A novel regulation of VEGF expression by HIF-1α and STAT3 in HDM2 transfected prostate cancer cells

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Abstract

On the basis of increasing roles for HDM2 oncoprotein in cancer growth and progression, we speculated that HDM2 might play a major role in hypoxia-induced metastatic process. For verification of this hypothesis, wild-type LNCaP prostate cancer cells and HDM2 transfected LNCaP-MST (HDM2 stably transfected) cells were studied. The data obtained from our experiments revealed that the HDM2 transfected LNCaP-MST cells possessed an ability to multiply rapidly and show distinct morphological features compared to non-transfected LNCaP cells. During exposures to hypoxia HDM2 expression in the LNCaP and LNCaP-MST cells was significantly higher compared to the normoxic levels. The LNCaP-MST cells also expressed higher levels of HIF-1α (hypoxia-inducible factor-1α) and p-STAT3 even under the normoxic conditions compared to the non-transfected cells. The HIF-1α and p-STAT3 expressions were increased several fold when the cells were subjected to hypoxic conditions. The HIF-1α and p-STAT3 protein expressions observed in HDM2 transfected LNCaP-MST cells were 20 and 15 folds higher, respectively, compared to the non-transfected wild-type LNCaP cells. These results demonstrate that HDM2 may have an important regulatory role in mediating the HIF-1α and p-STAT3 protein expression during both normoxic and hypoxic conditions. Furthermore, the vascular endothelial growth factor (VEGF) expression that is typically regulated by HIF-1α and p-STAT3 was also increased significantly by 136% ($P < 0.01$) after HDM2 transfection. The overall results point towards a novel ability of HDM2 in regulating HIF-1α and p-STAT3 levels even in normoxic conditions that eventually lead to an up-regulation of VEGF expression.

Keywords: HDM2 ● VEGF ● HIF-1α ● STAT3 ● angiogenesis ● hypoxia

Introduction

Overexpression of HDM2 in cancer cells generally leads to constitutive inhibition of p53, which allows the tumour cells to escape from p53-induced cell cycle arrest and apoptosis [1]. So far, HDM2 has been found to be overexpressed in more than 40 different types of malignancies, including solid tumours, sarcomas and leukaemias, which qualify HDM2 as a classical oncogene [2]. Although some conflicting data concerning the cellular effects of HDM2 in individual cancers already exist, the overall evidence is convincingly indicative of the fact that increased HDM2 expression is frequently related to aggressive cancer growth and poor prognosis [3]. While many studies suggest that the tumourigenicity of HDM2 is generally due to its negative regulation of p53 tumour suppressor function, p53-independent effects of HDM2 are also recognized as equally important. Numerous *in vivo* studies have addressed a p53-independent role for HDM2 in processes other than tumourigenesis [4–6]. So far, HDM2 amplification has been detected more frequently in metastatic or recurrent tumours than in primary tumours. It is speculated that HDM2 protein might play

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a vital role in increasing the metastatic ability of originally non-metastatic tumour cells [7]. Angiogenesis, the process of new blood vessels formation from a pre-existing vasculature, is considered to be essential for enhancing tumour growth and metastasis [8]. This angiogenic process is tightly regulated by hypoxia through the transcriptional control of pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, transforming growth factor, interleukin 8, *etc*. Amongst these, VEGF regulates both physiological and pathological angiogenesis seemed to be the most critical transcriptional target for both HIF-1α and STAT3 [9–11]. It has been reported that under hypoxic condition, both STAT3 and HIF-1α bind to the VEGF promoter and form a molecular complex with p300/CBP and Ref-1/APE, to eventually induce VEGF transcription [12]. In this paper, we are showing the influence of HDM2 on VEGF transcription through increasing the levels of HIF-1α and STAT3. To date some of our own experiments and the reports from other laboratories have clearly demonstrated that overexpression of VEGF could significantly enhance cancer cell survival [13, 14]. Accordingly, we have found a correlation for HDM2 in GI-101A, HL-60, MCF-7, A2780/CP70, OVCAR-3 and LNCaP cells with respect to proangiogenic regulatory mechanisms [15]. In general, loss of p53 activity in tumour cells enhances the levels of HIF-1α and STAT3 to augment gene transcription in response to hypoxia, including VEGF expression [11, 16]. The levels of HIF-1α, STAT3 and VEGF detected in HDM2 transfected LNCaP-MST cell lines during normoxia and hypoxia in our experiments presented us with an important finding related to tumour angiogenesis. The preliminary results of these findings along with our hypothesis are presented.

Materials and methods

Cell culture

The LNCaP prostate cancer cell line was a generous gift from Dr. Thomas Powell (Cleveland Clinic Foundation, Cleveland, OH, USA). The HDM2 transfected LNCaP (LNCaP-MST) cells were kindly provided by Dr. Alan Pollack (Fox Chase Cancer Center, Philadelphia, PA, USA). The LNCaP cells were maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% foetal bovine serum (Hyclone, Logan, UT, USA), 1% L-glutamine, 1% penicillin-streptomycin (Life Technologies, Gaithersburg, MD, USA). The LNCaP-MST cells were maintained in the above-mentioned medium additionally containing 500 μg Geneticin (G418)/ml (Life Technologies). The cancer cells were grown in a humidified air/CO_2 (19:1) atmosphere at 37°C and replenished with the respective growth media before each experiment.

Cell morphology and cell proliferation analysis

To show the distinct morphological differences between LNCaP and LNCaP-MST cells, they were photographed by using Leica DMI3000B microscope (LEICA, Buffalo Grove, IL, USA) after growing them to 70% confluency. To study the cell proliferation rate, cell counts were made at the time of seeding and after 48 hrs by using TC10 automated cell counter (Bio-Rad, Hercules, CA, USA).

Hypoxia stimulation

For all hypoxia experiments the cells were grown to 80–90% confluency and, on the day of experiments they were replenished with the respective growth medium containing 2% FBS. Culture dishes were then placed in air-tight modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) that was saturated with pre-analysed gas mixture 1% O_2, 5% CO_2, 94% N_2 (Praxair Inc., Miami, FL, USA) and humidity. This incubator was kept at 37°C for 12 hrs. The normoxic cells were placed at 37°C in a humidified air/CO_2 (19:1) atmosphere. Both hypoxia and normoxia plates were always assayed at the same time.

Western blot analysis of HDM2, HIF-1α, STAT3 and VEGF

At the end of 12-hr incubation period, both the normoxic and hypoxic cells were lysed by sonication and the cell lysates were subjected to Western blot experiments. Exactly 750 μg of protein from each sample was incubated with anti-HDM2 monoclonal antibody (Ab-1) at 4°C for 12 hrs, according to the method of Rathinavelu *et al.* [7]. At the end of incubation the immunoprecipitates were collected by centrifugation and resuspended in the homogenizing buffer. About 30 μg of the immunoprecipitated proteins were resolved on 7.5% SDS-polyacrylamide gel. The proteins were then transferred onto the nitrocellulose membrane and probed with 1:200 dilution of anti-HDM2 monoclonal antibody (Ab-1) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The immunoreactive HDM2 protein signals were detected using ECL blot-developing system (Amersham Corporation, Piscataway, NJ, USA).

For the detection of HIF-1α and VEGF protein levels, 30 μg aliquots of the protein samples were subjected to electrophoresis on 7.5% polyacrylamide gel and then they were transferred onto the nitrocellulose membrane. After blocking with 5% non-fat dry milk solution, the membranes were probed with (1:250 dilution) anti-HIF-1α monoclonal antibody (BD Transduction Laboratories, CA, USA), or anti-VEGF antibody (Santa Cruz Biotechnologies) or STAT3 polyclonal antibody (Proteintech, Chicago, IL, USA). The HIF-1α, VEGF and STAT3 protein bands were visualized using Amersham chemiluminescence kit after incubation of the blotted membrane with HRP conjugated secondary antibody (Amersham). As a control, β-actin Western blots were developed using a 1:2000 dilution of anti-β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), using the same protein samples.

Statistical analysis

The results were expressed as mean $\pm$ S.D. The statistical significance between groups were analysed by one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons tests. The *P* values <0.05 were considered significant and presented in the results.
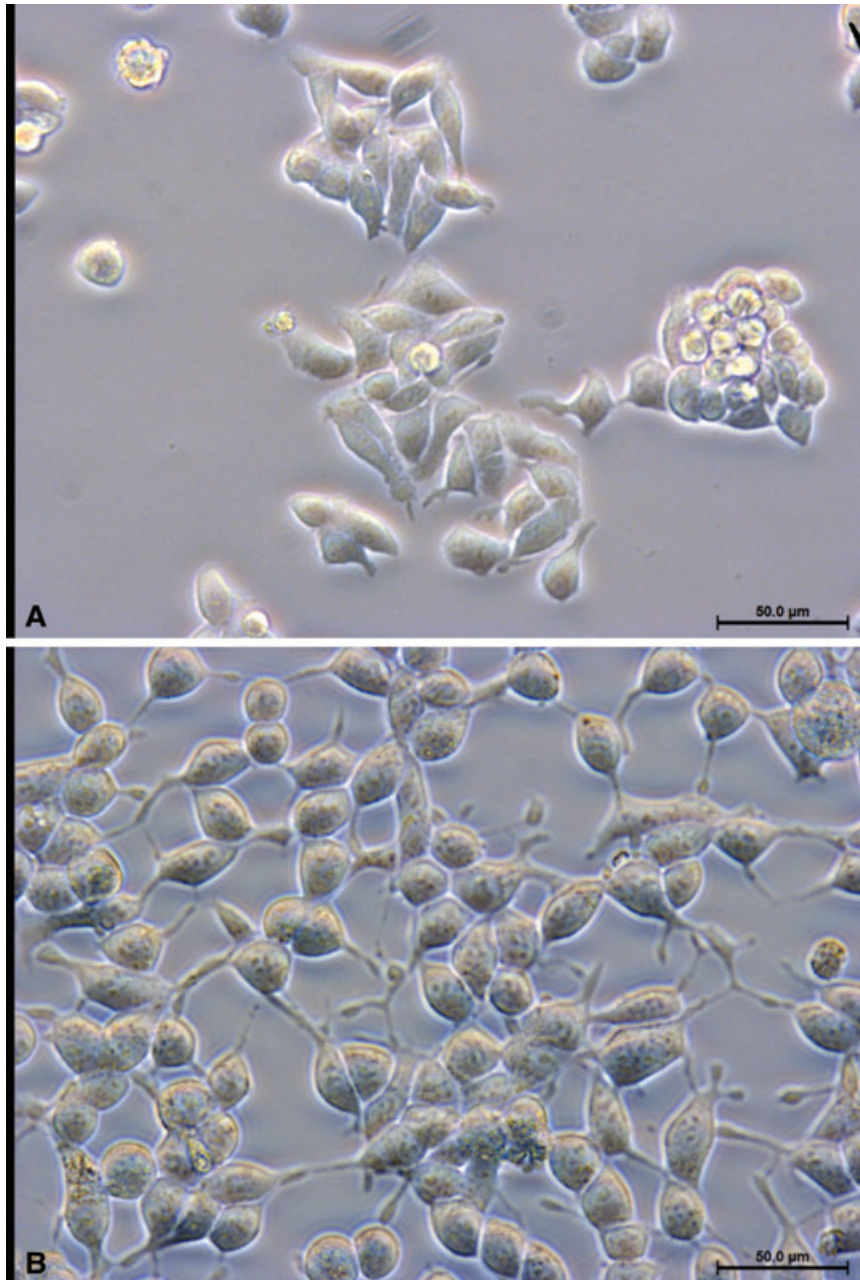
Fig. 1 (**A** and **B**) LNCaP and LNCaP-MST cells grown in complete RPMI medium with 10% FBS showing distinct morphological differences.

Results

Morphological differences and cell proliferation rate of LNCaP and LNCaP-MST cells

LNCaP cells are small and slightly elongated (Fig. 1A) in shape, whereas HDM2 transfected LNCaP-MST cells are larger in size, showed filamentous outgrowth and networking (Fig. 1B). Cell proliferation rate was studied by counting the cells at the time of seeding and after 48 hrs. Figure 2 shows the clear differences in cell growth rate between LNCaP and LNCaP-MST cells. HDM2 transfected LNCaP-MST cells were growing much faster than LNCaP cells by doubling in approximately 24 hrs. On the other hand, LNCaP cells doubled once in 48 hrs.
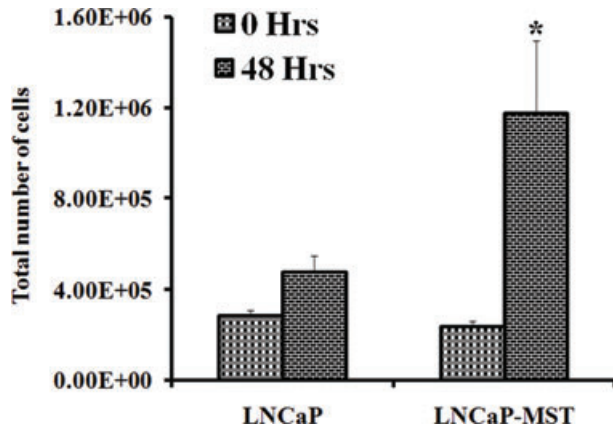
Fig. 2 Graph shows the counts of LNCaP and LNCaP-MST cells at the start of the cell doubling experiment (0 hrs) and at the end of 48 hrs. The * indicates $P < 0.05$ when compared with LNCaP.

Fig. 3 HDM2 protein expression in both LNCaP and LNCaP-MST cells. The wild-type LNCaP and HDM2 transfected cells were grown under normoxic and hypoxic conditions. After 12-hr exposure to hypoxia, the cells were lysed and immunoprecipitation of the cell extracts with anti-HDM2 antibody was carried out. Immunoprecipitated protein extracts were resolved using 7.5% SDS-PAGE and probed with anti-HDM2 antibody. The β-actin served as an internal control in our blotting experiments. The densitometry data from independent blots are given as mean ± S.D. ($n = 3$) and the * indicates $P < 0.05$ and ** indicates $P < 0.01$ when compared with respective controls.

Hypoxia increases HDM2 levels in LNCaP cells

HDM2 expression levels were determined in wild-type LNCaP and HDM2 transfected LNCaP-MST cells. As anticipated, the HDM2 transfected LNCaP-MST cells showed around 96% more HDM2 protein expression than the wild-type LNCaP cells ($P < 0.05$). Furthermore, to determine the effect of hypoxia on HDM2 protein expression, both LNCaP and LNCaP-MST cells were exposed to hypoxia for 12 hrs. In the wild-type LNCaP cells, hypoxia caused only 35% increase in HDM2 protein levels (Lane 1 *versus* Lane 2, Fig. 3) and 50% increase in HDM2 protein levels in HDM2 gene transfected LNCaP-MST cells (Lane 3 *versus* Lane 4, Fig. 3). The hypoxia-induced increase in HDM2 protein expression was much higher in LNCaP-MST cells compared to the non-transfected LNCaP cells ($P < 0.01$).

HDM2 transfection increases HIF-1α protein expression in LNCaP-MST cells

To investigate whether HDM2 transfection has any obvious influence on HIF-1α transcriptional control, the protein levels of HIF-1α in LNCaP and LNCaP-MST cells under both normoxic and hypoxic conditions were examined. HIF-1α is generally a short-lived protein with a half-life of less than 5 min. [17, 18]. The short half-life of HIF-1α is generally attributed to its ability to undergo quick degradation after rapidly inducing gene transcriptional responses. The Western blot analysis results revealed only barely detectable levels of HIF-1α protein (120-kD) expression in the wild-type LNCaP cells under normoxic condition. However, when the cells were subjected to hypoxia for 12 hrs, a notable increase in the expression of HIF-1α protein was detected even in non-transfected LNCaP cells. The HDM2 transfected LNCaP-MST cells showed a higher level expression of HIF-1α even under normoxic
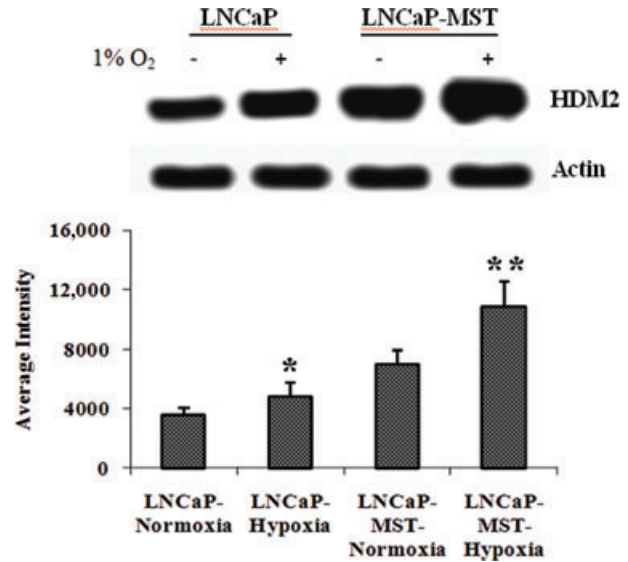
condition that was about 100% more than the wild-type LNCaP cell levels seen under normoxic conditions (compare Lane 1 *versus* Lane 3, Fig. 4). The hypoxic exposure of the HDM2 transfected LNCaP-MST cells showed a 9.5-fold increase in the levels of HIF-1α compared to the non-hypoxic LNCaP-MST cells ($P < 0.01$). In comparison, the above-mentioned increase was about 20-fold higher than the HDM2 non-transfected LNCaP cell levels maintained under hypoxia (Lane 2 *versus* Lane 4, Fig. 4).

HDM2 transfection increases VEGF expression in LNCaP cells under both normoxic and hypoxic condition

In order to show that, we probed the cell extracts for analysing the expression of VEGF. It was observed that VEGF protein expression was increased by 61% during the 12-hr period of hypoxia in wild-type LNCaP cells (Lane 1 *versus* Lane 2, Fig. 5). The HDM2 transfected LNCaP-MST cells showed significantly much higher VEGF expression, that was nearly 180% increase ($P < 0.05$) when compared to the non-transfected LNCaP cells maintained with normoxic exposure (Lane 1 *versus* Lane 3, Fig. 5). These results confirmed that the HIF-1α protein that was induced by hypoxia in
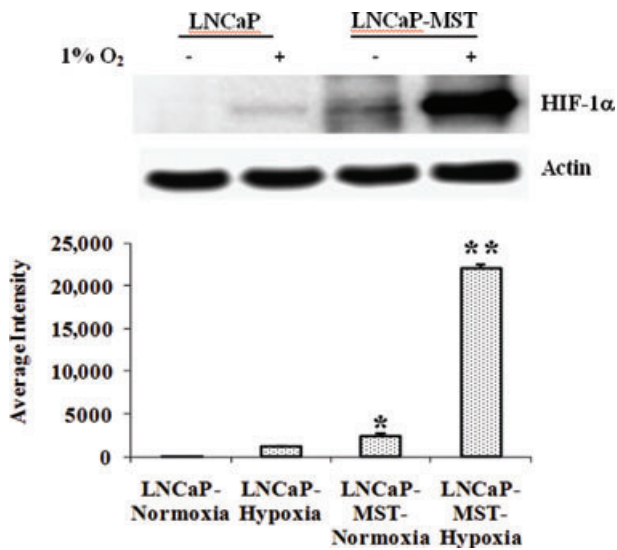
Fig. 4 Expression of HIF-1α in LNCaP and HDM2 transfected LNCaP-MST cells. A representative Western blot showing HIF-1α protein level in the cells exposed to normoxia and hypoxia. Cellular extracts were prepared and subjected to Western blot using an anti-HIF-1α monoclonal antibody. The blot was then stripped and probed for β-actin expression. The blot signals were quantified using Image J software. The densitometry data from independent blots are given as mean $\pm$ S.D. ($n = 3$) and the * indicates $P < 0.05$ and ** indicates $P < 0.01$ when compared with respective controls.

Fig. 5 Expression of VEGF in LNCaP and HDM2 transfected LNCaP-MST cells. The wild-type and HDM2 transfected cells were incubated under normoxic (Lane 1 and Lane 3, respectively), and hypoxic (Lane 2 and Lane 4, respectively) conditions. At the end of 12-hr hypoxic exposure, the protein lysates were analysed using Western blot with anti-VEGF antibody. Band intensities were quantified using Image J software and the results from three separate Western blot analyses are presented. The densitometry data from independent blots are given as mean $\pm$ S.D. ($n = 3$) and the * indicates $P < 0.05$ and ** indicates $P < 0.01$ when compared with respective controls.
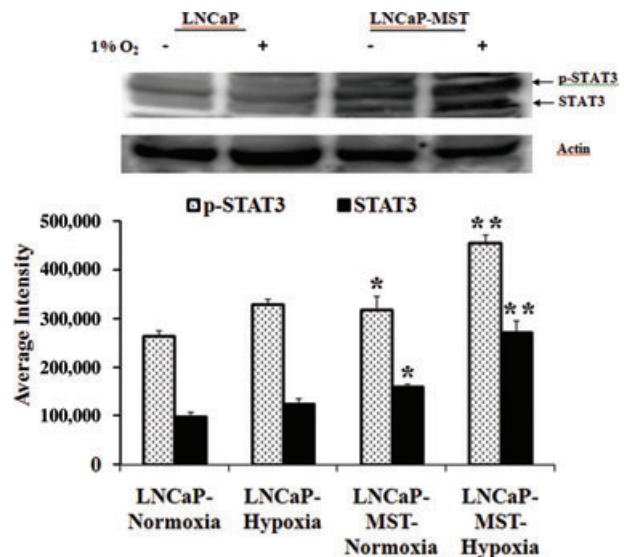

Fig. 6 Shows the levels of STAT3 and phospho STAT3 in LNCaP and HDM2 transfected LNCaP-MST cells. The representative Western blot showing STAT3 protein level in the cells exposed to normoxia and hypoxia. Cellular extracts were prepared and subjected to Western blotting using an anti-STAT3 monoclonal antibody. The blot was then stripped and probed for β-actin expression. The band intensities were quantified using UVP Image analysis software. The relative intensities of STAT3 and phospho STAT3 are shown in the bar graph. The densitometry data from independent blots are given as mean $\pm$ S.D. ($n = 3$) and the * indicates $P < 0.05$ and ** indicates $P < 0.01$ when compared with respective controls.
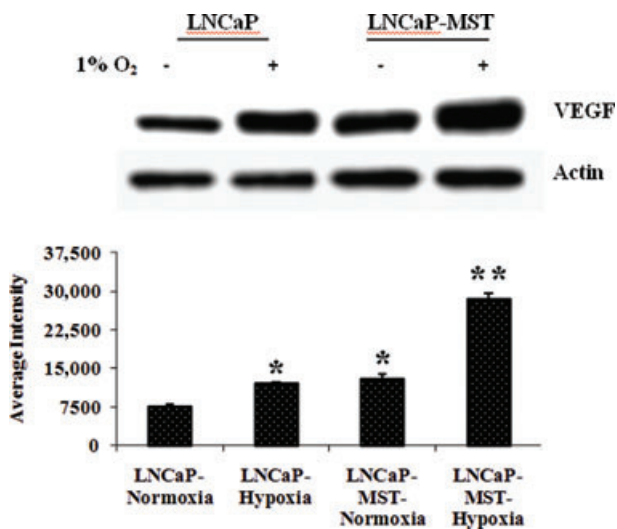
our experiments must have been fully functional in activating the hypoxic response element (HRE) and causing elevation in VEGF transcription. Furthermore, in the samples of HDM2 transfected LNCaP-MST cells that were exposed to hypoxia, the VEGF expression was found to be increased very significantly ($P < 0.01$) and resulted in a 136% increase over the non-transfected cells exposed to hypoxia (Lane 2 *versus* Lane 4, Fig. 5).

STAT3 expression under normoxic and hypoxic condition in LNCaP and LNCaP-MST cells

Previous reports have shown that STAT3 is also involved in the control of VEGF transcription. In this study, the levels of STAT3 in the cells were analysed under normoxic and hypoxic conditions to establish possible correlation between STAT3, HDM2 and VEGF. Figure 6 showed that the expressions of STAT3 and phospho STAT3 were higher in HDM2 transfected LNCaP-MST cells compared to the LNCaP cells. In addition, hypoxic condition also significantly increases the levels of STAT3 and p-STAT3 in both cell lines though the increase was much higher in LNCaP-MST cells ($P < 0.01$). These results suggested that STAT3 may also have an important role in elevating VEGF transcription in HDM2 overexpressing cells.

Discussion

It is recognized that HDM2 oncoprotein has profound effects on cell proliferation and tumour growth [3]. Also, cancer cells often encounter hypoxic environments during the growth phase that influence the progression of a tumour. In the present study, an attempt was made to determine the status of HDM2 in wild-type (LNCaP) and HDM2 transfected (LNCaP-MST) prostate cancer cells that were exposed to both normoxic and hypoxic conditions. Our results revealed up regulation of HDM2 expression in both LNCaP and LNCaP-MST cells during hypoxia, with significantly higher HDM2 expression levels in the LNCaP-MST cells ($P < 0.01$). This could be partially due to excessive HDM2 gene transcription resulting in activation of the stress response pathway. Furthermore, the observed enhancement of HDM2 expression in this study could activate p53-dependent or independent pathways in LNCaP cells, which can only be verified through a series of experiments with p53 null and p53 wild-type cells [19]. Overall, these findings demonstrate that HDM2 transfected LNCaP-MST cells are much more responsive to hypoxia than non-transfected LNCaP cells.

Induction of HIF-1α under hypoxic condition has been previously reported in many cancer cell lines, including colon, pancreas, breast and prostate cancer [16, 20–22]. Having initially demonstrated that hypoxic conditions could significantly increase HDM2 protein expression in both LNCaP and LNCaP-MST cells, our subsequent goal was to determine whether HDM2 level has any link to HIF-1α protein expression under any of the experimental conditions used. So far, the present results confirm that HDM2 transfection could significantly increase the HIF-1α expression during normoxia and augment that increase even more during hypoxic conditions. Accumulating literature indicates that HIF-1α could up-regulate VEGF expression and thereby induce tumour angiogenesis. Accordingly, HDM2 transfection increased HIF-1α levels and also increased the levels of VEGF in prostate cancer cells. Under normoxic conditions, two mechanisms are involved in the regulation of HIF-1α levels and its regulatory effects [23]. One mechanism is the proteosomal degradation of HIF-1α by pVHL following hydroxylation of proline residues 564 and 402 by prolyl hydroxylase that enables the recognition of HIF-1α [23]. Another mechanism is regulated by FIH (factor inhibiting hypoxia-inducible factor). In this process, FIH hydroxylates Asn803 in the C-terminal transactivation domain of HIF-1α, which interferes with its binding to p300 co-activator that eventually results in the inactivation of HIF-1α regulated transcription [24, 25]. As discussed earlier, the HDM2 transfected LNCaP-MST cells express higher than normal levels of HIF-1α, and this might be due to the inhibition of FIH binding to HIF-1α by HDM2. Inhibition of FIH by HDM2 leading to increased levels or activities of HIF-1α has been previously reported under both hypoxic and normoxic conditions [26].

In agreement with the present data, Zhong *et al.* [20] previously described an increase in the expression of HIF-1α protein in normoxic conditions, which supports the notion that hypoxia may not be the only underpinning cause for induction and stabilization of HIF-1α levels in cancer cells. Various other stimuli have been found to play critical roles in controlling HIF-1α expression in non-hypoxic environments [27]. Chun *et al.* [28] have demonstrated that phorbol esters could cause non-hypoxic induction of HIF-1α expression through a redox-dependent, mitogen-activated/extracellular signal-regulated kinase-1 (MEK-1) pathway. It was also reported that human hepatoma (HepG2) cells grown under normoxic conditions, when exposed to elevated temperatures, could increase nuclear translocation and induction of HIF-1α protein expression [29]. Furthermore, the same group demonstrated that during hyperthermia, it is the chaperone activity of HSP90 that is responsible for HIF-1α stabilization under normoxic conditions rather than the transcriptional activity increase [29]. Thus, an elevated expression of HIF-1α observed in HDM2 transfected LNCaP-MST cells under both normoxic and hypoxic conditions strongly suggests that HDM2 brings about post-translational regulation of HIF-1α through increases in protein stability as was reported in many other cell lines [30]. However, the possibility that HDM2 directly modulating HIF-1α protein synthesis cannot be ruled out completely since HDM2 can bind to L5 ribosomal proteins that regulate processes such as ribosomal biogenesis and protein translation [31].

Up-regulation of VEGF observed in LNCaP and LNCaP-MST cells suggests that HDM2 could be directly involved in promoting VEGF transcription by elevating HIF-1α protein as well as phospho STAT3 levels. The role of STAT3 in VEGF transcription has been previously reported in prostate, breast, ovarian, head, neck and other cancers [32–35]. Activation of the c-Src, a non-receptor tyrosine kinase, by cytokines and growth factors during hypoxia leads to tyrosine phosphorylation and STAT3 activation [12, 36]. Activation of STAT3 by phosphorylation and increased STAT3 levels have been associated with cell proliferation and cancer cell survival [37, 38]. In the present experiment, the level of p-STAT3 was found to be greater in HDM2 transfected LNCaP-MST cells compared to LNCaP cells. This indicates a significant influence of HDM2 on STAT3 and p-STAT3 levels. Hypoxia was also shown to increase the STAT3 and p-STAT3 levels much more in LNCaP-MST cells compared to LNCaP cells. In this context, previous reports have indicated that p53 can down-regulate STAT3 phosphorylation [39], which will lead to inactivation of the STAT3. Therefore, it is possible that HDM2 mediated degradation of p53 in LNCaP-MST cells might be a primary reason for the presence of p-STAT3 in HDM2 transfected cells compared to LNCaP.

Finally, we are speculating that p-STAT3 and HIF-1α triggered pathways leading to VEGF transcriptional control is reasonable since it is widely recognized as a common mechanism during tumourigenesis. Furthermore, complexation of HIF-1α and HIF-1β to make the active heteromeric protein complex that can bind to HRE is also essential for promoting VEGF transcription [40]. This complex binds to the core sequence 5′-RCGTG-3′ within HREs that are present in target genes, including VEGF, and up-regulates their transcription [40]. In addition, activated STAT3 can also translocate from cytoplasm to the nucleus and bind with VEGF promoter to induce transcription [12]. Since, these regulatory processes seem to be notably pronounced in LNCaP-MST cells, it is probable that HDM2 gene transfection was responsible for the up-regulation of both HIF-1α and p-STAT3 expressions in cancer cells, thereby increasing the levels of VEGF in normoxic

conditions. The levels of HIF-1α, p-STAT3 and VEGF got enhanced further when the transfected cells were exposed to hypoxic conditions. This appears to confirm HDM2 as a critical regulatory factor in the process of angiogenesis *via* impacting HIF-1α, p-STAT3 and VEGF expressions.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

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