

# Hypoxia upregulates the gene expression of mitochondrial aconitase in prostate carcinoma cells

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## Abstract

Hypoxia induces metabolic alteration in cancer cells by stabilizing hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$  (HIF1A)), which regulates the bioenergetic genes of glycolysis and lipid metabolic pathways. However, the target genes of hypoxia-induced metabolic alterations in the prostate remain uncertain. Mitochondrial aconitase (mACON) (ACONM) is an enzyme that is central to carbohydrate and energy metabolism and is responsible for the interconversion of citrate to isocitrate as part of the citric acid cycle in the human prostate. We evaluated the effects of the molecular mechanisms of hypoxia on *mACON* gene expression in PC-3 and LNCaP human prostate carcinoma cells. Immunoblotting assays revealed that hypoxia modulated mACON and lactate dehydrogenase A (LDHA) protein expression, while these effects were attenuated when *HIF-1 $\alpha$*  was knocked down. Hypoxia induced fatty acid synthase (FASN) in PC-3 cells while hypoxia blocked *FASN* gene expression in LNCaP cells after 24-h incubation. Results of real-time RT-qPCR, immunoblotting, and transient gene expression assays revealed that hypoxia treatment or co-transfection with *HIF-1 $\alpha$*  expression vector enhanced gene expression of *mACON*, implying that hypoxia modulated *mACON* at the transcriptional level. Hypoxia-induced *mACON* promoter activity is dependent on the DNA fragment located at –1013 to –842 upstream of the translation initiation site. L-mimosine, an iron chelator, stabilized HIF-1 $\alpha$  but downregulated *mACON* gene expression, suggesting that iron chelation blocked the hypoxia-induced *mACON* gene expression. These results suggest that hypoxia dysregulates the expressions of *LDHA*, *FASN*, and *mACON* genes, and the hypoxia-induced *mACON* gene expression is via the HIF-1 $\alpha$ -dependent and iron-dependent pathways in prostate carcinoma cells.

## Key Words

- ▶ prostate
- ▶ metabolism
- ▶ signal transduction
- ▶ gene regulation

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## Introduction

Metabolic anomalies in altered forms of flux through key metabolic pathways are primary hallmarks of many malignant tumors including prostate tumors (Bertilsson *et al.* 2012, Costello & Franklin 2012). Most cancer cells

fulfill their energy needs primarily via glycolysis, which is regulated by p53, c-Myc, and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$  (HIF1A); Yeung *et al.* 2008). Under certain hypoxic conditions, anaerobic glycolysis, instead of

aerobic glycolysis, which increases lactate accumulation or lipid metabolism pathways or both, enhances fatty acid synthase (*FASN*) expression and provides metabolic advantages for the tumor cells (Hu *et al.* 2011). Tumor cells engaged in glycolysis do not convert their pyruvate completely into lactate when the availability of oxygen to the cell is insufficient to support cellular oxidative metabolism. Rather, a measurable fraction of the pyruvate metabolized in the tricarboxylic acid cycle provides energy and precursors for the biosynthetic pathway that consumes tricarboxylic acid cycle intermediates (DeBerardinis 2008). In prostate tissue, the low-citrate phenotype reflects a hypoxia-defense adaptation and citrate levels are important to defining the oncogenic condition (Costello & Franklin 2005). The malignant prostate cells are more O<sub>2</sub> limited than in the normal phenotype and become even more O<sub>2</sub> limited as their malignancy state advances (Hochachka *et al.* 2002). The hypoxia adaptation in the prostate alters HIF-1 $\alpha$  levels and energy metabolism (Bourdeau-Heller & Oberley 2007, Higgins *et al.* 2009). However, the metabolic alternations in the prostate carcinoma cells induced by hypoxia are still not well delineated.

Mitochondrial aconitase (aconitase hydratase, EC4.2.1.3; *mACON*) is regarded as the key enzyme in citrate oxidation in human prostate epithelial cells and its abnormal expression is implicated in tumorigenesis of the prostate (Costello & Franklin 1994, Juang 2004a, Bertilsson *et al.* 2012). The *mACON* gene is located in chromosome 22q13.2 and its expression in the prostate is regulated by androgen, prolactin, cholesterol, iron, zinc, and p53 (Juang 2004b,c, Juang *et al.* 2004, Feng *et al.* 2005, Tsui *et al.* 2006,2011). Several researchers have suggested that hypoxia modulates the metabolic phenotype of human prostate carcinoma cells (Hochachka *et al.* 2002, Higgins *et al.* 2009); however, there is no study so far that discusses the modulation of hypoxia and its impact on *mACON* gene expression.

Fatty acid metabolism rather than glycolysis is reported as the energy fuel for prostate carcinoma cells (Liu 2006). The glycolysis–citrate–lipogenesis pathways in providing the synthetic and bioenergetic requirement are essential for the growth and proliferation of prostate tumor cells (Costello & Franklin 2012). *FASN*, a key regulator of *de novo* lipid biosynthesis, is highly over-expressed in prostate carcinoma cells and constitutes a therapeutic target in this disease (Baron *et al.* 2004). Previous studies of ectopic expression of *FASN* in prostate cells or transgenic expression of *FASN* in mice indicated that *FASN* acts as an oncogene of prostate cancer (Migita *et al.* 2009). However, the effect of hypoxia on

gene expression of *FASN* in prostate cells is still not clearly understood.

It is well known that many human cancers have higher lactate dehydrogenase A (LDHA) levels than normal tissues, and the inhibition of LDHA blocks tumor progression (Le *et al.* 2010). The serum LDH level has been regarded as a prognostic factor for prostate cancer, and a high serum LDH level means a poor outcome for prostate cancer patients (Yamada *et al.* 2011). Hypoxia induces expression of *LDHA*, which converts pyruvate into lactate in several cell types (Semenza *et al.* 1996). Increased levels of LDH within the cells are believed to be largely due to the upregulation by the transcription factor *HIF-1 $\alpha$* , as an earlier study explored hypoxia response elements in the promoter of the *LDHA* gene (Semenza 2007). Nonetheless, no report has yet described the regulation of *LDHA* by hypoxia in prostate carcinoma cells.

The objectives of this study were to determine the regulatory effects of hypoxia on the expressions of *LDHA*, *FASN*, and *mACON* in human prostate carcinoma cells. The mechanism of the hypoxic effect on *mACON* gene expression was also investigated.

## Materials and methods

### Cell culture and chemicals

PZ-HPV-7, CA-HPV-10, LNCaP, PC-3, and DU145 cell lines were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan) and were maintained as described previously (Tsui *et al.* 2012). Cells were incubated under normoxia using a standard CO<sub>2</sub> incubator at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% room air (21% O<sub>2</sub>) until the cells grew to 70–80% confluence in RPMI-1640 medium with 10% FCS. On the day of hypoxic treatment (1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub>), growth medium was switched to fresh medium with 10% FCS that had been pre-equilibrated in the hypoxic incubator (APM-30D, Astec, Fukuoka, Japan). Cells were then incubated in the hypoxic incubator for another 24 h. L-mimosine, chetomin, and ferric ammonium citrate were purchased from Sigma Chemical Company, FCS was purchased from HyClone (Logan, UT, USA), and RPMI-1640 medium was purchased from Gibco, Invitrogen Corporation.

### Immunoblotting assay

Equal quantities of cell extract (40  $\mu$ g) were loaded onto 12% SDS–PAGE and analyzed using an electrochemiluminescence detection system and were viewed

using the ChemiGenius image capture system (Syngene, Cambridge, UK). Polyclonal mACON antiserum was prepared as described previously (Tsui *et al.* 2011). The blot membranes were probed with 1:500 HIF-1 $\alpha$  (610958, BD Biosciences, San Jose, CA, USA), 1:3000 diluted  $\beta$ -actin antiserum (I-19, Santa Cruz Biotechnology), 1:100 diluted vascular endothelial growth factor (VEGF; A-20, Santa Cruz Biotechnology), 1:500 diluted LDHA (E-9, Santa Cruz Biotechnology), 1:1000 diluted FASN (A-5, Santa Cruz Biotechnology), 1:1000 diluted heme oxygenase 1 (HO-1; Hsp32, Stressgen, Victoria, BC, Canada), 1:100 p53 (DO-1, Santa Cruz Biotechnology), and 1:12 500 diluted mACON antiserum. The intensities of the different bands were analyzed using the GeneTools program of ChemiGenius (Syngene).

#### Lactate secretion assay

Cells were incubated under normoxic or hypoxic conditions in 0.5 ml RPMI medium with 10% FCS in a 24-well plate ( $1 \times 10^5$  cells/well) for 24 h. Following incubation, the supernatants of the conditioned media from each well were collected for lactate secretion assays using a lactate assay kit (K627-100, Bio Vision, Milpitas, CA, USA) according to the protocol described by the manufacturer. The lactate level in each sample was adjusted by the concentration of protein in the whole cell extract, which was measured using a bicinchoninic acid protein assay kit (Pierce Protein Research, Rockford, IL, USA).

#### HIF-1 $\alpha$ expression vector

The human *HIF-1 $\alpha$*  cDNA (MGC: 10483) vector was purchased from Invitrogen. Human *HIF-1 $\alpha$*  cDNA was linearized by cutting with BamHI and XbaI and ligated with the overexpression vector pcDNA3 (Invitrogen) as described previously (Chung *et al.* 2011).

#### Knockdown HIF-1 $\alpha$

LNCaP and PC-3 cells were transduced with *HIF-1 $\alpha$*  shRNA lentiviral particles (sc-35561-V, Santa Cruz Biotechnology) as described previously (Chung *et al.* 2012). The cells (LN-HIF-1 $\alpha$ si and PC-HIF-1 $\alpha$ si) were selected by incubation with 10  $\mu$ g/ml puromycin dihydrochloride for at least five generations. The mock-transfected LNCaP (LN-COLsi) and PC-3 (PC-COLsi) cells were transduced with control shRNA lentiviral particles (sc-10808-V, Santa Cruz Biotechnology) and were clonally selected in the same manner as the *HIF-1 $\alpha$*  knockdown cells.

#### Real-time RT-PCR

Total RNA was isolated using the Trizol reagent, and cDNA was synthesized using the Superscript III preamplification system (Invitrogen). Real-time PCR (qPCR) was performed as described previously (Chung *et al.* 2012). FAM dye-labeled TaqMan MGB probes and PCR primers were purchased for human mACON (HS00426616\_g1) from Applied Biosystems. For the internal positive control,  $\beta$ -actin (HS016060665\_g1) was used with a FAM reporter dye-labeled TaqMan MGB probe. The amplification conditions were as follows: 40 cycles at 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 1 min. Mean cycle threshold ( $C_t$ ) values for mACON were normalized against the  $\beta$ -actin control probe to calculate  $\Delta C_t$  values using StepOne software v2.0 (Applied Biosystems). All reactions were performed in triplicate, and each experiment was conducted on at least three independent occasions.

#### Reporter vectors, transient transfection, and reporter assay

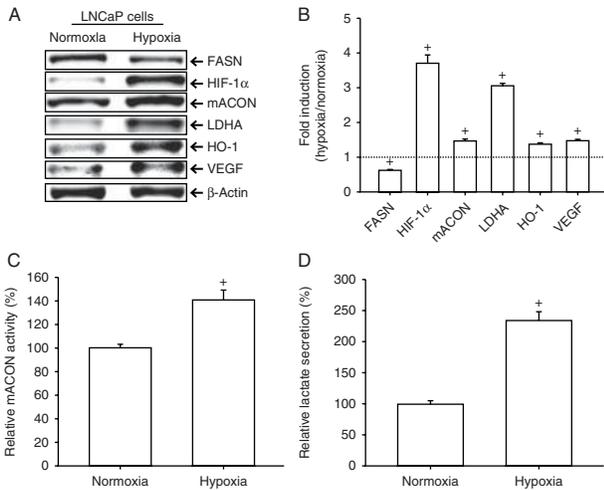
The reporter vectors containing the 5'-flanking region of the *mACON* gene were constructed as described previously (Tsui *et al.* 2011). LNCaP cells or PC-3 cells were plated onto 24-well plates 1 day before transfection. Cells were transiently transfected using TransFast transfection reagent (Promega BioScience) as described previously (Tsui *et al.* 2012). The luciferase activity was adjusted for transfection efficiency using the normalization control plasmid pCMVSPORT $\beta$ gal (Invitrogen). For the transient co-transfection of HIF-1 $\alpha$  experiments, cells were transfected with the same amount of plasmid in each well by adding pcDNA3 vector to eliminate the variable degrees of efficiency of reporter activities.

#### Statistical analysis

Results are expressed as the means  $\pm$  S.E.M. of at least three independent replications of each experiment. Statistical significance was determined using Student's *t*-test and one-way ANOVA using the SigmaStat program for Windows, version 2.03 (SPSS, Inc.).

#### Results

The immunoblotting assays revealed that hypoxia, in comparison to normoxia, significantly upregulated the protein levels of HIF-1 $\alpha$  in both LNCaP and PC-3 cells (Figs 1A and 2A). Results also showed that hypoxia

**Figure 1**

Hypoxia modulates protein expression of mACON, FASN, and LDHA in LNCaP cells. (A) LNCaP cells were cultured under hypoxic or normoxic conditions for 24 h. The expression of HIF-1 $\alpha$ , mACON, FASN, LDHA, HO-1, VEGF, and  $\beta$ -actin were determined by immunoblotting assay. (B) The quantitative analysis was done by determining the intensity of each band for target genes and  $\beta$ -actin from three independent experiments. Data are presented as the fold-induction ( $\pm$  s.e.m.,  $n=3$ ) of the relative density of the target gene/ $\beta$ -actin in relation to the normoxia treatment group. (C) Mitochondria were extracted and the enzymatic activity of mACON was analyzed in the LNCaP cells. Data are presented as the mean percentage ( $\pm$  s.e.m.,  $n=6$ ) of mACON enzymatic activity relative to that of the normoxia-treated group. (D) Supernatants of the conditioned medium were collected and the lactate levels were analyzed in the LNCaP cells. Data are presented as the mean percentage ( $\pm$  s.e.m.,  $n=6$ ) of lactate secretion relative to that of the normoxia-treated cell group;  $^+P<0.01$ .

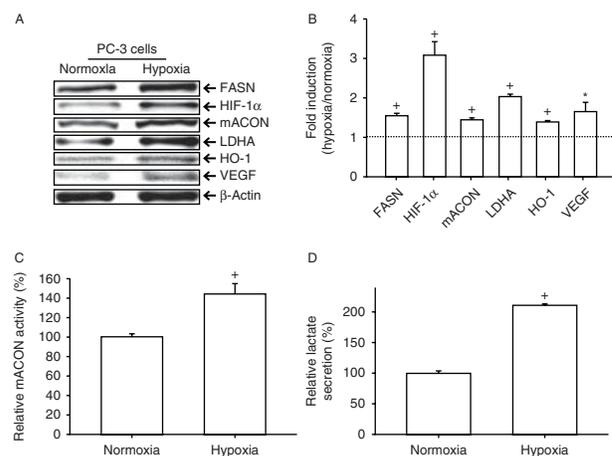
upregulated mACON, LDHA, HO-1 (HMOX1), and VEGF (VEGFA) protein levels (Fig. 1A). Similar results were found in the hypoxia-treated PC-3 cells (Fig. 2A). The quantitative analysis was done by determining the intensity of each band for different genes and  $\beta$ -actin from three independent experiments (Figs 1B and 2B). Interestingly, the quantitative results indicated that FASN levels were downregulated in LNCaP cells by 39% after 24 h of hypoxic culture in comparison with LNCaP cells incubated under normoxic condition (Fig. 1B). However, we did not find a similar phenomenon in the PC-3 cells. Hypoxic treatment significantly increased FASN levels (1.53-fold) in PC-3 cells (Fig. 2B).

Because we found that hypoxia upregulated mACON protein levels in both LNCaP and PC-3 cells, we sought to determine whether its enzymatic activities were induced by hypoxia. Results revealed that hypoxia, in comparison with normoxia, significantly upregulated 1.41- and 1.44-fold mACON enzymatic activities respectively in LNCaP and

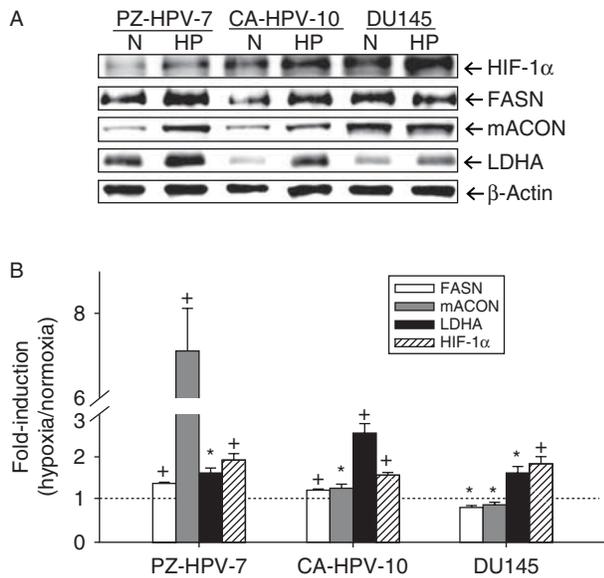
PC-3 cells (Figs 1C and 2C). We further determined the cytosolic aconitase (cACON) activities in the cells. However, results showed that hypoxia has no significant enzymatic effects on cACON in either LNCaP ( $P=0.148$ ) or PC-3 ( $P=0.213$ ) cells (Supplementary Figure S1, see section on supplementary data given at the end of this article).

We analyzed the lactate levels in the supernatant of the culture media after hypoxic or normoxic culture for 24 h. Results showed that hypoxic treatment enhanced lactate secretion in both LNCaP (2.34-fold) and PC-3 (2.11-fold) cells after 24 h of hypoxic culture in comparison with PC-3 cells incubated under normoxia (Figs 1D and 2D).

As we found the divergent effect of hypoxia on the FASN expression in LNCaP and PC-3 cells, we continued to determine the effects of hypoxia on FASN, mACON, LDHA, and HIF-1 $\alpha$  protein levels in other prostate cell lines including PZ-HPV-7, CA-HPV-10, and DU145 cells. The results of immunoblotting assays revealed that hypoxia significantly upregulated protein levels of HIF-1 $\alpha$ , FASN, mACON, and LDHA in PZ-HPV-7 and CA-HPV-10 cells; however, hypoxia downregulated minimally but significantly protein expressions of FASN and mACON while upregulated HIF-1 $\alpha$  and LDHA protein expressions on DU145 cells (Fig. 3).

**Figure 2**

Hypoxia modulates protein expression of mACON, FASN, and LDHA in PC-3 cells. (A) PC-3 cells were treated and then the expression of proteins were analyzed as described in Fig. 1A. (B) The quantitative analysis and data presentation are described as in Fig. 1B ( $^*P<0.05$ ,  $^+P<0.01$ ). (C) Mitochondria were extracted and the enzymatic activity of mACON was analyzed in the PC-3 cells. (D) Supernatants of the conditioned medium were collected and the lactate levels were analyzed in the PC-3 cells. Data are presented as the mean percentage ( $\pm$  s.e.m.,  $n=6$ ) of mACON enzymatic activities and lactate secretion relative to that of the normoxia-treated cell group ( $^+P<0.01$ ).

**Figure 3**

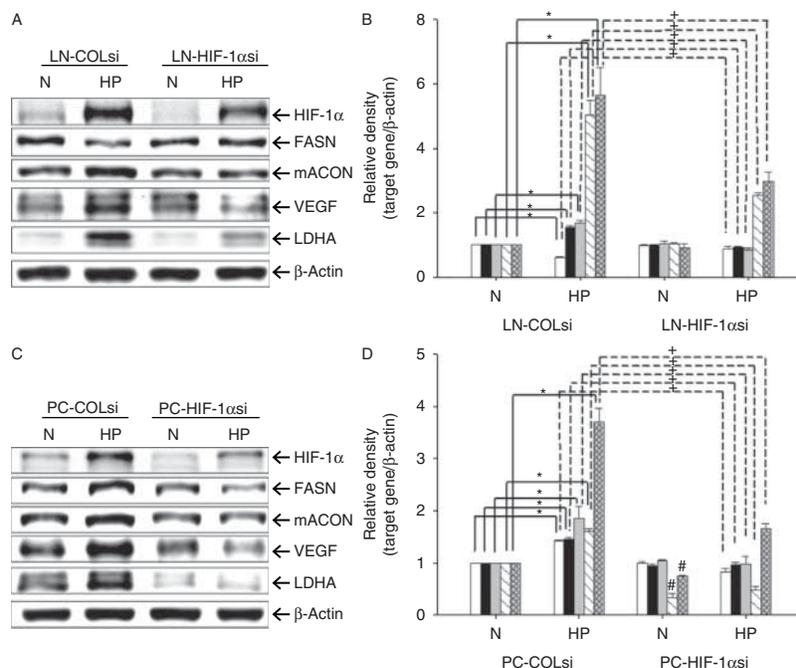
Hypoxia modulates protein expression of mACON, FASN, and LDHA in PZ-HPV-7, CA-HPV-10, and DU145 cells. Cells were cultured under hypoxic or normoxic conditions for 24 h. The expression of HIF-1 $\alpha$ , FASN, LDHA, mACON, and  $\beta$ -actin were determined by immunoblotting assay. (B) The quantitative analysis and data presentation are described as in Fig. 1B (\* $P < 0.05$ , + $P < 0.01$ ).

In order to determine that the hypoxic dysregulation of mACON, LDHA, VEGF, and FASN in prostate carcinoma cells was HIF-1 $\alpha$  dependent, we knocked down *HIF-1 $\alpha$*  in LNCaP (LN-HIF-1 $\alpha$ si) and PC-3 (PC-HIF-1 $\alpha$ si) cells by transduction with *HIF-1 $\alpha$*  shRNA lentiviral particles. The immunoblotting assays revealed that hypoxia, in comparison with normoxia, significantly upregulated ( $P < 0.01$ ) protein levels of HIF-1 $\alpha$ , mACON, LDHA, and VEGF in mock-transfected LNCaP (LN-COLsi) and PC-3 (PC-COLsi) cells. However, the increase in protein expressions of the genes under hypoxia was significantly attenuated ( $P < 0.01$ ) when the *HIF-1 $\alpha$*  gene was knocked down in the LNCaP (LN-HIF-1 $\alpha$ si) and PC-3 (PC-HIF-1 $\alpha$ si) cells (Fig. 4A and C). The quantitative analysis was done by determining the intensity of each band for different genes and  $\beta$ -actin from three independent experiments (Fig. 4B and D). As expected, in comparison with normoxia, hypoxia significantly downregulated ( $P < 0.01$ ) protein levels of FASN in the mock-transfected LNCaP (LN-COLsi) cells but upregulated protein levels of FASN in the mock-transfected PC-3 (PC-COLsi) cells. Moreover, dysregulation of FASN protein levels was attenuated when the *HIF-1 $\alpha$*  gene was knocked down from both (LN-HIF-1 $\alpha$ si and PC-HIF-1 $\alpha$ si) cells.

As our results showed that hypoxic treatment upregulated mACON protein levels in both LNCaP and PC-3 cells, we determined the effects of the hypoxia mimetic, L-mimosine, on gene expression of *mACON*. In contrast to the results of hypoxic treatments, L-mimosine treatment downregulated mACON protein levels in LNCaP cells even though L-mimosine indeed stabilized HIF-1 $\alpha$  (Fig. 5A). Interestingly, mACON protein levels were upregulated to 1.55-fold under hypoxia in comparison with normoxia. The quantitative results indicated that 800  $\mu$ M L-mimosine induced a 1.89-fold increase in HIF-1 $\alpha$  protein levels but downregulated mACON levels by 25% after 24 h of incubation in comparison with solvent control treatment groups (Fig. 5B). Results of transient gene expression assays revealed that 200, 400, and 800  $\mu$ M L-mimosine downregulated the promoter activity of the human *mACON* gene by 20.1, 24, and 20% respectively (Fig. 5C). The immunoblotting assays showed that L-mimosine did not affect P53 (TP53) expression in LNCaP cells, indicating that decreasing *mACON* gene expression by L-mimosine is not dependent on the p53 pathway (Fig. 5D).

Immunoblotting results also showed that L-mimosine treatment upregulated HIF-1 $\alpha$  but downregulated mACON protein levels in PC-3 cells (Fig. 5E). We determined whether blocking of *mACON* gene expression by L-mimosine was due to the iron chelation nature of L-mimosine as *mACON* is an iron-upregulated gene. The immunoblotting assays revealed that ferric ammonium citrate, an iron donor, increased *mACON* gene expression, and this increase was blocked after co-treatment with L-mimosine in PC-3 cells (Fig. 5F). Similar results were found in the transient gene expression assays, which indicated that L-mimosine blocked the ferric ammonium citrate-induced increases in *mACON* promoter activity in PC-3 cells (Fig. 5G).

The RT-qPCR analyses showed that expression of the *mACON* gene was significantly induced by hypoxia in the LNCaP cells. Hypoxia induced  $5.1 \pm 0.7$ -fold increases in *mACON* expression after incubation for 12 h, while hypoxia (vs normoxia) did not significantly increase *mACON* mRNA levels after 24-h incubation (Fig. 6A). The transient gene expression assay revealed that hypoxia, in comparison with normoxia, upregulated the promoter activity of the *mACON* gene in LNCaP cells by about twofold. Transient overexpression of HIF-1 $\alpha$  significantly induced *mACON* promoter activities in LNCaP cells under both normoxic and hypoxic conditions (Fig. 6B). Results of immunoblotting assays showed that hypoxia induced  $5.8 \pm 0.5$ -,  $2.2 \pm 0.1$ -,  $1.5 \pm 0.1$ -, and  $3.6 \pm 0.4$ -fold increases

**Figure 4**

*HIF-1 $\alpha$*  knockdown attenuates the activation of hypoxia on gene expression of *mACON* in prostate carcinoma cells. The mock-knockdown LNCaP (LN-COLsi) and the *HIF-1 $\alpha$* -knockdown LNCaP cells (LN-HIF-1 $\alpha$ si) (A) and the mock-knockdown PC-3 (PC-COLsi) and *HIF-1 $\alpha$* -knockdown PC-3 cells (PC-HIF-1 $\alpha$ si) (C) were cultured under normoxic (N) or hypoxic (HP) conditions for 24 h. The expressions of *HIF-1 $\alpha$* , *mACON*, *FASN*, *LDHA*, *VEGF*, and  $\beta$ -actin were determined by immunoblotting assay. Quantitative

results were analyzed by determining the intensity of each band for the target genes (white bars, *FASN*; black bars, *mACON*; gray bars, *VEGF*; white coarse bars, *LDHA*, and gray cyclone bars, *HIF-1 $\alpha$* ) and  $\beta$ -actin (B and D). Data are presented as the fold-induction ( $\pm$ S.E.M.,  $n=3$ ) of the relative density of the target gene/ $\beta$ -actin in relation to the mock-knockdown normoxia-treated group (\*, #, +  $P<0.01$ ).

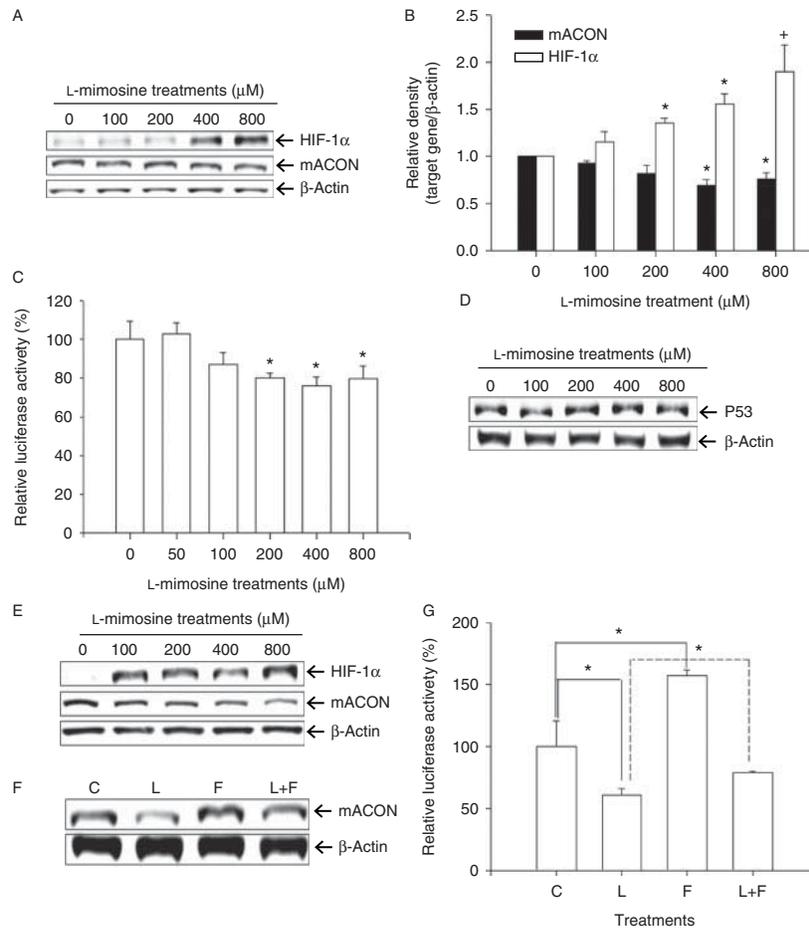
in *HIF-1 $\alpha$* , *LDHA*, *mACON*, and *VEGF* protein levels respectively. The 100 nM chetomin co-treatment did not significantly block the *HIF-1 $\alpha$*  protein levels; however, co-treatment with chetomin attenuates hypoxia-induced *LDHA*, *mACON*, and *VEGF* protein expressions. Co-treatment with chetomin attenuates hypoxia-blocked *FASN* protein expression in LNCaP cells (Fig. 6C). Induction of transient gene expression using a 5'-deletion assay indicated that increased *mACON* promoter activities induced by *HIF-1 $\alpha$*  is dependent on the region  $-1013$  to  $-841$  upstream of the translational initiation site of the *mACON* gene (Fig. 6D).

## Discussion

Hypoxia is regarded as a potent tumor-induced shield against destruction in prostate cancer (Marignol *et al.* 2008). The first response of oxygen limitation in the cells is to coordinate the regulation of glycolytic enzyme genes in order to maintain ATP synthesis (Warburg 1956, Kroemer & Pouyssegur 2008, Hu *et al.* 2011). One of the major

hypoxia adaptations to prevent excessive reactive oxygen species is to induce expression of *LDHA*, which converts pyruvate into lactate (Semenza *et al.* 1996). In this study, we demonstrated that hypoxia induced *LDHA* protein expression in LNCaP and PC-3 prostate carcinoma cells in support of the theory of increase in the 'anoxic glycolytic pathway' in prostate carcinoma cells when tumor cells are exposed to hypoxia proposed by Costello & Franklin (2005). Our results showed that hypoxia enhanced lactate secretion in prostate carcinoma cells, which is consistent with an other report (Higgins *et al.* 2009). Our results further showed that *HIF-1 $\alpha$*  knockdown in prostate carcinoma cells attenuated the activation of *LDHA* protein expression by hypoxia; moreover, chetomin blocked hypoxia-induced *LDHA*. These results are in agreement with previous reports, indicating an essential binding site for *HIF-1 $\alpha$*  in the promoter of the human *LDHA* gene (Semenza 2007).

Unlike other organs, the prostate produces, accumulates, and releases large amounts of citrate (Costello & Franklin 1994). The study indicates that prostate carcinoma cells metabolize cytoplasmic citrate via *mACON*

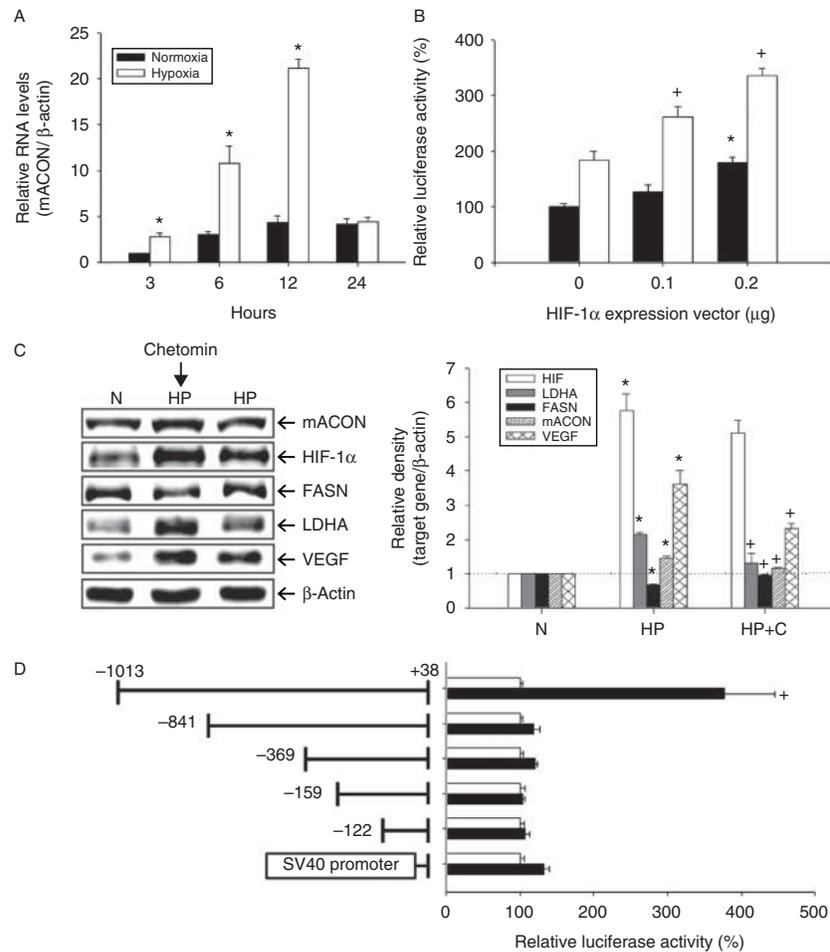
**Figure 5**

L-mimosine downregulates the expression of human *mACON* gene via iron chelation. (A) Immunoblots of HIF-1 $\alpha$ , *mACON*, and  $\beta$ -actin using lysates from varying concentrations of L-mimosine-treated LNCaP cells. (B) The quantitative analysis and data presentation are described as in Fig. 1B (\* $P < 0.05$ , + $P < 0.01$ ). (C) The *mACON* reporter vector-transfected LNCaP cells were treated with varying concentrations of L-mimosine as indicated for 24 h. Data are presented as the mean percentage  $\pm$  s.e.m. ( $n = 6$ ) of the luciferase activity induced by L-mimosine relative to the control solvent group (\* $P < 0.05$ ). (D) Immunoblotting for P53 and  $\beta$ -actin using lysates from varying concentrations of L-mimosine-treated LNCaP cells.

(E) Immunoblotting for HIF-1 $\alpha$ , *mACON*, and  $\beta$ -actin using lysates from varying concentrations of L-mimosine-treated PC-3 cells. (F) PC-3 cells were treated with 400  $\mu$ M L-mimosine and/or 100  $\mu$ g/ml ferric ammonium citrate for 24 h. The expressions of *mACON* and  $\beta$ -actin were determined by immunoblotting assays. (G) The *mACON* reporter vector-transfected PC-3 cells were treated with L-mimosine (400  $\mu$ M) and/or ferric ammonium citrate (100  $\mu$ g/ml) for 24 h. Data are presented as the mean percentage  $\pm$  s.e.m. ( $n = 6$ ) of the luciferase activity in relation to the control solvent-treated group (\* $P < 0.05$ ; C, control solvent; L, L-mimosine; F, ferric ammonium citrate).

and FASN to enhance their metastatic behavior (Mycielska *et al.* 2006). FASN is a key metabolic enzyme that catalyzes the synthesis of palmitate from the condensation of malonyl-CoA and acetyl-CoA *de novo* and plays a central role in energy homeostasis by converting excess carbon intake into fatty acids for storage. Elevated expression of FASN is linked with prostate tumorigenesis and progression (Migita *et al.* 2009, Flavin *et al.* 2011). Costello & Franklin (2005) proposed a 'glycolysis–citrate–lipogenesis' metabolic pathway indicating that a net increase in lipid biosynthesis and incorporation of citrate for lipogenesis is essential for proliferation in tumor cells. Our study

showed that hypoxia enhances *FASN* gene expression in highly metastatic prostate carcinoma PC-3 cells. Another study also indicated hypoxia upregulated *FASN* gene expression in breast cancer cells (Furuta *et al.* 2008). However, we found that hypoxia decreased *FASN* gene expression in the human prostate carcinoma LNCaP cells, which is consistent with a previous report of hypoxia decreasing *FASN* mRNA in LNCaP cells (Higgins *et al.* 2009). LNCaP cells, in comparison with PC-3 cells and P53 wild-type cells are androgen-dependent and express a more oxidative phenotype, as assessed by energy metabolism and metabolic gene expression (Higgins *et al.* 2009,

**Figure 6**

Hypoxia modulates the gene expression of *mACON* of prostate carcinoma cells. (A) LNCaP cells were cultured under normoxic (black bars) or hypoxic (white bars) conditions for various time periods. The *mACON* mRNA levels were determined by RT-qPCR. The mRNA levels of normoxia-treated cells for 3 h were set at 1. Data are presented as the mean-fold ( $\pm$ s.e.m.,  $n=3$ ) of the mRNA levels in relation to the normoxia-treated group for 3 h ( $*P<0.01$ ). (B) The *mACON* reporter vector-transfected LNCaP cells were co-transfected with varying concentrations of HIF-1 $\alpha$  expression vector and then incubated in normoxic (black bars) or hypoxic (white bars) condition for another 24 h. Data are presented as the mean percentage ( $\pm$ s.e.m.,  $n=6$ ) of the luciferase activity in relation to the mock-transfected group ( $*P<0.05$ ,  $^+P<0.01$ ). (C, left) LNCaP cells were cultured under normoxic (N), hypoxic (HP), or hypoxia with 100 nM of chetomin (HP+C) conditions for 24 h. The expressions of HIF-1 $\alpha$ , *mACON*, LDHA, FASN, VEGF, and  $\beta$ -actin

were determined by immunoblotting assay (C, right). The protein levels of normoxia-treated cells were regarded as 1. Data are presented as the fold-induction ( $\pm$ s.e.m.,  $n=3$ ) of protein levels of the hypoxia-treated group in relation to the normoxia-treated group ( $*P<0.05$ ) and hypoxia with chetomin-treated group in relation to the hypoxia-treated groups ( $^+P<0.05$ ). (D) Nested deletion constructs of *mACON* reporter vectors were co-transfected with control expression vector (pcDNA3; white bars) or HIF-1 $\alpha$  expression vector (black bars) into LNCaP cells. The SV40 promoter represents pGL3-promoter vector (Promega Bioscience). Data are presented as the mean percentage  $\pm$ s.e.m. ( $n=6$ ) of the luciferase activity of the reporter vectors induced by the HIF-1 $\alpha$  expression vector relative to the luciferase activity associated with group that was co-transfected with control expression vector ( $^+P<0.01$ ).

Tsui *et al.* 2011). Results from the present study indicate that the effect of hypoxia on *FASN* gene expression seems to be dependent on cell type, although the mechanisms of divergent effects of hypoxia on *FASN* in prostate carcinoma cells are still unknown.

The issue of adaptive response to hypoxia suggests that the limitation of cellular oxygen availability is the direct cause of the failure of the tricarboxylic acid cycle

activity and citrate oxidation (Semenza *et al.* 1996). Nonetheless, other hypotheses suggest that hypoxia augments *mACON* in the Krebs cycle, leading to a gain of two thirds of the potential energy available from either glucose or fat-derived acetyl-CoA flux into the tricarboxylic acid cycle in the prostate (Hochachka *et al.* 2002, Costello & Franklin 2005). Our results demonstrate that hypoxia enhances the gene expression of *mACON* in

prostate carcinoma cells in a HIF-1 $\alpha$ -dependent manner. Hypoxia enhances gene expression of *LDHA*, *FASN*, and *mACON* in PC-3 cells, which suggests that hypoxia not only enhances the conversion of pyruvate into lactate but also promotes citrate in prostate carcinoma cells for both oxidation for energy production and export for lipogenesis. Our study is the first report to reveal HIF-1 $\alpha$ -induced *mACON* gene expression at the transcriptional and translational levels in human prostate carcinoma cells, although the detailed mechanisms of HIF-1 $\alpha$ 's effect on *mACON* gene expression in prostate carcinoma cells still require further investigation. Interestingly, a study using the human embryonic kidney 293 cells indicated that hypoxia increased cACON but did not regulate mACON protein accumulation or activity (Schneider & Leibold 2003). The other study indicated that hypoxia (0.2% O<sub>2</sub>) for 48 h decreased total aconitase activity in primary human pulmonary arterial endothelial cells (Chang *et al.* 2009). However, our results showed that hypoxia enhances *mACON* gene expression but does not affect the enzymatic activity of cACON in either PC-3 or LNCaP cells. Therefore, it seems that hypoxia-induced *mACON* gene expression is specific to prostate cells.

L-mimosine is a prolyl 4-hydroxylase inhibitor, which culminates in the stabilization of the transcriptional activator HIF-1 $\alpha$  and increases expressions of prostate-specific antigen, B-cell translocation gene 2, and N-myc downstream-regulated gene 1 gene products that mediate hypoxic adaptation *in vitro* in human prostate carcinoma cells (Chung *et al.* 2011, 2012). Our results indicate that L-mimosine induces increases in HIF-1 $\alpha$  protein levels but reduces *mACON* gene expression. An early report indicated that L-mimosine induced P53 expression in human RKO colorectal cancer cells (Ji *et al.* 1997), while our recent study indicated that P53 downregulates *mACON* gene expression (Tsui *et al.* 2011). Interestingly, results of immunoblotting assays in the present study imply that L-mimosine does not increase P53 protein expression in wild-type P53-expressing LNCaP cells, suggesting that the decrease in *mACON* gene expression by L-mimosine may not occur through the p53 pathway. L-mimosine also acts as iron chelator, which reversibly blocks prostate cell proliferation at the late G1 phase (Chung *et al.* 2012). Our results indicate that L-mimosine blocks the increase in *mACON* gene expression by the iron donor, ferric ammonium citrate, suggesting that inhibition of mACON by L-mimosine is due to iron deprivation even though HIF-1 $\alpha$  induces *mACON* gene expression. These results are in agreement with our previous study, which indicated that mACON is not only an iron-dependent

enzyme but is also modulated by iron at both the transcriptional and translation levels (Juang 2004b).

HO-1, the inducible isoform of the rate-limiting enzyme in heme degradation, has cytoprotective effects against oxidative damage (Gozzelino *et al.* 2010). Immunohistochemical analysis of prostate tissue reveals significant elevation of HO-1 expression in epithelial cancer cells compared with those of normal or benign prostate tissues (Alaoui-Jamali *et al.* 2009, Li *et al.* 2011). Although the functions of HO-1 in prostate carcinoma cells are still not well delineated, our results show that hypoxia induces HO-1 expression in prostate (LNCaP and PC-3) cells *in vitro*. These results are consistent with previous research on the human microvascular endothelial cell line HMEC-1 (Ockaili *et al.* 2005).

Results of RT-qPCR and transient gene expression assays in the present study indicated that modulation of hypoxia in *mACON* occurs at the transcriptional levels. Moreover, our immunoblotting data showed that chetomin, an epidithiodioxopiperazine known to disrupt the HIF-1 $\alpha$  pathway (Kung *et al.* 2004), attenuates hypoxia-induced mACON expressions, suggesting that hypoxia-induced *mACON* gene expression is via the HIF-1 $\alpha$ -dependent pathway in prostate carcinoma cells. Induction of transient gene expression by the 5'-deletion assay indicated that enhancement of HIF-1 $\alpha$  on *mACON* promoter activity is dependent on the -1013 to -841 region upstream of the translational initiation site of the *mACON* gene. However, we did not find the conserved DNA sequence (5'-RCGTG-3') of HIF-1 $\alpha$  response element (Wang *et al.* 1995) but the putative Egr1 and Oct4 binding sites in the *mACON* promoter region (-1013 to -841). Studies indicated that hypoxia indirectly regulates target gene expression by modulating microRNAs, chromatin-modifying enzymes, and transcription factors including *EGR1* and *OCT4* (*POU5F1*) (Zhang *et al.* 2007, Semenza 2012). However, we cannot rule out the possibility that the consensus hypoxia response elements may locate at the 5'-upstream of the DNA fragment (-1013 to +38), which we used in this study, as an other study indicated that 60% of HIF-1 binding sites are located >2.5 kb upstream of the transcription start site (Schodel *et al.* 2011). Whether *mACON* gene expression by hypoxia enhances directly and/or indirectly via the HIF-1 $\alpha$ -dependent pathway remains to be further elucidated.

In conclusion, we suggest that hypoxia enhances not only the 'anoxic glycolytic pathway' by inducing *LDHA* gene expression, the 'glycolytic-lipogenic pathway' by inducing *FASN* gene expression, but also the 'promoter citrate oxidation pathway' by increasing *mACON* gene

expression. The greater induction of LDHA than FASN and mACON by hypoxia further suggests that the major metabolic adaptation of hypoxia is the 'anoxic glycolytic pathway' in prostate carcinoma cells. The modulation of hypoxia in LDHA, FASN, and mACON is via the HIF-1 $\alpha$ -dependent pathway in prostate carcinoma cells. Although both L-mimosine and hypoxia treatments stabilize HIF-1 $\alpha$  protein expression, hypoxia enhances expression of mACON at the transcriptional level, while L-mimosine blocks mACON expression by iron chelation. The hypoxia-induced mACON gene expression is dependent on the DNA fragment (–1013 to –841) upstream of the translation initiation site of the mACON gene. Hypoxia-induced mACON gene expression is via the HIF-1 $\alpha$ -dependent and iron-dependent pathways in prostate carcinoma cells.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0090>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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