Androgen receptor (AR) signaling is involved in the development and progression of prostate cancer. Tumor microvasculature contributes to continual exposure of prostate cancer cells to hypoxia–reoxygenation, however, the role of hypoxia–reoxygenation in prostate cancer progression and modulation of AR signaling is not understood. In this study, we evaluated the effects of hypoxia–reoxygenation in LNCaP cells, a line of hormone responsive human prostate cancer cells. Our results demonstrate that hypoxia–reoxygenation resulted in increased survival, higher clonogenicity and enhanced invasiveness of these cells. Moreover, hypoxia–reoxygenation was associated with an increased AR activity independent of androgens as well as increased hypoxia inducible factor (HIF-1α) levels and activity. We also observed that the activation of p38 mitogen-activated protein (MAP) kinase pathway was a early response to hypoxia, and inhibition of p38 MAP kinase pathway by variety of approaches abolished hypoxia–reoxygenation induced increased AR activity as well as increased survival, clonogenicity and invasiveness. These results demonstrate a critical role for hypoxia-induced p38 MAP kinase pathway in androgen-independent AR activation in prostate cancer cells, and suggest that hypoxia–reoxygenation may select for aggressive androgen-independent prostate cancer phenotype.

**Introduction**

Prostate cancer is a multifocal disease that requires androgens for development and finally develops into solid tumor. In prostate tissue, androgens are involved in differentiation, development and normal functioning (Culig and Bartsch, 2006). Initial stages of erroneous prostate cancer growth can be controlled by reducing the availability of androgens to the prostate cells (Huggins and Hodges, 1941). However, over time prostate cancer cells attain androgen independence. Though there are many different ways leading to progression of cancer and ultimately to androgen-independent cancer (Asirvatham et al., 2006), mechanisms underlying the emergence to an androgen receptor (AR)-independent aggressive prostate cancer are not completely understood.

Tumor microenvironment usually consists of disorganized and hemorrhagic vasculature (Carmeliet and Jain, 2000; McDonald and Choyke, 2003), which can lead to low oxygen (hypoxia) and nutrient supply to cells. Clinical studies with withdrawal of androgens have demonstrated reduction in hypoxia in tumor regions of prostate cancer patients (Milosevic et al., 2007), suggesting that hypoxia may be involved in the development of androgen independence in these patients, however, mechanisms for this androgen independence have not been identified.

Tissue hypoxia is known to initiate multiple events that allow cells to continue proliferating, mainly by activating hypoxia inducible factor 1 (HIF-1), a transcription factor activating downstream targets responsible for angiogenesis and increased survival (Kimbro and Simons, 2006). HIF-1 is composed of two subunits, one of which is HIF-1α, an oxygen sensor and has been associated with malignant progression and resistance to radiotherapy and chemotherapy (Zhong et al., 1998; Vleugel et al., 2005). Interestingly, HIF-1α has been found to be overexpressed in prostate cancer tumors even in nonhypoxic conditions and signaling pathways, commonly induced in cellular stress like c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (JNK) and p38 MAPK, respectively.
protein kinase (MAPK) have been implicated in the activation and control of HIF-1α in cancer (Berra et al., 2000; Baek et al., 2001). Many studies have concentrated on the downstream effects of HIF-1α in hypoxic conditions, however, little is known about the signaling events involved in the activation of HIF-1α under hypoxia, in prostate cancer.

p38 MAPK is known to be activated by oxidative and nitrosative stress (Sumbayev and Yasinska, 2005) and is involved in regulating such activities in the cell as differentiation, survival and apoptosis. Inhibition of p38 MAPK has been shown to increase cellular injury when lung cells are exposed to hypoxia (Powell et al., 2004) and in prostate cancer, p38 MAPK has been implicated in androgen-independent progression of prostate cancer (Shida et al., 2007). Accumulating data suggests an important role for p38 MAPK in the stabilization of AR, independent of androgens, by involving chaperons most notably heat-shock protein (HSP)27 (Zoubeidi et al., 2007).

Most of the understanding about androgen response in prostate cancer has been derived from the androgen responsive human prostate cancer cell line, LNCaP (Horoszewicz et al., 1983) that can be adapted to grow even in the absence of androgens (Kokontis et al., 1998). In our study, we used LNCaP cell line to demonstrate the upstream signaling events that may lead to androgen independence in prostate cancer. We demonstrate for the first time the critical role of hypoxia in activating p38 MAPK and androgen-independent AR stabilization and activity, causing increased aggressiveness of LNCaP cells.

Results

Incubation of LNCaP cells in hypoxia resulted in the stabilization of HIF-1α

It is well known that the primary response of cells to lower levels of oxygen is the stabilization and increased activity of HIF-1α. As hypoxia response is mediated by HIF-1α, we investigated the changes in HIF-1α and its downstream target vascular endothelial growth factor (VEGF) upon exposing LNCaP cells to hypoxia. In as less as 4h in hypoxia, the amount of HIF-1α protein was increased, whereas it was undetectable in normoxic condition (Figure 1a) in LNCaP cells. We used the hypoxia response element (HRE) driven luciferase reporter 

\[ 3 \times \text{HRE-luc} \] to measure HIF-1α activity in LNCaP cells and observed a significant increase in the luciferase activity (Figure 1b), which indicated higher levels of active HIF-1α in LNCaP cells under hypoxia. As expected, higher luciferase activity was observed in LNCaP cells exposed to hypoxia for 24h than for 12h.

As a response to hypoxic conditions, cells are known to express proteins that lead to increased vasculature in the tumor. Our studies indicated increased levels of stable VEGF mRNA in LNCaP cells in as little as 4h exposure to hypoxia (Figure 1c). Taken together, these results suggest that HIF-1α is stabilized and activated in LNCaP cells that are under hypoxic stress.
expressed in prostate epithelial cells and prostate cancer cells as a result of specific AR binding (Cheng et al., 2003). LNCaP cells in androgen-free medium, exposed to hypoxia for 4 h showed increased AR activity and this was comparable to AR activity in LNCaP cells stimulated with 10 nM dihydroxytestosterone (DHT; Figure 2b) for the same duration.

Further, we investigated the changes occurring in the activity of signaling molecules involved in AR signaling in LNCaP cells because of hypoxia exposure. We did not observe any significant or consistent activation of extracellular signal-regulated kinase or JNK in LNCaP cells in our study. LNCaP cells incubated in hypoxia for 4 h showed significantly increased phosphorylation of p38 MAPK (Figure 2c). However, the level of phospho-p38 MAPK was reduced upon longer exposure (12 h) suggesting that activation of p38 MAPK may be an early response to low oxygen tension in LNCaP cells. Even then, we observed that the amount of phospho-p38 was higher in the cells incubated in hypoxia compared to cells in normoxia for the same duration (Figure 2c, lower panel).

In conjunction with increased p38 MAPK activity, we also observed increased HSP27 phosphorylation in...
LNCaP cells exposed to hypoxia for 4 h, further confirming the involvement of p38 MAPK (Figure 2d). Much higher levels of phospho-HSP27 as well as stable AR were seen upon reexposing LNCaP cells to atmospheric oxygen after 4 h exposure to hypoxia (Figure 2d, lower panel). HSP27 phosphorylation and AR levels were much higher when LNCaP cells are reoxygenated for longer time after hypoxia stimulation (24 h compared to 12 h reoxygenation) indicating that once activated, HSP27 can activate AR for longer time.

Taken together these results suggest that hypoxia increases the amount and activity of AR in LNCaP cells, independent of androgens and this may be mediated by HSP27 as a result of increased p38 MAPK activity.

**Hypoxia–reoxygenation selects for aggressive phenotype in LNCaP cells**

Cells in a tumor experience inconsistent levels of oxygen and nutrient supply because of disorganized vasculature. This results in periods of oxygen availability and hypoxia. Our observations showed that LNCaP cells are better adapted to survive in low oxygen environment compared to normal prostate epithelial cells (RWPE-1). LNCaP cells incubated in hypoxia for 24 h showed only a modest loss of cell viability (Figure 3a, lower panel) compared to more than 50% loss in case of RWPE-1. Even with 12 h incubation in hypoxia, there was about 25% cell death in RWPE-1 cells, whereas LNCaP-100 have comparable survival to cells in normoxia (Figure 3a, upper panel). Similar differences in the rate of proliferation of LNCaP and RWPE-1 cells under hypoxia were observed following 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown).

In addition to cell viability, we also investigated changes in the invasiveness and clonogenicity of LNCaP cells when subjected to hypoxia. LNCaP cells incubated for 8 h in hypoxia have significantly increased invasion through basement membrane matrix (Figure 3b) in the presence of androgens. Surprisingly, the number of cells that invaded through the basement membrane matrix was much higher when the cells are incubated in hypoxia for 8 h in the absence of androgens (Figure 3b, lower panel). We detected an increase in the amount of secreted matrix metalloproteinase (MMP)-9 during reoxygeneration after hypoxia exposure in LNCaP cells (Figure 3c). In addition to invasion, another important determinant for metastasis is the ability of cancer cells to establish clonal growth at new sites. LNCaP cells subjected to hypoxia for 12 h showed a significantly increased ability to form colonies in soft agar compared to normoxic cells (Figure 3d). In addition, the average colony size was also much higher in hypoxia treated cells.

Taken together these results suggest that hypoxia may be involved in developing and aggressive phenotype in LNCaP cells even in the absence of androgens.

**Inhibition of p38 MAPK leads to reduced AR protein levels and activity and reduced HIF-1α activity**

LNCaP cells transfected with small interfering RNA (siRNA) against p38 MAPK protein showed significantly lower levels of AR protein when subjected to hypoxia for 12 h compared to control cells transfected with nonspecific siRNA (Figure 4a). In addition, LNCaP cells pretreated with 10 μM SB203580 and incubated under hypoxic conditions for 12 h showed reduced levels of total AR (Figure 4b, upper panel) as well as nuclear AR (Figure 4b, lower panel). However, inhibition of p38 MAPK during reoxygeneration after 12 h hypoxia did not affect the levels of stable AR in LNCaP cells (Figure 4b). Inhibition of p38 MAPK during hypoxia treatments also significantly reduced the phosphorylation of HSP27 suggesting that HSP27 be involved in p38 MAPK signaling pathway in LNCaP cells. In addition to the total protein levels, transfecting LNCaP cells with p38 siRNA or preincubation with 10 μM SB203580 significantly reduced AR activity (as determined by TARPp/PSAe luciferase activity) during hypoxia treatment (Figure 4c).

**Figure 2**: Hypoxia–reoxygenation increases androgen receptor protein levels and activity in LNCaP cells. (a) LNCaP cells were subjected to hypoxia in a modular incubator for 4 and 12 h. Western blot analysis for protein levels of hypoxia inducible factor (HIF)-1α, total p38 mitogen-activated protein kinase (MAPK) and androgen receptor was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein as a loading control (upper panel). Representative gels from three individual experiments are shown. Protein amounts were calculated by determining band intensities using densitometry and normalizing them to GAPDH levels used as a loading control (lower panel). Each data point is represented as mean ± s.d. of triplicate experiments (* indicates P < 0.05 compared to respective normoxia controls, n = 3). (b) LNCaP cells maintained in androgen-free medium (10% CSFBS) were transfected with pGL3-TARPp/PSAe plasmid and pRL-CMV as a transfection control. After transfection (36 h), cells were incubated either in 10 nm dihydroxytestosterone (DHT) or in hypoxia for 4 h. Luciferase activity was measured with Dual luciferase assay kit as before. Each data point is represented as mean ± s.d. of duplicate experiments carried out in duplicate (*** indicates P < 0.01 compared to normoxic and unstimulated control, n = 4). (c) LNCaP cells were subjected to hypoxia in a modular incubator for 4 and 12 h. Western blot analysis for protein levels of HIF-1α, total p38 mitogen-activated protein kinase (MAPK) and phospho-p38 MAPK was performed using GAPDH protein as a loading control (upper panel). Representative gel images from three individual experiments are shown. Protein amounts were calculated as before (lower panel). Each data point is represented as mean ± s.d. of triplicate experiments (* indicates P < 0.05, ** indicates P < 0.01 compared to respective normoxia controls, n = 3).
To further confirm the role of HSP27 in p38 MAPK-mediated AR signaling, we transfected LNCaP cells with a dominant-negative mutant of MAPKAP kinase 2 (MK2). MK2 is known to be a downstream effector of p38 MAPK and is present upstream to HSP27 in the pathway. Dominant-negative mutant of MK2 greatly reduced HSP27 phosphorylation when LNCaP cells are subjected to hypoxia for 4h, compared to wild-type plasmid (Figure 4d). Similar reduction in HSP27 phosphorylation was seen even after 12h of reoxygenation when the dominant-negative MK2 was expressed.

Figure 3  Hypoxia–reoxygenation promotes aggressive phenotype. (a) RWPE-1 and LNCaP cells were incubated for 12h (upper panel) and 24h (lower panel) in hypoxia. Cells were stained with crystal violet to determine survival. Data points represent mean ± s.d. of duplicate experiments carried out in triplicate (** indicates P<0.01 and *** indicates P<0.001 compared to normoxia control, n = 6). (b) LNCaP cells were incubated in hypoxia for 8h in complete medium in the presence or absence of androgens. Equal numbers of cells were seeded in Boyden chambers and 48h later, invaded cells were stained with crystal violet. Cells that invaded through the matrigel were quantified using ImageJ Software (lower panel) and each data point represents mean ± s.d. of duplicate experiments carried out in duplicate (* indicates P<0.05 compared to normoxia control, n = 4). Representative images are shown. (c) LNCaP cells were subjected to hypoxia for 8h in serum-free medium and cultured for further 12h in normoxia. Equal volume of conditioned medium was collected after hypoxia or after reoxygenation, concentrated and equal volume of concentrated medium was used for western blot analysis to determine the secreted matrix metalloproteinase (MMP)-9 protein. (d) LNCaP cells were incubated in hypoxia for 12h and colony formation in soft agar was assayed (left panel). Representative images from duplicate experiments carried out in triplicate are shown. Number of colonies was counted using ImageJ Software and quantified (right panel). Each data point represents mean ± s.d. (* indicates P<0.05 compared to control, n = 6).
In addition to inhibition of AR, reduction of p38 MAPK activity also influenced HIF-1α activity in LNCaP cells under hypoxia. HIF-1α activity, as determined by HIF-1 protein binding to HRE, was reduced by about 40% in LNCaP cells incubated for 2 h in hypoxia in the presence of 10 μM SB203580 (Figure 4e, left panel). Much higher reduction was observed when 20 μM SB203580 was used. LNCaP cells transfected with siRNA targeted against p38 MAPK and exposed to hypoxia for 12 h showed lower levels of VEGF mRNA (Figure 4e, right panel).

Taken together these results suggest that p38 MAPK may be involved in regulating AR and HIF-1α activities under hypoxic conditions in LNCaP cells.

Inhibition of p38 MAPK reduces survival, invasiveness and clonogenic potential of LNCaP cells subjected to hypoxia

To understand the significance of p38 MAPK activity in LNCaP cells during hypoxia, we assessed the survival, invasion and clonogenic potential of LNCaP cells incubated in hypoxia, after inhibiting p38 MAPK activity. Addition of 10 μM SB203580 during 12 h hypoxia incubation significantly reduced the survival of LNCaP cells (Figure 5a). A similar reduction in the proliferation of these cells was observed with MTT assay (data not shown). Invasion of LNCaP cells incubated in hypoxia for 8 h in androgen-free medium was comparable to the number of cells that invaded through matrigel matrix in presence of 10 nM DHT in normoxia (Figure 5b). Significantly, the number of cells that invaded was reduced when 10 μM SB203580 was included in the medium during 8 h hypoxia incubation as well as during the invasion assay in Boyden chambers. In addition to invasiveness, clonogenic potential was also significantly reduced in cells subjected to 12 h hypoxia after inhibiting p38 MAPK (Figure 5c). Moreover, the size of established colonies was also greatly reduced compared to the cells in which p38 MAPK was active during hypoxia treatment.

Taken together, these results complement the earlier observations of increased survival and invasion of LNCaP cells during hypoxia and the essential role of p38 MAPK in this process.

Chronic hypoxia–reoxygenation enhances aggressiveness of LNCaP cells by selecting for androgen independence

In an effort to simulate the intermittent hypoxia–reoxygenation conditions that exist in a tumor, LNCaP cells were grown in a cyclic 12 h hypoxia and 12 h reoxygenation environment for 3 weeks, passing the cells as required. Surviving cells (named as hrLNCaP) were collected and equal numbers of cells were seeded in androgen-free medium to determine the growth potential of hrLNCaP cells compared to parent LNCaP cells. We observed that continuous cycles of hypoxia–reoxygenation selected for cells with a highly significant faster growth rate compared to the control LNCaP cells (Figure 6a) as determined by MTT assay. hrLNCaP cells also showed higher invasiveness even under normoxic conditions (Figure 6b). The number of cells that invade through the basement matrix was comparable irrespective of the presence or absence of DHT (Figure 6b, right panel).

Taken together, these results suggest that continuous hypoxia–reoxygenation cycles select for a population of LNCaP cells (hrLNCaP) that have enhanced survival and invasiveness even in the absence of androgens.

Discussion

As a consequence of the formation of solid tumors, cells inside a tumor mass are subjected to low oxygen and nutrient environment. Hypoxia has been shown to activate many signaling pathways in prostate cancer cells, which ultimately are responsible for angiogenesis, anaerobic metabolism and for progression of the disease. Cancer cells are required to tolerate the hypoxic conditions occurring inside a tumor and also adapt to changes in androgen levels as a result of reduced blood flow and finally attain androgen independence and aggressive growth. Our study focused on the role of hypoxia in AR function and the signaling pathways that may finally lead to androgen independence in prostate cancer.

The initial response of LNCaP cells to hypoxia is the activation of the hypoxia responsive transcription factor; HIF-1 (Milosevic et al, 2007) and constitutive expression of HIF-1α has been shown to increase apoptosis resistance in pancreatic cancer cells (Akakura et al, 2001). One of the major adaptive responses to hypoxia in a tumor is increased neovascularization and tumor angiogenesis, essential for survival and metastasis (Zetter, 1998). VEGF is involved in angiogenesis and its transcriptional upregulation has been shown to be mediated by HIF-1α in prostate cancer cells (Zhong et al, 2000; Calvani et al, 2008). In this study, we observed increased VEGF mRNA levels within 4 h exposure to hypoxia.

Prostate cells require androgens for transcription of many genes, which are involved in maintaining normal prostate health. AR signaling in prostate cancer influences the progression of the disease and treatment options. Studies by Park et al (2006) showed increased binding of AR to androgen response element in response to hypoxia and suggested that this response was independent of HIF-1α signaling. Other studies have shown the activation of different signaling pathways in hypoxia, notably the MAPK pathways JNK and p38 MAPK (Kwon et al, 2005). We observed activation of p38 MAPK in LNCaP cells subjected to hypoxia for 4 h, possibly as an initial response to lower oxygen tension.

Our studies show increased levels of AR protein as well as AR activity in LNCaP cells during hypoxia exposure, consistent with increased p38 MAPK activity. As, recent reports suggest an indirect role for p38 MAPK in increased AR activity (Zoubedi et al., 2007), we rationalized that increased AR activity in hypoxia may be mediated through p38 MAPK. These
observations are supported by a study by Horii et al. (2007), which suggested that increased AR activity may not be a result of increased transcription of AR. We also observed increased phosphorylation of HSP27 under hypoxia, which suggests that the increased AR activity may be a result of increased cooperative interaction of AR and HSP27 as suggested by Zoubeidi et al. (2007). In this study, the authors suggested that p38 MAPK phosphorylates HSP27 and leads to its nuclear translocation and increased AR activity. Indeed, we observed reduced nuclear localization of AR when p38 MAPK activity is inhibited in LNCaP cells under hypoxia.

We also observed increased AR levels and sustained phosphorylation of HSP27 even after 12 h of reoxygenation post hypoxia, which suggests that once HSP27 is activated by hypoxic stimulation of p38 MAPK, LNCaP cells can maintain AR stability even in reoxygenated conditions. Similar results were observed by Park et al. (2006) where in maximal AR activity was observed by 4h of reoxygenation post hypoxia treatment. These observations thus, strengthen our hypothesis that the continuous hypoxia–reoxygenation environment of a tumor is highly relevant in increased AR activity and possibly in promoting aggressive tumor growth and androgen independence.

Earlier studies involving LNCaP cells with over-expressed HIF-1α, suggested increased invasiveness of these cells (Luo et al., 2006). We observed a similar increase in invasive potential of LNCaP cells incubated in hypoxia for 8h. Surprisingly, we observed much
higher invasiveness of hypoxia treated LNCaP cells in the absence of androgens. Recent studies using cancer cells have suggested changes in MMP during hypoxia (Pouysségur et al., 2006; Miyazaki et al., 2008). We observed increased secretion of MMP-9 into the external medium in cells subjected to reoxygenation after hypoxia treatment indicating that the hypoxia–reoxygenation conditions in the tumor microenvironment may be more relevant in increasing the metastatic potential of prostate cancer cells.

Previous studies show the involvement of p38 MAPK in HIF-1α induction in pulmonary artery fibroblasts (Mortimer et al., 2007) and in pancreatic cancer cells (Kwon et al., 2005). Moreover, AR was shown to be involved in the regulation of VEGF levels during hypoxia (Boddy et al., 2005). As inhibition of p38 MAPK caused reduction in AR levels in our study, we rationalized that p38 MAPK may also be involved in the HIF-1α activity during hypoxia. We observed a significantly reduced HRE-binding activity upon treating LNCaP cells with SB203580, suggesting that p38 MAPK may also be involved in regulating HIF-1α activity during hypoxia in LNCaP cells.

Though p38 MAPK has not been directly implicated with increased transcription of VEGF, many studies suggest the involvement of p38 MAPK in increased VEGF levels during hypoxia (Murata et al., 2006; Yoshino et al., 2006) probably by increasing mRNA stability (Págès et al., 2000). We observed reduced VEGF mRNA levels in LNCaP cells incubated in hypoxia, upon inhibition of p38 MAPK by siRNA, suggesting that p38 MAPK be involved in the hypoxic regulation of VEGF possibly because of its role in HIF-1α regulation. Even though, similar results were obtained by Murata et al. (2006) in human articular chondrocytes, we believe that this is the first time that the role of p38 MAPK in hypoxic regulation of HIF-1α has been demonstrated in prostate cancer cells.

These observations prompt a renewed view of the combined action of HIF-1α and AR in promoting aggressive growth in hypoxia and eventual emergence of androgen independence in prostate cancer. We hypothesize that p38 MAPK be involved proactively in stimulating AR activity by increasing the interaction of HSP27 and AR and subsequent stabilization and nuclear translocation of AR (Figure 6c). In another arm, activated p38 MAPK may be involved in increasing HIF-1 activity probably by stabilizing the HIF-1α subunit.

Xu et al. (2006) suggested that MK2 and HSP27, which are downstream effectors of p38 MAPK, have been involved in the regulation of mRNA expression and invasion of prostate cancer PC-3 cells. The observed increase in survival, invasiveness, and clonogenicity of LNCaP in our study may be a direct manifestation of increased p38 MAPK activity during hypoxia–reoxygenation. These observations are further supported by the reduction in survival and invasion of LNCaP cells during hypoxia when p38 MAPK was inhibited. The study thus highlights an important role played by p38 MAPK in promoting aggressiveness of prostate cancer cells independent of androgens, in a hypoxia–reoxygenation environment. Therefore, the activation of p38 MAPK during the hypoxia–reoxygenation cycles occurring in a tumor microenvironment may ultimately lead to the selection of a subpopulation of cells that are independent of androgen control and may serve as a nidus for androgen-independent prostate cancer. The generation of hrLNCaP cells in our study, which show androgen-independent aggressive growth and invasion may offer an in vitro model system to better understand the events occurring in an active prostate tumor.
observations are also supported by a recently published study by Butterworth et al. (2008) showing increased aggressiveness of LNCaP cells repeatedly subjected to long periods (more than 24 h) of hypoxia and reoxygenation.

In summary, this study highlights a role for p38 MAPK in the stabilization of HIF-1α and also in activating AR, independent of androgens in LNCaP cells under hypoxia. Increased p38 MAPK activity, may thus contribute to increased survival, invasiveness and clonogenicity of androgen-dependent prostate cancer cells subjected to continuous hypoxia–reoxygenation in a tumor microenvironment. Activation of p38 MAPK may thus promote aggressive growth of prostate cancer cells and the aberrant AR activity in the absence of androgens may thus promote the onset of androgen independence.

Figure 5 Inhibition of p38 mitogen-activated protein kinase (MAPK) reduces survival and invasion of LNCaP cells. (a) LNCaP cells were pretreated with 10 μM SB203580 for 1 h and then incubated in hypoxia for 12 h. Cells were stained with crystal violet to determine survival. Data points represent mean ± s.d. of duplicate experiments carried out in triplicate (⁎ indicates P < 0.05 compared to normoxia control, n = 6). (b) LNCaP cells were incubated in hypoxia for 8 h either in absence of androgens either with or without 10 μM SB203580. Equal numbers of cells were seeded in Boyden chambers and one set of cells not subjected to hypoxia were allowed to invade in presence of 10 nM dihydroxytestosterone (DHT). Later (48 h) invaded cells were stained with crystal violet (upper panel). Representative images from two independent experiments are shown. Cells that invaded through matrigel were quantified (lower panel) as before and each data point represents mean ± s.d. (⁎ indicates P < 0.05 compared to cells incubated in hypoxia in androgen-free medium and uninhibited p38 MAPK, n = 4). (c) LNCaP cells were incubated in hypoxia either with or without 10 μM SB203580 for 12 h and colony formation in soft agar was assayed (upper panel). Representative images from duplicate experiments carried out in triplicate are shown. Number of colonies was quantified as before (lower panel). Each data point represents mean ± s.d. (⁎ indicates P < 0.05 compared to control, n = 6).

Materials and methods

Chemicals and reagents

Anti-HIF-1α antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA). All other primary antibodies used in this study were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The specific inhibitor of p38 MAPK activity; 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), was procured from EMD Biosciences (San Diego, CA, USA). All chemicals used in this study were procured from Sigma (St Louis, MO, USA). Centrifugation YM-10 centrifugal concentrations used to concentrate conditioned medium were from Millipore Corporation (Billerica, MA, USA).

Cells and cell culture

LNCaP and RWPE-1 (immortalized epithelial cells derived from normal human prostate) cells were obtained from
Chronic hypoxia–reoxygenation selects for LNCaP cells with aggressive phenotype. (a) LNCaP cells were cultured in complete medium and incubated in hypoxia for 12 h and reoxygenated for 12 h, for 3 weeks. Equal numbers of surviving cells (hrLNCaP) were seeded in androgen-free medium and proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay over 3 days. Each data point represents mean ± s.d. of two individual experiments carried out in quadruplicate (** indicates $P < 0.001$, $n = 8$). (b) Equal number of hrLNCaP cells and LNCaP cells were seeded in Boyden chambers in androgen-free medium and cells were allowed to invade either in the presence or absence of 10 nM dihydroxytestosterone (DHT). Later (48 h), invaded cells were stained with crystal violet (left panel). Representative images from two individual experiments carried out in duplicate are shown. Invaded cells were quantified as before and each data point represents mean ± s.d. (*) indicates $P < 0.05$ compared to LNCaP cells in androgen-free medium, $n = 4$; right panel). (c) Plausible mechanism of dual control of p38 mitogen-activated protein kinase (MAPK) to promote aggressive growth of prostate cancer cells in hypoxia. When subjected to hypoxia, androgen receptor (AR) can be activated in an androgen-independent manner by p38 MAPK and heat-shock protein (HSP)27 and can translocate into the nucleus. On the other hand, activated p38 MAPK can help stabilize hypoxia inducible factor (HIF)-1α and promote HIF-1-mediated gene transcription. Together the active AR and HIF-1 promote aggressive growth and may in due course lead to androgen-independent prostate cancer.
American Type Culture Collection and cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and antibiotics at 37°C in 5% CO₂ environment. For all the experiments, cells were plated to simulate high density environment (Sung et al., 2006). Androgen-free FBS (CSFBS) was prepared by treating FBS overnight with activated charcoal. Charcoal was removed by centrifugation and serum was sterilized by filtering through 0.2 μm filter before use.

**RNA isolation and reverse transcriptase PCR**
RNA isolated from cells using the RNeasy kit (Qiagen, Valencia, CA, USA) was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Reverse transcription PCR was performed with gene specific primers using Platinum Taq Polymerase (Invitrogen, Carlsbad, CA, USA) and separating the products on a 1% agarose gel. Primers were procured from Integrated DNA Technologies (Coralville, IA, USA) and primer sequences for VEGF-C and glyceraldehyde-3-phosphate dehydrogenase are described in Table 1.

**Small interfering RNA transfection**
siRNA designed to target p38 MAPK and scrambled siRNA control were purchased from Ambion (Austin, TX, USA). Cells were transfected with these siRNA using HiPerFect Transfection Reagent (Qiagen) according to manufacturer’s recommendation experiments with gene silenced cells were carried out 60–72 h after transfection.

**Plasmids and reporter assay**
Plasmids bearing HRE (−1202 to −1159) in pTK-Luc (Blachly et al., 1999), the AR responsive Luciferase Vector ARPP1PSAe (Cheng et al., 2003), plasmids expressing MK2 wt (pcDNA3mycMK2WT) and dominant-negative (pcDNA3mycMK2K76R) forms (Winzen et al., 1999) have been described previously. LNCaP cells were transiently transfected with 0.5 μg of plasmid and 10 ng of the Renilla luciferase expression vector pRL-CMV as a transfection control (Promega Corporation, Madison, WI, USA), using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s instructions. 36–48 h after transfection, the medium was replaced with hypoxic medium and incubated in hypoxic environment for the required duration. Luciferase activity was measured using Dual luciferase assay kit according to the manufacturer’s protocol (Promega Corporation) with Monolight 2010 Luminometer (Analytical Luminescence Laboratory).

**Cell viability and proliferation assay**
For viability assay, cells were stained with crystal violet and incorporated dye was extracted in 2-ethoxyethanol and optical density was read at 595 nm as a relative indicator of cell number, as described previously (Maroni et al., 2005). Proliferation assay was quantified with a colorimetric method based on the metabolic reduction of soluble yellow MTT dye to its insoluble formazan (Kumar et al., 2008).

**Boyden chamber assay for invasion**
Invasiveness of cells through matrigel (BD Biosciences) was performed according to the protocol of Rizzi (1987). Cells were transfected with 0.5 μg of plasmid and 10 ng of AR responsive Luciferase Vector ARPP1PSAe/TARPp and Professor Matthias Gaestel (Institute of Biochemistry, Medical School, Hannover, Germany) for MK2 expression plasmids.

**Table 1** List of primers used in the study

| Primer name | Sequence |
|-------------|----------|
| VEGF-C f    | 5′-CGATGCTAGCCACCATGAACTTCTG-3′ |
| VEGF-C r    | 5′-CTACCACCCCATGCTGTA-3′ |
| GAPDH f     | 5′-ACCACAGCTCATGACCATAC-3′ |
| GAPDH r     | 5′-TCCACACCCATGGCTGTA-3′ |

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor.
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