Targeting VEGF receptor expressing cells is effective in controlling the progression of cancer [3].

Elevated reactive oxygen species (ROS) generation, was found in several tumors and has been associated with malignant transformation and the regulation of angiogenesis [1]. The induction of heme oxygenase 1 (HO-1), the rate limiting enzyme in heme degradation, represents a key event in cellular responses to pro-oxidative and pro-inflammatory insults [4]. It participates in the maintenance of cellular homeostasis, by reducing oxidative injury, attenuating inflammation and regulating cell proliferation [5]. There are differences in HO-1 basal expression profiles among cells and tissues and its pleiotropic effects to restore homeostasis. Thus, HO-1 has been proposed to act as a biosensor regulating cell destination [6]. However, the role of HO-1 in angiogenesis may vary depending on the tissue specific underlying conditions [7].

Prostate cancer (PCa) is one of the leading cause of cancer-associated death in men. It has been recognized that inflammation increases PCa tumorigenesis [8] and that a chronic inflammatory microenvironment in cancer cells is a decisive factor in the evolution of the disease. The molecular mechanisms that underlie the pathogenesis of cancer-associated inflammation are complex and involve a delicate interplay between tumor and its microenvironment [9]. In PCa tumors, the switch to an angiogenic phenotype is known to be critical for its progression. Unless a tumor can stimulate the formation of new blood vessels, it remains restricted to a microscopic size [10]. Inflammation and hypoxia are widely accepted as key elements in the induction of angiogenesis. Although it is well accepted that PCa dissemination and its capacity to survive in a distant organ is affected by angiogenic factors [8], the earliest molecular events that dictate the angiogenic switch remain elusive in PCa, probably because they occur before a clinical diagnosis can be established [11]. In tissues undergoing angiogenesis, the process of neovascularization involves a complex series of sequential events targeted in the activation of matrix degrading enzymes and motility [12].

One targeted approach for the control of prostate carcinogenesis should rely on the understanding of the molecular events that govern PCa associated angiogenesis. In this regard, our previous studies have shown that HO-1 is expressed in human primary "naive" treatment prostate carcinomas [13] and other authors have reported its expression in castrate-resistant PCa [14]. We have recently demonstrated that HO-1 inhibits cell proliferation, migration and invasion in prostate cancer cells and impairs tumor growth in vivo [15]. We also identified MMP9, a promoter of angiogenesis [16], as a novel downstream target of HO-1 [15]. However, the role of HO-1 in PCa angiogenesis is unexplored.

Increasing evidence suggests that inhibition of nuclear factor κB (NF-κB) activity in PCa cells could suppress the angiogenesis, invasion and metastasis by down-regulating the expression of NF-κB downstream target genes, such as VEGF, uPA, and MMP-9 [17].

Here, we found that PC3HO-1 xenografts displayed impaired angiogenesis compared to control tumors which established a novel role of HO-1 in angiogenesis. We identified several pro-angiogenic factors downmodulated by HO-1 in PC3 cells and proposed a mechanism mediated by NF-κB inhibition. These findings provide cogent evidence that HO-1 overexpression in PCa has a strong in vivo anti-angiogenic effect challenging tumor development.

Results

Comparative gene expression analysis shows HO-1 down-modulation of pro-angiogenic genes in PC3 cells

In our original analysis of a comparative gene array study between PCa cells over-expressing HO-1 and controls (RT-qPCR Oligo GEArray® Human Angiogenesis Microarray analysis), we found that HO-1 repressed the expression of MMP9 [15], which has been correlated with PCa invasion, angiogenesis and metastasis. This comparison also revealed decreased expression of several pro-inflammatory and angiogenic genes, which appeared to be downstream targets of HO-1, including ANGPT1, ANGPTL3, FIGF, IL6, VEGF A, VEGF C, VEGF D, THBS1 [15]. Here we decided further our analyzes to include some other genes involved in angiogenesis (e.g., integrins [18]). Currently, $\alpha5\beta1$ integrin is the best characterized integrin for its function in cancer and its contribution to angiogenesis [18, 19]. In accordance with our previous findings [15], mRNA levels of the $\alpha5$ integrin subunit were found to be significantly reduced (50%, $P < 0.05$) in PC3 HO-1 over-expressing cells (PC3HO-1) compared to controls (Fig. 1a). We also found a 60% ($P < 0.05$) reduction in the expression of HIF1α mRNA levels in PC3 cells over-expressing HO-1 compared to PC3βGal (Fig. 1b). Of all the pro-angiogenic factors induced by HIF1α, VEGF is particularly noteworthy, because it has potent angiogenic properties and is expressed in a large number of human cancers including prostate tumors [20, 21]. Accordingly, PC3HO-1 cells showed a reduction of both VEGF C (70%,

$P < 0.05$) and VEGF A (30%, $P < 0.05$) compared to PC3βGal (Fig. 1c, d), key players throughout the development of the tumor, helping vessels establish, grow and survive. To examine the effect of HO-1 induction in VEGF transcriptional activation, the cells were transfected with a full-length human VEGF promoter cloned upstream of the luciferase gene. HO-1 overexpression strongly inhibited the VEGF reporter activity (Fig. 1e).

Previous studies provide strong evidence that endogenous ROS play important roles in cancer cell induced angiogenesis and tumor growth through VEGF expression [17]. Thus, it is possible that ROS mediate HO-1 regulation of VEGF transcripts. We used intracellular DCFHDA staining method to measure ROS levels in PCa cells. When cells were exposed to a stressor as H_2O_2, ROS levels were significantly less in PC3HO-1 ($P < 0.05$) compared to controls as assessed by cell by cell immunofluorescence quantification (Fig. 2). These data suggest that HO-1 might regulate VEGF transcriptional activation by blocking ROS generation.

Forced-expression of HO-1 inhibits in vivo angiogenesis in nude mice

We next wanted to assess whether HO-1 impaired the angiogenic process in vivo. Tumor microvessel density is regarded as an important prognostic marker and an independent predictor of pathological stages and malignant potential of PCa [22]. We have previously demonstrated that HO-1 overexpression inhibited prostate tumor growth in vivo [15]. To assess whether the above mentioned decrease in the expression of pro-angiogenic genes correlated with changes in new blood vessel formation, we performed an in vivo angiogenesis assay [23] and assessed the adjacent tumor microvasculature density in PCa xenografts. PC3HO-1 and control cells were injected intradermally in the right flank of athymic *nu/nu* male mice together with a drop of trypan blue, in order to localize the centre of the injected area, 6 days later. The left (contralateral) flank was injected with vehicle. Small tumors developed in all mice injected after 6 days post-inoculation (early stage xenografts). No significant difference was observed in body weight for mice bearing PC3HO-1 and PC3βGal tumors (data not shown). Microvessel density (MVD) of tumor sections was determined as an index of tumor angiogenesis. Six days after xenograft generation, the vasculature on the adjacent skin was photographed, quantified and compared with the microvasculature of the contralateral flank. Capillary tubular formation was visualized in both PC3HO-1 and PC3βGal xenografts (Fig. 3a, b). Although no significant difference was observed in the number of medium vessels (Fig. 3c), HO-1 overexpression caused a significant disruption on the tubular formation ability of small capillary-like vessels (number of small vessels per area (mm^2) in the PC3HO-1 group (0.59 ± 0.11, mean $\pm$ SEM) versus the control group (1.53 ± 0.11, mean $\pm$ SEM) (Fig. 3d). Furthermore, Masson's trichrome staining, that highlights vessel walls confirmed these results (Fig. 3b). The microscopic sections showed well-preserved subcutaneous tumors in PC3βGal group and

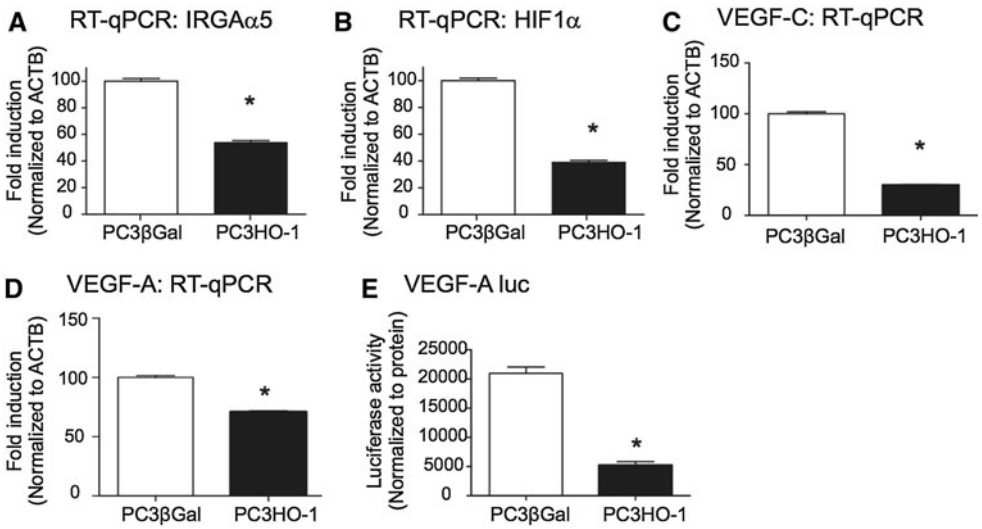

Fig. 1 HO-1 downmodulates pro-angiogenic genes in PC3 cells. PC3 cells were stably transfected with pcDNA3HO1 (PC3HO-1) or empty vector (PC3βGal). mRNA expression levels of **a** α5 integrin subunit **b** HIF1α, **c** VEGF C, **d** VEGF A, were analyzed by RealTime-PCR. Data were normalized to β-actin. One representative from at least three independent experiments is shown. *Significant difference, $P < 0.01$. **e** PC3HO-1 and PC3βGal cells were transfected with the VEGF luciferase reporter plasmid. Then cells were lysed and luciferase activity assay was performed. Data were normalized to protein values. One representative from at least three independent experiments is shown. *Significant difference, $P < 0.05$

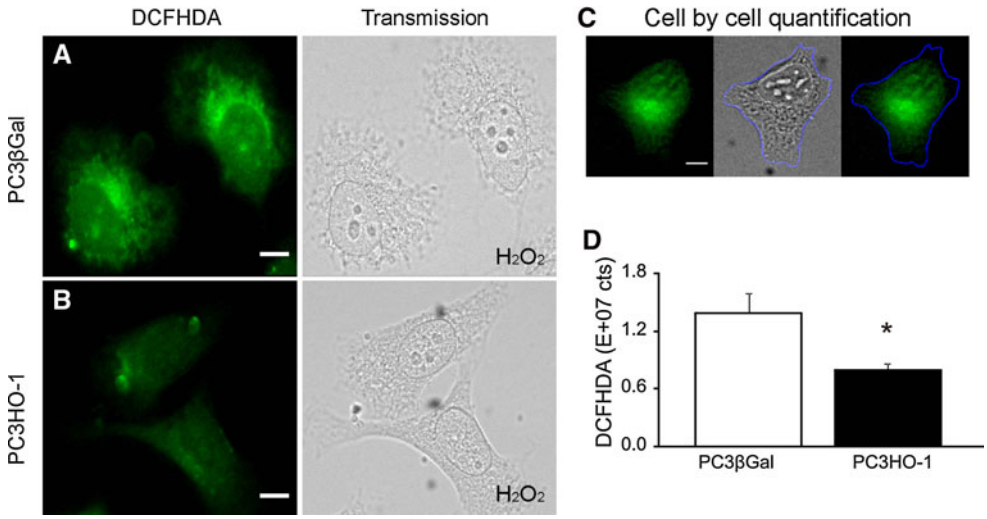

Fig. 2 HO-1 abrogates ROS generation. **a** PC3HO-1 and **b** PC3βGal cells were treated with (H_2O_2 200 μM, 1.5 h), stained with CM2-DCFHDA (10 μM, 1 h), sensitive to ROS. Imaging was performed in PBS by wide field microscopy (Olympus IX71). All images were acquired with the same exposure time to allow comparison of signal intensities. *Scale bars* 10 μm. **a** and **b** *Left Panels* represent fluorescent images, an indicative of the presence of ROS. **a** and **b** *Right Panels* represent bright field images. **c** Segmentation of cell region (*blue line*). **d** Cell to cell quantification of DCFHDA levels by the generation of a Matlab routine. Cells with similar sizes were considered for the statistical analysis. The results are shown as the mean ± SEM. *Significant difference, $P < 0.01$

numerous large calibre vessels were observed in the surrounding normal tissue adjacent to tumor. Control xenografts also displayed intratumoral medium calibre vessels, predominantly of peripheral disposition (Fig. 3b). In accordance, PC3HO-1 xenografts showed a decrease in vessel number and size in both intratumoral and adjacent normal tissue (Fig. 3b).

To confirm the anti-angiogenic effect of HO-1 in vivo, we investigated intratumoral microvessel density by immunohistochemistry analysis of the endothelial cell specific marker CD34. The microscopic examination showed only sporadic CD34 positive cells in the PC3HO-1 xenografts (Fig. 4a, b) but numerous positive cells in the control group of tumors (Fig. 4c, d). In agreement with our previous findings in long-term xenografts (grown s.c. for 23 days) [15], PC3HO1 tumors showed weak nuclear and cytoplasmatic MMP9 immunostaining in few cells arranged in a diffuse pattern (Fig. 4e, f). Meanwhile, control tumors showed intense nuclear and cytoplasmatic immunostaining for MMP9 in numerous neoplastic cells with predominance of those located in the peripheral area (Fig. 4g, h). Altogether, these results further confirm that HO-1 rises as a novel in vivo modulator of angiogenesis in PCa.

HO-1 induction abrogates NF-κB signaling pathway

One other factor that regulates angiogenesis is NF-κB, possibly by inducing expression of pro-angiogenic factors such as VEGF [17]. Activation of NF-κB is also a central event in the initiation and amplification of inflammatory responses and tumor progression [24]. TNF-α, an upstream regulator of NF-κB, was found to be downregulated by genetic induction of HO-1 (82%, $P < 0.01$) compared to control cells (Fig. 5a). Since several of the HO-1 downregulated genes are NF-κB target genes, we sought to assess NF-κB activity in PC3 cells under HO-1 modulation. We then analyzed whether HO-1 controls NF-κB activity using a reporter construct containing five repeats of the NF-κB consensus binding sequence cloned upstream of the luciferase gene (pNFκB-luc). Hemin significantly repressed (36.6%, $P < 0.05$) the transcriptional activity of the pNFκB-luc in PC3 cells (Fig. 5b). Moreover, a similar response was observed in PC3 cells transiently transfected with HO-1 (45.6% reduction, $P < 0.05$) (Fig. 5c). Overexpression of RelA/p65 was used as a positive control of NF-κB activation, resulting in a significant threefold increase ($P < 0.05$) of NF-κB luciferase activity. In order to determine whether the effect of HO-1 on NF-κB-mediated transcription had the same effect that a specific repressor of this pathway, we used the mutated inhibitor of NF-κB (IκBss). This mutant is not susceptible to phosphorylation and, for that reason, constitutively suppresses NF-κB activation. As shown in Fig. 5c, overexpression of IκBss entirely inhibited NF-κB transactivation (72%, $P < 0.05$) to a similar extent as HO-1 overexpression. It is note worthy the fact that the PC3 cell line (androgen receptor null) displays high constitutive NF-κB activation consistent with that observed in other androgen-insensitive PCa cells compared with their androgen-responsive counterparts [25, 26].

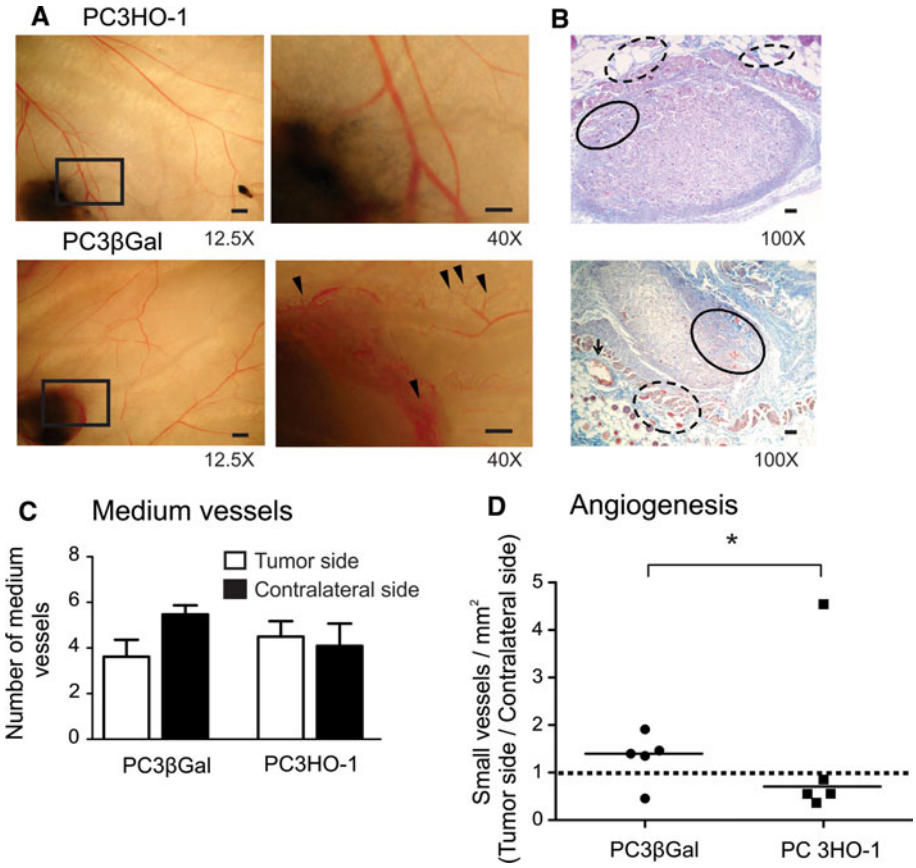

Fig. 3 HO-1 inhibits angiogenesis in vivo. PC3HO-1 and PC3βGal cells were injected intradermally in the right flank of *nu/nu* mice. Vehicle was injected in the left flank (contralateral side). After 6 days the skin was removed and photographs were taken under a stereo microscope. Small and medium vessels were measured. **a** Images of skin adjacent to PC3HO-1 (*upper panels*) and PC3βGal (*lower panels*) tumors (Original magnification: *Left* ×12.5, *scale bars* 200 μm; *Right* ×40, *scale bars* 100 μm). *Arrow heads* indicate small vessels. **b** Trichrome staining of tumor samples (PC3HO-1 *upper panel* and PC3βGal *lower panel*; Original magnification: ×100, *scale bars* 50 μm). Few small vessels are observed in PC3HO-1 tumor (*circle with solid line*) and in the surrounding tissue (*circle with dashed line*). Medium-sized vessels are observed in the PC3βGal tumor periphery (*circle with solid line*) and in peri-tumor tissue (*circle with dashed line*); large vessel in adjacent tissue is indicated (*arrow*). **c** Number of medium vessels in the tumors (*open bar*) and in the contralateral side (*full bar*). **d** Quantification of small vessels. Relativization of small blood vessels in tumor side to contralateral side in each animal. Fisher test. *Significant difference, $P < 0.05$

NF-κB is normally sequestered in the cytoplasm by the NF-κB inhibitor (IκB). The IκB kinase (IKK), involved in propagating the cellular response to inflammation, specifically phosphorylates the inhibitory IκB protein. This phosphorylation results in the dissociation of IκBα from NF-κB and thereby NF-κB activation. In accordance with the repressed NF-κB-mediated activation observed under HO-1 modulation, we also detected increased expression levels of IκB mRNA in PC3 cells when HO-1 was induced genetically or pharmacologically (Fig. 5d) and significantly repressed mRNA levels of IKK (36%, $P < 0.05$) in PC3HO-1 cells (Fig. 5e). Taken together these results suggest that the anti-angiogenic effect of HO1 are at least mediated by the downregulation of NF-κB activity.

HO-1 overexpression inhibits VEGFR2 in PC3 long-term xenografts

To further explore the anti-angiogenic role of HO-1 in vivo, we examined VEGFR2 expression, a specific angiogenic marker which mediates almost all of the known cellular responses to VEGF [27]. Long-term PC3HO-1 tumors (grown *s.c.* for 23 days) showed a significant lower VEGFR2 expression with a weak positive cytoplasmic immunostaining in scanty tumor cells compared to PC3βGal tumors with intense positive cytoplasmic immunostaining for VEGFR2 in many tumor cells with a cell membrane pattern (Fig. 6a). The differential staining intensity between both tumor groups was quantitatively assessed (Fig. 6b). Accordingly, VEGFR2

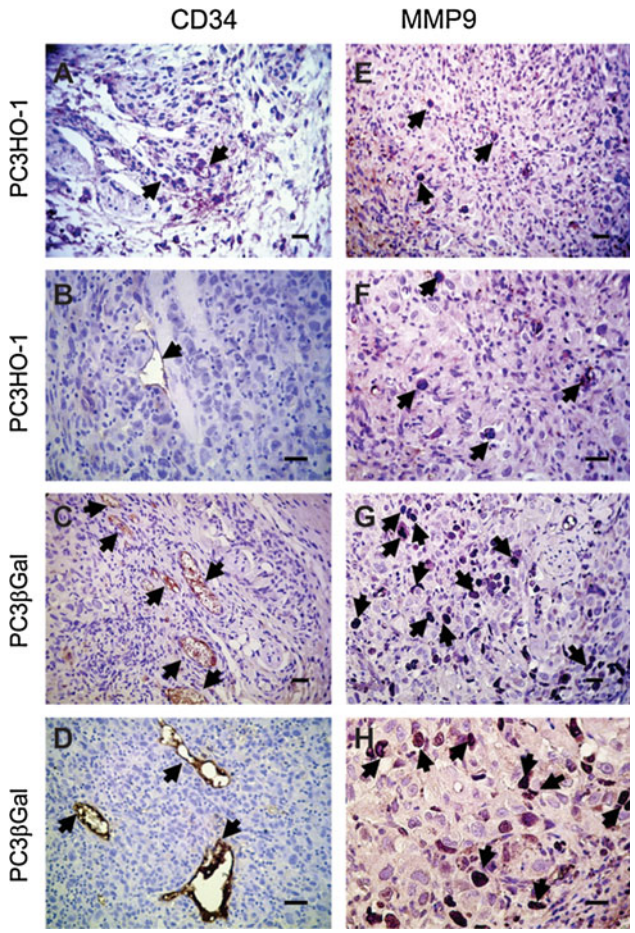

Fig. 4 Decreased expression of CD34 and MMP9 in PC3HO-1 xenografts. Tumors were generated as described in "Methods" and Legends to Fig. 3. Tissue sections of PC3HO-1 (*upper panels*) and PC3βGal (*lower panels*) were immunostained for CD34 (**a**–**d**) and MMP9 (**e**–**h**). **a** Subcutaneous tumor edge with few, medium caliber, vascular structures. **b** Intratumor isolated vascular structure of medium size. **c** Subcutaneous tumor edge with numerous vascular structures of medium and large calibre. **d** Intratumor vascular structures of medium and large calibre with prominent endothelium. Arrows indicate positive staining for CD34. **e** Few tumor cells with mild positive immunostaining. **f** Mild nuclear immunostaining. **g** Numerous tumor cells with intense positive immunostaining. **h** Nuclear and cytoplasmatic immunostaining pattern. *Arrows* indicate positive staining for MMP9. **a**, **c**, **e** and **g** Original magnification $\times$250. **b**, **d**, **f** and **h** Original magnification $\times$400. *Scale bars* 20 μm

mRNA levels were found to be significantly reduced (32%, $P < 0.01$) in PC3HO-1cells compared to controls (Fig. 6c). PC3HO-1 tumors grown for 23 days, not only were smaller [15] but also clearly less vascularized as it can be macroscopically and quantitative determined (Fig. 6d, e).

These results provide strong evidence in support of the anti-angiogenic activity of HO-1 in vivo, which could be partially responsible for its anti-tumor activity previously reported in PCa [15].

Discussion

PCa cancer is one of the leading cause of cancer in men [28]. Several molecular alterations leading to constitutively active mitogenic and cell survival signaling, as well as loss of apoptotic response, are involved in uncontrolled growth of PCa, leading to castrate-resistant progression, apoptotic resistance and increased expression and secretion of angiogenic factors [29]. The conversion to the angiogenic phenotype, known as the "angiogenic switch", is thought to contribute to the growth and progression of prostate tumors from latent and localized focal carcinomas to invasive carcinomas. Thus, anti-angiogenic therapy emerges as an essential strategy for the treatment of cancer [3]. However, angiogenesis is a complex process mediated by multiple factors (e.g., hypoxia, growth factors, cytokines and oxidative stress [30]) and thus it is essential to decipher the critical steps involved in this process in prostate cancer. We believe that our studies had identify new players in this process and thus open new opportunities for therapy development for prostate cancer.

ROS function as signaling molecules to mediate several angiogenic responses such as cell proliferation, migration and gene expression in endothelial and cancer cells [1]. Moreover, high ROS production in cancer cells are implicated in the regulation of angiogenesis and tumor growth, probably regulating HIF1α and VEGF expression [31]. In agreement with these reports, we document here that ROS levels are diminished when PC3 cells over-expressing HO-1 are exposed to a stressor (Fig. 2), likely as one of the pathways favouring reduced angiogenesis.

It is well accepted that HO-1 confers cytoprotection against oxidative stress and inflammation and several signaling molecules are implicated in the cytoprotection [31]. In addition, this protein exerts vital metabolic functions limiting the axis of heme degradation and maintaining the cellular homeostasis. Although classical recognized as a microsomal protein, its presence has been detected in other subcellular compartments [13, 32–34] such as the nucleus, with a non-catalytic canonical function [35], thus implying that there are HO-1 functions that remain to be elucidated.

Pathological angiogenesis is a hallmark of cancer [36]. Unlike physiological forms of angiogenesis that occur during embryonic development and normal wound healing, pathological, tumor-induced angiogenesis often ensues due to imbalances in either angiogenic activators or inhibitors [37]. Recent pre-clinical and clinical studies have employed strategies to target tumor vasculature as a mean to impede tumor growth and eventual metastasis [38]. However, anti-angiogenic therapies in human subjects have proven to be limited due to marginal effectiveness, toxicities, and relative lack of specificity [38]. Therefore, further

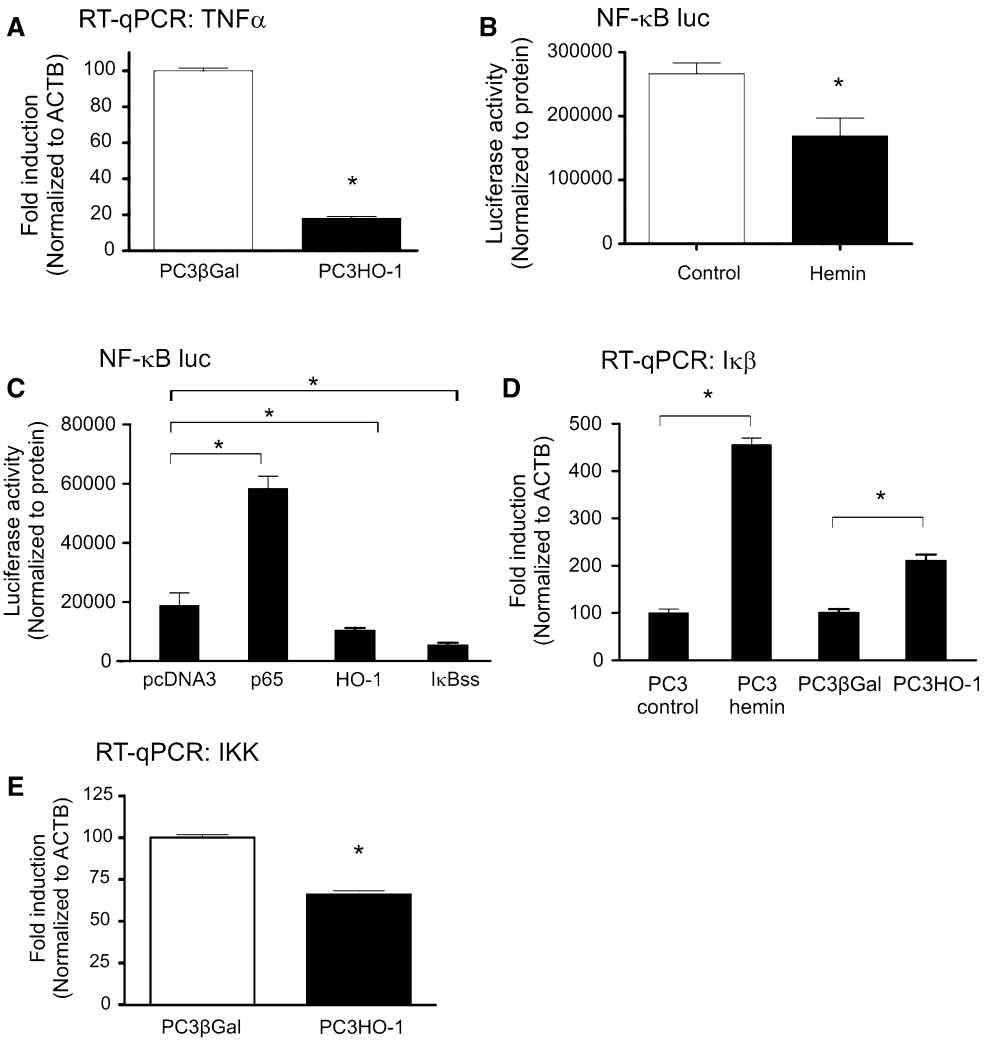

Fig. 5 HO-1 abrogates NF-κB signaling pathway. PC3 cells were transiently transfected with pcDNA3HO1 (PC3HO-1) or empty vector (PC3βGal). **a** TNFα mRNA expression levels. Data was normalized to β-actin. One representative from at least three independent experiments is shown. *Significant difference, $P < 0.01$. PC3 cells, **b** treated or not with hemin (70 μM, 24 h), **c** transiently transfected with pcDNA3HO-1, empty vector, p65 expression vector or a specific repressor of NF-κB (ssIκB), were transfected along with the NF-κB reporter construct containing five repeats of the NF-κB consensus binding sequence linked to the luciferase gene (pNFκB-luc). Cells were then lysed and luciferase activity assay was performed. Data were normalized to protein values. One representative from at least three independent experiments is shown. *Significant difference, $P < 0.05$. **d** IκB and **e** IKK mRNA levels were analyzed by RealTime-PCR. *Significant difference, $P < 0.05$

exploration is currently underway for more specific drugs that target tumor angiogenesis.

The role of HO-1 in both, tumor growth and angiogenesis is controversial [5]. While it has been reported that HO-1 accelerates tumor angiogenesis of human pancreatic cancer [39] and urothelial carcinoma of the urinary bladder [40], in colon cancer, inhibition of the HO pathway increases metastasis to the liver [41]. Our results together with those reports suggest that HO-1 function is context-dependent and demonstrate once again the complexity of HO-1 signaling. The controversial results indicate that HO-1 exhibits distinct effects on tumorigenesis, which may represent different phases of the multistep carcinogenesis process. In turn, HO-1 effect may be related to its relative expression levels and their synergistic/antagonistic interactions with other signaling molecules and angiogenic players. Thus, cancer cells and the surrounding stroma are exposed in a milieu and received several signalling stimuli, where HO-1 may enforce or restrict tumour-promoting functions, according to the selected tumoral microenvironment. Furthermore, Bussolati et al. [4, 42] proposed a dual role for HO-1: whereas VEGF induced angiogenesis required HO-1 activity, blood vessel formation induced by inflammation was attenuated by HO-1 overexpression. Therefore, the mechanism of HO-1 in pathological angiogenesis is not clear and in cancer it may depend on the type of tumor or other still not defined factors.

We previously reported the nuclear expression of HO-1 in human primary prostate carcinomas [13]. In PCa cell lines we further confirmed that HO-1 up-regulation induced its nuclear localization and inhibited cell proliferation, migration and invasion. Moreover, it impaired tumor growth in vivo and downregulated the expression of target genes associated with inflammation and angiogenesis [15]. In the present report we confirmed that HO-1 forced-expression in PC3 cells, a highly aggressive and invasive PCa cell line, repressed VEGFA, VEGFC and HIF1α at the transcriptional level (Fig. 1). HO-1 overexpression greatly inhibited the VEGF promoter activity (Fig. 1). Furthermore, in vivo studies showed that HO-1 overexpression

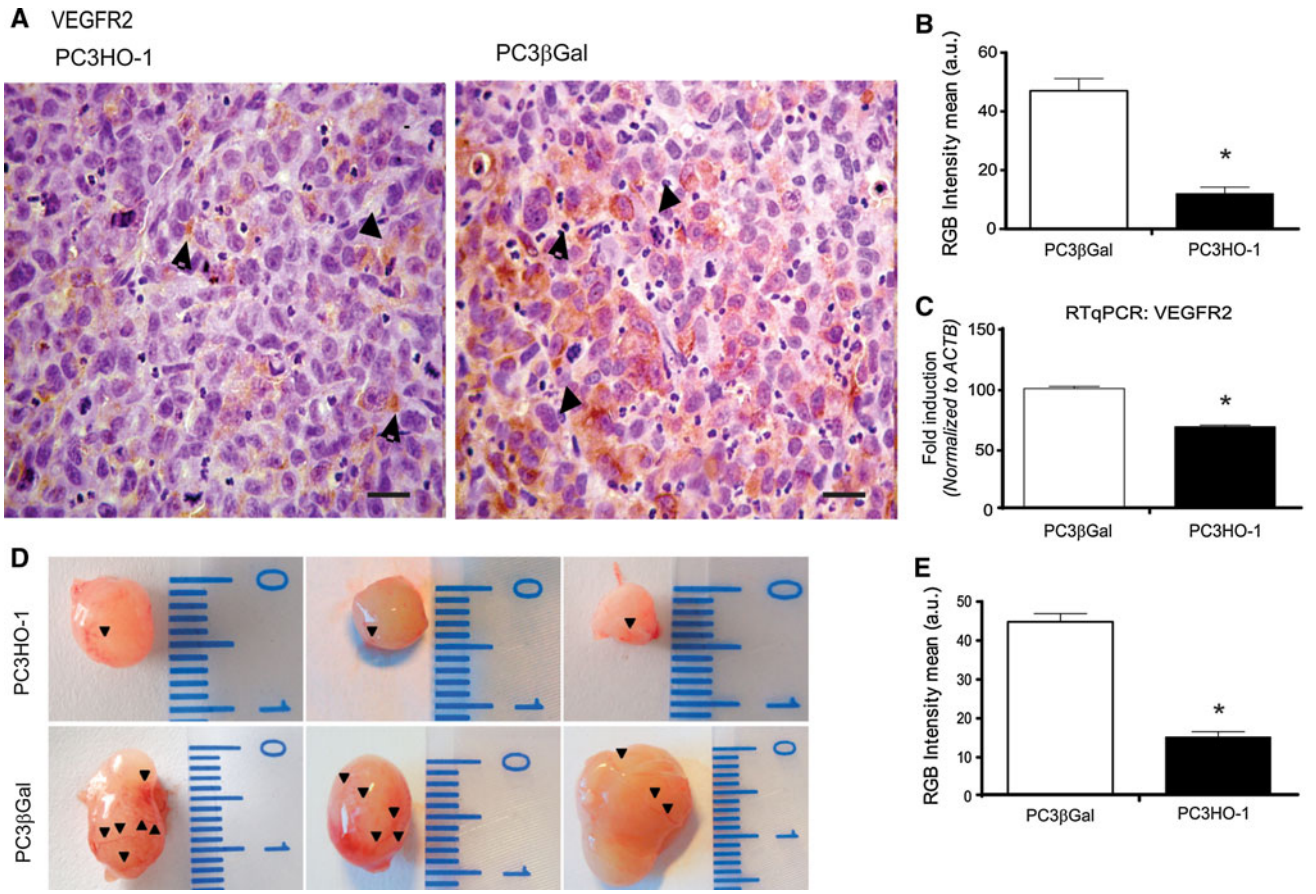

Fig. 6 Impaired angiogenesis in long-term xenografts. Athymic nude (*nu/nu*) mice were injected *s.c.* in the right flank with PC3 cells overexpressing HO-1 (PC3HO-1) or control (PC3βGal). Animals were sacrificed after 23 days and tumors excised. **a** Histological analysis of tumors showing VEGFR2 immunostaining. PC3HO-1 (*left panel*) showed few cells with weak positive cytoplasmic stain, while PC3βGal (*right panel*) presented numerous cells with intense cytoplasmatic positive stain and cell membrane pattern. *Arrow heads* indicate positive VEGFR2 immunoreactivity. Magnification ×400, *scale bars* 20 µm. **b** Cell area was segmented in each image for quantification of the mean immunostaining intensity. The results were expressed as mean ± SEM. *Significant difference, $P < 0.05$. **c** VEGFR2 mRNA expression levels were analyzed by RealTime-PCR in PC3HO-1 and PC3βGal cells. Data was normalized to β-actin. *Significant difference, $P < 0.01$. **d** Representative images of tumors excised at day 23 showing the reduced growth and vascularization of HO-1 overexpressing xenografts. **e** Blood vessel formation was quantified using Image J. Tumor area was segmented in each image and were converted to grey scale. Grey mean intensity was assessed. The difference between this value and the background was considered and normalized to tumor volume. The results were expressed as mean ± SEM. *Significant difference, $P < 0.05$

significantly impaired the ability of early-stage PC3HO-1 xenografts to form vascular structures, with a marked decrease in the number of small vessels, microscopically corroborated by Masson trichrome staining (Fig. 3). Finally, the expression of CD34 and MMP9, markers of angiogenesis, was significantly impaired in PC3HO-1 xenografts (Fig. 4), further confirming the anti-angiogenic capability of HO-1.

The process of tumor cell invasion and metastasis requires the degradation of connective tissue associated with vascular basement membranes and interstitial connective tissue [17]. In addition, VEGF stimulation can enhance the motility of targeted tumor cells in cooperation with MMPs and urokinase plasminogen activator (uPA)-mediated pathways [43]. In accordance, early stage PC3HO-1 xenografts displayed a striking lower MMP9 expression compared with control xenografts (Fig. 4), coincident with reduced microvessel density (Fig. 3). It is note worthy that MMP9 expression did not increase over time, and that tumor volume remained significantly small in PC3HO-1 xenografts compared to controls [15]. Immunohistochemical analysis also revealed decreased expression of VEGFR2 in PC3HO-1 long-term xenografts (Fig. 6). VEGF and VEGFR are expressed at high levels by prostate carcinoma cells and their expression correlates with increasing grade, vascularity, and tumorigenicity [44]. These relationships have been observed in humans as well as in animal models of PCa. Evidence from clinical studies have also shown that high VEGF levels in PCa are associated with poor prognosis [45] and that plasma levels of VEGF increase with PCa progression to skeletal metastases.

Given that inflammation is a critical component of tumor growth and progression [46], and that several molecular and cellular linking pathways have been proposed but yet not fully elucidated, we next sought to identify a possible mechanism by which HO-1 impairs angiogenesis, with an eye toward the NF-κB pathway. NF-κB activation was found to stimulate angiogenesis, possibly by inducing expression of pro-angiogenic factors as VEGF [47]. Classical activation of NF-κB proceeds by the degradation of IκB proteins, which is mediated by the activity of the IκB kinase complex (IKK). Here we demonstrated that HO-1 overexpression in PC3 cells repressed NF-κB activation, induced the accumulation of IκB and decreased IKK mRNA levels (Fig. 5). Our results are in line with evidence that points to a pathophysiologic role for NF-κB in PCa [48]. This factor was also associated with high expression of MMP9 and VEGF in PCa [49].

Moreover, NF-κB was found to be involved in the integrin-coupled ECM signaling following oxidative stress. It is of particular significance that many genes which are regulated by oxidative stress are targets of NF-κB such as integrins [19]. In this context, in our study HO-1 overexpression reduced α5 integrin subunit transcript levels (Fig. 1).

NF-κB is constitutively activated in human prostate adenocarcinoma and correlates with disease progression [49]. It is worthy to remark that NF-κB blockade results in decreased angiogenesis in several PCa models [50, 51]. Given that NF-κB transcriptionally regulates the expression of genes involved in angiogenesis and that our previous findings show that HO-1 reduces tumor growth in PCa xenografts [15], these data strongly supports the notion that the HO-1-inhibitory effect on NF-κB signaling is perhaps partially critical in mediating the growth-suppressive effects of HO-1. Additionally, we cannot rule out the direct inhibitory effects of HO-1 on angiogenic targets. Figure 7 provides a schematic representation of the different angiogenic targets downmodulated by HO-1 overexpression, which is supported by our in vitro and in vivo studies and give ground to HO-1 anti-angiogenic capability.

In summary, we have demonstrated that HO-1 induction attenuates tumor angiogenesis in vivo and propose a novel role for HO-1 in prostate cancer progression providing a target for state of the art therapeutic strategies and a new sight in prostate carcinogenesis.

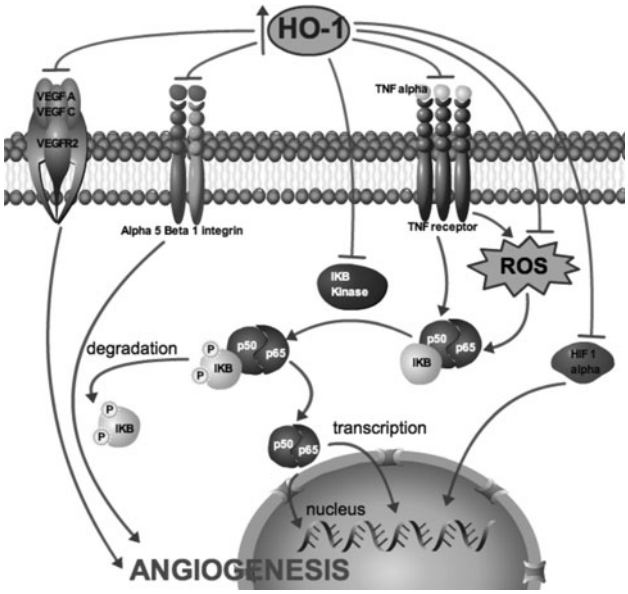

Fig. 7 Schematic representation of the anti-angiogenic effect of HO-1 in PCa. HO-1 overexpression impairs angiogenesis by downmodulating pro-angiogenic factors expression, inhibiting NF-κB activation and reducing ROS generation. In turn NF-κB inactivation and lower ROS levels also contribute to decrease angiogenesis in PC3 cells

Methods

Cell culture and antibodies

PC3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were routinely cultured in RPMI1640 (Invitrogen) supplemented with 10% FBS. PC3HO-1 and PC3pcDNA3 cells were obtained previously described [15]. Antibodies: anti-HO-1 was from Stressgen Biotechnologies Corp. (San Diego, CA, USA), anti-MMP9 (Santa Cruz Biotechnology, USA), anti-βactin from Sigma (UK), anti-mouse secondary antibody from Amersham Ltd (UK), anti-VEGFR2 (Cell Signaling, Technology, Beverly, MA, USA) and anti-mouse CD34 (eBioscience, San Diego, CA, USA).

Plasmids

VEGF luciferase reporter plasmid was kindly provided by Dr. Omar Coso (Laboratory of Physiology and Molecular Biology, School of Sciences, University of Buenos Aires, Bs As, Argentina). The human pcDNA3HO-1 expressing vector was kindly provided by Dr. M. Mayhofer (Clinical Institute for Medical and Chemical Laboratory Diagnostics, Vienna).

NF-κB luciferase (luc) plasmid, IκBss expression vector carrying mutated Ser 32 and Ser 36 to prevent phosphorylation and proteolysis of IκB, and p65 expression vector were kindly provided by Dr. Mónica Costas, Laboratory of Molecular Biology and Apoptosis, Institute of Medical Research Alfredo Lanari, Buenos Aires, Argentina [52].

Luciferase assay

PC3βGal and PC3HO-1 cells were transfected using Lipofectamine 2000 (Invitrogen) with the human wild-type promoter region of VEGF A or NF-κB luciferase vector. After transfection, cells were incubated in Reporter Lysis Buffer (Promega, Madison, WI, USA) and luciferase activity was determined by the Luciferase Assay system (Promega, Madison, WI, USA) in a HIDEX luminometer. Each transfection was done in triplicate and each experiment was repeated at least three times. Data were normalized to total protein determined by the Bradford assay.

RNA isolation and RT-qPCR (reverse transcription quantitative PCR)

Total RNA was isolated with the RNeasy Mini Kit (Qiagen). cDNAs were synthesized with Omniscript Reverse Transcriptase (Qiagen) and amplified by real-time PCR amplification with Taq HotStart master mix kit (Qiagen). Primers were designed to amplify a 100 bp region present in the fully mature RNA species of HO-1 (5′-GAGTGT AAGGACCCATCGGA-3′ and 5′-GCCAGCAACAAAGT GCAAG-3′); MMP9 (5′-AGACCTGGGCAGATTCCAA ACC-3′ and 5′-GCAAAGGCGTCGTCA ATCACC-3′); VEGFA (5′-GCCTTGCCTTGCTGCTCTACC-3′ and 5′-G TGATGATTCTGCCCTCCTCCTTC-3′); VEGF C (5′-AG GCTGGCAACATAAC AGAGAAC-3′ and 5′-GCGACTC CAAACTCCTTCCC-3′); ACTB (5′CGGTTGGCC TTA GGGTTCAGGGGGG-3′ and 5′-GTGGGCCGCTCTAGG CACCA-3′); HIF1α: (5′-AGCCCTAACGTGTTATCTGT CGCT-3′; 5′-GCTGCATGATCGTCTGGCTGCT-3′; IRGA α5 5′-CTGGCACCCCAAGGACAGAGGT-3′ and 5′-TC GGGGGCTTCAACTTAGACGCG-3′; IKK 5′-CCCACA GCTATGACACCGGAAG-3′ and 5′-TCCCCAGAGGA-GACTCTTCGCC-3′ and IκB 5′-TCCCCTCGTCTTCGGC TACGTC-3′ and 5′-TCAGTGCCGGCCGAGAAGCCT AG-3′; VEGFR2 5′-GCAGAGCCATGTGGTCTCTCTG G-3′ and 5′-TGGCGCACTCTTCCTCCAACTGC-3′. Each PCR was performed in duplicate and three biological independent experiments were performed.

Intracellular ROS detection and cell by cell quantification of DCFHDA

PC3βGal and PC3HO-1 cells were seeded in 12-well plate at density of 1×10^5 cells per well on a coverslip overnight. Cells were treated with 200 μM H_2O_2 for 1.5 h, washed twice with PBS and then stained with 10 μM 2′,7′-Dichlorofluorescein diacetate (CM2-DCFHDA; Invitrogen-Molecular Probes™) for 1 h at 37°C. Cells were then washed with PBS thrice and fixed with methanol.

Imaging was performed with a wide field microscope Olympus IX71 using a water immersion objective UApo 40 × 1.15 NA and a Hamamatsu Orca CCD camera (C4742-95, Japan). Samples were imaged in PBS. Filters were as follow: excitation filter, band pass (BP): 447/40 nm; dicroic mirror, 495 nm; emission filter, band pass: 520/50 nm. The same acquisition parameters were used during all the imaging process (light potency and exposure time). Quantification was performed by creation of a routine with DIPimage for Matlab (TU Delft, The Netherlands, http://www.ph.tn.tudelft.nl/DIPlib/index.html) as described: Green channel background (median) was subtracted and a median filter (size: 7 pixels, shape: elliptic) was applied. Segmentation was performed for each cell using the transmission image and we generated the region denominated cell. For the estimation of the DCFHDA levels values in cell were summed for green channel, and also sizes were measured. We considered cells with similar sizes for the statistical analysis. Images for qualitative presentation were prepared using Image J (http://www.rsb.info.nih.gov, NIH, USA). Background of green channel (mean of empty region) was subtracted and a median filter was applied (radius: 1 pixel).

In vivo angiogenesis assay

Six- to eight-week-old male athymic nude (*nu/nu*) mice, each weighing at least 20 g, were utilized in accordance with "Guidelines for the Welfare of Animals in Experimental Neoplasia" (United Kingdom Coordinating Committee on Cancer Research) and the local ethical review committee. Mice were randomized into two groups: PC3HO-1 and PC3βGal. In vivo angiogenesis assay was carried out as previously described [23]. Briefly, 2×10^5 PC3βGal or PC3HO-1 cells together with a drop of Trypan Blue were injected intradermally into the right flank of mice. Vehicle (culture medium with a drop of Trypan Blue) was injected in the contralateral flank. After 6 days, mice were sacrificed; the skin was dissected out, the inoculated sites were photographed under a magnifying lens (Carl Zeiss, Stemi 2000-C), attached to a Canon digital power shot A-640 camera (10 megapixels and optic zoom 4×). The individual small and medium vessels adjacent to the tumoral area and in the contraletral flank were counted at ×40 magnification. The MVD was calculated according to the mean number of microvessels mm^2.

Blood vessel quantification in human prostate cancer xenografts

PC3HO-1 and PC3βGal harvested tumors were photographed, and blood vessel formation was quantified using Image J. Tumor area was segmented in each image and

RGB images were converted to grey scale. Grey mean intensity was assessed. The difference between this value and the background was considered and normalized to tumor volume. The results were expressed as the mean ± SEM. The *P* values were estimated using Student's T test (two tails).

Human prostate cancer xenograft model

Long term mice xenografts using PC3HO-1 and PC3βGal cell lines were generated as previously described [15].

Histological and immunohistochemical analysis

All tumors were processed and fixed using a routinely established protocol and stained as previously described [13]. Briefly, the skin and underlying tumors were fixed in buffered formalin and embedded in paraffin. Microscopic staggered cuts were stained with H&E; Masson's trichrome technique was also performed to highlight vascular structures. IHC technique was performed starting with the inhibition of the endogenous peroxide activity using hydrogen peroxide in methanol for 30 min (3%). Antigen retrieval was done by microwaving (4 cycles of 5 min each in 0.1 M citrate buffer, 750 W). Tissue slides were incubated overnight with the following primary antibodies: monoclonal mouse anti-MMP9 (1:400) from Santa Cruz Biotechnology, rabbit monoclonal anti-VEGFR 2 (Cell Signaling, Technology, Beverly, MA, USA) (1:200); monoclonal anti-mouse CD34 (eBioscience, San Diego, CA, USA) (1:100). Detections were performed with the Vectostain Elite ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA, USA) in accordance with the manufacturer's instructions. The peroxidase reaction was conducted, under microscope, using 3,3′-diaminobenzidine. Slides were counterstained with Mayer's hematoxylin and analyzed by standard light microscopy. Negative control slides were prepared by substituting primary antiserum with PBS. For semiquantitative analysis, the degree of staining was rated as high, moderate, low, or not detectable (3+, 2+, 1+, and 0, respectively); the staining was also observed for localization. For quantitative analysis cell area was segmented in each image (five sections from each group of tumors were examined). Mean intensity was assessed. The difference between this value and the background was considered. The results were expressed as the mean ± SEM. The *P* values were estimated using Student's t-Test (two tails). Three independent experiments were performed with five tumors in the PC3HO-1 group and five tumors in the PC3βGal group for early-stage (6 days) and long-term (23-days) xenografts.

Statistical analysis

All results are given as mean ± SD of 'n' separate independent experiments unless stated otherwise. Student's *t*-Test was used to ascertain statistical significance with a threshold of $P < 0.05$ and $P < 0.01$. Comparisons for in vivo experiments were made with one-way ANOVA followed by Dunnett's test, with $P < 0.05$ as the criterion for statistical significance. Fisher's test was used for the angiogenesis in vivo assay, statistical significance $P < 0.05$.

Acknowledgments This work was supported by grants from the University of Buenos Aires, Argentina, UBACyT (2011- 00179), AGENCIA (PICT RAICES 2006-00367 and 2010-0431). We thank Prof. Gabriel Rabinovich (M. Phil, Ph.D.), Prof. Omar Coso and Dr. Monica Costas for kindly providing us with some reagents used in this paper. We are also very grateful to Pablo Vallecorsa (Instituto de Estudios Oncológicos, Academia Nacional de Medicina) for his technical support in the IHQ analysis.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Ushio-Fukai M, Nakamura Y (2008) Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. Cancer Lett 266(1):37–52
2. Hiratsuka S (2011) Vasculogenensis, angiogenesis and special features of tumor blood vessels. Front Biosci 16:1413–1427
3. Mohamedali KA, Li ZG, Starbuck MW, Wan X, Yang J, Kim S, Zhang W, Rosenblum MG, Navone NM (2011) Inhibition of prostate cancer osteoblastic progression with VEGF121/rGel, a single agent targeting osteoblasts, osteoclasts, and tumor neovasculature. Clin Cancer Res 17(8):2328–2338
4. Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO, Mason JC (2004) Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. Blood 103(3):761–766
5. Jozkowicz A, Was H, Dulak J (2007) Heme oxygenase-1 in tumors: is it a false friend? Antioxid Redox Signal 9(12):2099–2117
6. Wegiel B, Chin BY, Otterbein LE (2008) Inhale to survive, cycle or die? Carbon monoxide and cellular proliferation. Cell Cycle 7(10):1379–1384
7. Dulak J, Deshane J, Jozkowicz A, Agarwal A (2008) Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis. Circulation 117(2):231–241
8. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG (2007) Inflammation in prostate carcinogenesis. Nat Rev Cancer 7(4):256–269
9. Finger EC, Giaccia AJ (2010) Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. Cancer Metastasis Rev 29(2):285–293
10. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6(4):273–286
11. Huss WJ, Hanrahan CF, Barrios RJ, Simons JW, Greenberg NM (2001) Angiogenesis and prostate cancer: identification of a molecular progression switch. Cancer Res 61(6):2736–2743

12. Jodele S, Blavier L, Yoon JM, DeClerck YA (2006) Modifying the soil to affect the seed: role of stromal-derived matrix metalloproteinases in cancer progression. Cancer Metastasis Rev 25(1):35–43
13. Sacca P, Meiss R, Casas G, Mazza O, Calvo JC, Navone N, Vazquez E (2007) Nuclear translocation of haeme oxygenase-1 is associated to prostate cancer. Br J Cancer 97(12):1683–1689
14. Li Y, Su J, Dingzhang X, Zhang J, Yoshimoto M, Liu S, Bijian K, Gupta A, Squire JA, Alaoui Jamali MA, Bismar TA (2011) PTEN deletion and heme oxygenase-1 overexpression cooperate in prostate cancer progression and are associated with adverse clinical outcome. J Pathol 224(1):90–100
15. Gueron G, De Siervi A, Ferrando M, Salierno M, De Luca P, Elguero B, Meiss R, Navone N, Vazquez ES (2009) Critical role of endogenous heme oxygenase 1 as a tuner of the invasive potential of prostate cancer cells. Mol Cancer Res 7(11): 1745–1755
16. Mira E, Lacalle RA, Buesa JM, de Buitrago GG, Jimenez-Baranda S, Gomez-Mouton C, Martinez AC, Manes S (2004) Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface. J Cell Sci 117(Pt 9):1847–1857
17. Kong D, Li Y, Wang Z, Banerjee S, Sarkar FH (2007) Inhibition of angiogenesis and invasion by 3,3′-diindolylmethane is mediated by the nuclear factor-κB downstream target genes MMP-9 and uPA that regulated bioavailability of vascular endothelial growth factor in prostate cancer. Cancer Res 67(7):3310–3319
18. Akalu A, Cretu A, Brooks PC (2005) Targeting integrins for the control of tumour angiogenesis. Expert Opin Investig Drugs 14(12):1475–1486
19. Eliceiri BP (2001) Integrin and growth factor receptor crosstalk. Circ Res 89(12):1104–1110
20. Rankin EB, Giaccia AJ (2008) The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 15(4):678–685
21. Tuomela J, Valta M, Seppanen J, Tarkkonen K, Vaananen HK, Harkonen P (2009) Overexpression of vascular endothelial growth factor C increases growth and alters the metastatic pattern of orthotopic PC-3 prostate tumors. BMC Cancer 9:362
22. Joseph IB, Nelson JB, Denmeade SR, Isaacs JT (1997) Androgens regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. Clin Cancer Res 3(12 Pt 1):2507–2511
23. Davel LE, Rimmaudo L, Espanol A, de la Torre E, Jasnis MA, Ribeiro ML, Gotoh T, de Lustig ES, Sales ME (2004) Different mechanisms lead to the angiogenic process induced by three adenocarcinoma cell lines. Angiogenesis 7(1):45–51
24. Liu SF, Malik AB (2006) NF-κB activation as a pathological mechanism of septic shock and inflammation. Am J Physiol Lung Cell Mol Physiol 290(4):L622–L645
25. Shukla S, Maclennan GT, Marengo SR, Resnick MI, Gupta S (2005) Constitutive activation of P I3 K-Akt and NF-κB during prostate cancer progression in autochthonous transgenic mouse model. Prostate 64(3):224–239
26. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, Rabson AB (2002) Mechanisms of constitutive NF-κB activation in human prostate cancer cells. Prostate 52(3):183–200
27. Holmes K, Roberts OL, Thomas AM, Cross MJ (2007) Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. Cell Signal 19(10):2003–2012
28. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90
29. Ramsay AK, Leung HY (2009) Signalling pathways in prostate carcinogenesis: potentials for molecular-targeted therapy. Clin Sci (Lond) 117(6):209–228
30. Berz D, Wanebo H (2011) Targeting the growth factors and angiogenesis pathways: small molecules in solid tumors. J Surg Oncol 103(6):574–586
31. Tertil M, Jozkowicz A, Dulak J (2010) Oxidative stress in tumor angiogenesis-therapeutic targets. Curr Pharm Des 16(35): 3877–3894
32. Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J Biol Chem 282(28):20621–20633
33. Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X, Lee JS, Postma DS, Kauffman HF, Choi AM (2007) Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. Am J Respir Cell Mol Biol 36(4):409–417
34. Converso DP, Taille C, Carreras MC, Jaitovich A, Poderoso JJ, Boczkowski J (2006) HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism. FASEB J 20(8):1236–1238
35. Lin QS, Weis S, Yang G, Zhuang T, Abate A, Dennery PA (2008) Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress. Free Radic Biol Med 44(5):847–855
36. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
37. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407(6801):249–257
38. Zayed MA, Yuan W, Chalothorn D, Faber JE, Parise LV (2010) Tumor growth and angiogenesis is impaired in CIB1 knockout mice. J Angiogenes Res 2:17
39. Sunamura M, Duda DG, Ghattas MH, Lozonschi L, Motoi F, Yamauchi J, Matsuno S, Shibahara S, Abraham NG (2003) Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer. Angiogenesis 6(1):15–24
40. Miyake M, Fujimoto K, Anai S, Ohnishi S, Kuwada M, Nakai Y, Inoue T, Matsumura Y, Tomioka A, Ikeda T, Tanaka N, Hirao Y (2011) Heme oxygenase-1 promotes angiogenesis in urothelial carcinoma of the urinary bladder. Oncol Rep 25(3):653–660
41. Zhu X, Fan WG, Li DP, Lin MC, Kung H (2010) Heme oxygenase-1 system and gastrointestinal tumors. World J Gastroenterol 16(21):2633–2637
42. Bussolati B, Mason JC (2006) Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis. Antioxid Redox Signal 8(7–8): 1153–1163
43. McCawley LJ, Matrisian LM (2001) Tumor progression: defining the soil round the tumor seed. Curr Biol 11(1):R25–R27
44. Chen J, De S, Brainard J, Byzova TV (2004) Metastatic properties of prostate cancer cells are controlled by VEGF. Cell Commun Adhes 11(1):1–11
45. Latil A, Bieche I, Pesche S, Valeri A, Fournier G, Cussenot O, Lidereau R (2000) VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. Int J Cancer 89(2):167–171
46. Bonecchi R, Locati M, Mantovani A (2011) Chemokines and cancer: a fatal attraction. Cancer Cell 19(4):434–435
47. Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ (2000) Blockade of nuclear factor-κB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res 60(19):5334–5339
48. Rettig MB, Heber D, An J, Seeram NP, Rao JY, Liu H, Klatte T, Belldegrun A, Moro A, Henning SM, Mo D, Aronson WJ, Pantuck A (2008) Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-κB-dependent mechanism. Mol Cancer Ther 7(9):2662–2671

49. Shukla S, MacLennan GT, Fu P, Patel J, Marengo SR, Resnick MI, Gupta S (2004) Nuclear factor-κB/p65 (Rel A) is constitutively activated in human prostate adenocarcinoma and correlates with disease progression. Neoplasia 6(4):390–400
50. Gasparian AV, Yao YJ, Kowalczyk D, Lyakh LA, Karseladze A, Slaga TJ, Budunova IV (2002) The role of IKK in constitutive activation of NF-κB transcription factor in prostate carcinoma cells. J Cell Sci 115(Pt 1):141–151
51. Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ (2001) Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene 20(31):4188–4197
52. Werbajh S, Nojek I, Lanz R, Costas MA (2000) RAC-3 is a NF-κB coactivator. FEBS Lett 485(2–3):195–199